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Proteins and Proteomics of *Leishmania* and *Trypanosoma*

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Editorial

The so-called neglected tropical diseases comprise a group of infections that is especially endemic in low-income population in developing countries of Africa, Asia and the Americas. In this group, the American (*Trypanosoma cruzi*) and African (*Trypanosoma brucei* complex) trypanosome species and microorganisms belonging to the *Leishmania* genus are the etiologic agents of major human and animal parasitic diseases worldwide, being responsible for large socio-economic losses, especially in developing countries. The effective treatment of diseases caused by trypanosomatids is still an open issue, nevertheless of paramount importance. Chemotherapy still relies on drugs developed decades ago, showing limited efficacy and possibility of toxic side effects. Nowadays, new approaches have been employed to improve treatment, and researches conducted to discover strategies that are safer, more efficacious and accessible. These strategies include the use of lipid formulations such as amphotericin B or miltefosine to treat the leishmaniasis, and chemotherapeutic combinations for other parasitic diseases. However, this still remains expensive considering the target population. Moreover, the emergence of resistance has been reported. Efforts to tackle these diseases require research on the molecular components that regulate the infection initiation, which is critical for a better understanding of the diseases' pathogeneses. Considering that these infections represent a major health concern worldwide, the development of a new generation of chemotherapeutic agents is of extreme importance. For that, research on the relevant aspects of targeted drug development is a critical priority. Several proteins/glycoproteins are being explored as targets for chemotherapy development, since they play key roles in different phases of the life cycle of trypanosomatids, including: hydrolytic enzymes, surface proteins with adhesive properties and extracellular components capable in helping the parasites to evade the host immune responses. Better biochemical and/or molecular characterisation of these (glyco)proteins can help to decipher their real relevance in the life cycle of human pathogenic trypanosomatids. In this context, without doubt, proteomic techniques are an interesting approach to discover novel potential parasite molecules to be used as target to generate novel anti-trypanosomatid compounds. With regarding to all these issues,

the present book focuses on the description of relevant proteins that participate in key steps of biological events and virulence of parasites belonging to the *Leishmania* and *Trypanosoma* genera.

The authors believe that this book will serve as a basis for consultations by specialists in the field of microbiology, in particular parasitologists, because it contemplates matters of the utmost importance in the proposed area. The chapters are written by experts who actively contribute to international scientific literature, generating knowledge about protein molecules synthesised by pathogens belonging to the genera *Leishmania* and *Trypanosoma*. We also believe that the theme "Proteins and proteomics of *Leishmania* and *Trypanosoma*" is a very attractive proposal and the compilation of interesting data will benefit authors around the world. This fact can be corroborated by a simple inspection in scientific papers' databases, where a large number of papers have been published over the last years on this subject in order to unravel the mechanisms of pathogenicity of human pathogenic trypanosomatids, which still constitute a major public health problem worldwide.

The book *Proteins and proteomics of* Leishmania *and* Trypanosoma contains (1) an integrated view about the biochemistry of parasites belonging to the *Leishmania* and *Trypanosoma* genera; (2) an updated review on the expression of biologically relevant proteins by human pathogenic trypanosomatids and their possible role in the interaction with host cells and (3) several pictures, diagrams and tables that can be used in both undergraduate and postgraduate teaching as well as scientific lectures. Briefly, Chap. 1 by *Juliany Rodrigues* and co-workers opens the book providing an update on the biology of *Leishmania* and *Trypanosoma*, focusing in epidemiology, life cycle and ultrastructural aspects of special structures and organelles found exclusively in trypanosomatid cells that contain unique proteins responsible for crucial metabolic pathways. Chapter 2 presented by *Despina Smirlis* and *Milena Soares* critically discusses the tools for exploiting, predicting and selecting novel and potential protein targets for rational drug design. In Chap. 3, *Ana Paula Fernandes* and co-workers discuss about visceralising proteins from *Leishmania*, with emphasis on the amastigote-specific antigen A2, which participates in pathogenesis and is a promising target for the development of vaccine against visceral leishmaniasis. Chapter 4 by *Maria Fernanda Silva* and *Lucile Maria Floeter-Winter* describes the relevance of arginine uptake and arginase activity in the establishment and maintenance of *Leishmania* infection. In Chap. 5, *Turán Ürményi* and co-workers review the relevance of heat shock proteins in *Trypanosoma cruzi*, since this parasite experiences several kinds of stress during its complex life cycle. *Cristian Cortez* and co-workers (Chap. 6), *Eliciane Mattos* and co-workers (Chap. 7), *Isadora Oliveira* and co-workers (Chap. 8) and *Sergio Rubin* (Chap. 9) elucidate biochemical, molecular, structural and topological aspects of the well-known gp85/sialidase superfamily that are expressed at the surface of clinically relevant trypomastigote forms of *T. cruzi,* such as gp82 (Chap. 6), gp85 (Chap. 7) and *trans-sialidase* (Chaps. 8 and 9), which participate in the attachment and invasion of both extracellular matrix components and host cells. In Chap. 10, *Anita Freitas-Mesquita* and *José Roberto Meyer-Fernandes* provided details about the occurrence and physiological roles of two ecto-enzymes, ecto-nucleotidases and

ecto-phosphatases, in parasites belonging to *Leishmania* and *Trypanosoma* genera. Chapter 11 presented by *Claudia d'Avila-Levy* and co-workers critically analyses the function of gp63 (leishmanolysin) from the perspective of the interaction of trypanosomatids with the invertebrate host. *Carla Polycarpo* (Chap. 12) provides some important clarifications about the aminoacyl-tRNA synthetases from trypanosomatids, exploring their structural diversity as a rational target for the design of novel drugs. The last three chapters discuss trypanosomatids from an entirely proteomic point of view of trypanosomatids. *Rubem Menna-Barreto* and *Jonas Perales* (Chap. 13), *José Batista de Jesus* and co-workers (Chap. 14) and *Fabricio Marchini* and co-workers (Chap. 15) summarise proteomic and phosphoproteomic maps of different morphotypes of both *T. cruzi* and *Leishmania* as well as describe unique metabolic pathways and parasite-specific molecules with potential participation in essential physiological/pathological events from *T. cruzi* and *Leishmania*, identifying alternative candidates for drug interventions.

The editors really hope that the reading of each chapter and, of course, the book as a whole, will arouse enthusiasm and scientific curiosity in young students and researchers around the world to learn more about these intriguing microorganisms, which continue to challenge us and excite our curiosity. With this proposal in mind, new perspectives need to be envisioned and employed. In this context, we need to better understand the physiology of these microorganisms in order to find new cellular targets and new drugs for alleviating the discomfort of millions of individuals who, unfortunately, live with the suffering inflicted by infections caused by *Leishmania* and *Trypanosoma*.

Finally, the editors are extremely grateful to all the contributing authors for their enthusiasm and valuable cooperation and to the consulting editors for their expert and exhaustive scientific review.

Contents

Chapter 1
Biology of Human Pathogenic Trypanosomatids: Epidemiology, Lifecycle and Ultrastructure

Juliany Cola Fernandes Rodrigues, Joseane Lima Prado Godinho, and Wanderley de Souza

Abstract *Leishmania* and *Trypanosoma* belong to the Trypanosomatidae family and cause important human infections such as leishmaniasis, Chagas disease, and sleeping sickness. Leishmaniasis, caused by protozoa belonging to *Leishmania*, affects about 12 million people worldwide and can present different clinical manifestations, i.e., visceral leishmaniasis (VL), cutaneous leishmaniasis (CL),

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mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL), and post-kala-azar dermal leishmaniasis (PKDL). Chagas disease, also known as American trypanosomiasis, is caused by *Trypanosoma cruzi* and is mainly prevalent in Latin America but is increasingly occurring in the United States, Canada, and Europe. Sleeping sickness or human African trypanosomiasis (HAT), caused by two sub-species of *Trypanosoma brucei* (i.e., *T. b. rhodesiense* and *T. b. gambiense*), occurs only in sub-Saharan Africa countries. These pathogenic trypanosomatids alternate between invertebrate and vertebrate hosts throughout their lifecycles, and different developmental stages can live inside the host cells and circulate in the bloodstream or in the insect gut. Trypanosomatids have a classical eukaryotic ultrastructural organization with some of the same main organelles found in mammalian host cells, while also containing special structures and organelles that are absent in other eukaryotic organisms. For example, the mitochondrion is ramified and contains a region known as the kinetoplast, which houses the mitochondrial DNA. Also, the glycosomes are specialized peroxisomes containing glycolytic pathway enzymes. Moreover, a layer of subpellicular microtubules confers mechanic rigidity to the cell. Some of these structures have been investigated to determine their function and identify potential enzymes and metabolic pathways that may constitute targets for new chemotherapeutic drugs.

1 Introduction

Leishmania and *Trypanosoma* belong to the Trypanosomatidae family and cause important human infections; e.g., *Leishmania* causes leishmaniasis, and *Trypanosoma* is responsible for Chagas disease and human African trypanosomiasis (HAT), otherwise known as sleeping sickness. Together, these illnesses are among the most important neglected tropical diseases (NTDs), and they affect about 22 million people worldwide (Figs. 1.1 and 1.2). These protozoan parasites alternate between invertebrate and vertebrate hosts throughout their lifecycles, and different developmental stages are capable of living inside the host cells or circulating in the bloodstream or the insect gut. Another important feature of these parasites is the presence of special organelles that are absent in other eukaryotic organisms or have functions exclusively found in trypanosomatids. As an example, mitochondrion is unique and ramified and has a particular region localized close to the basal body and flagellar pocket; this specific region is known as the kinetoplast and houses the mitochondrial DNA. In this chapter, we will briefly review the most relevant data on the epidemiology, lifecycle, and structural organization of *Leishmania*, *Trypanosoma cruzi* (i.e., causative agent of Chagas disease), and *Trypanosoma brucei* (i.e., causative agent of HAT).

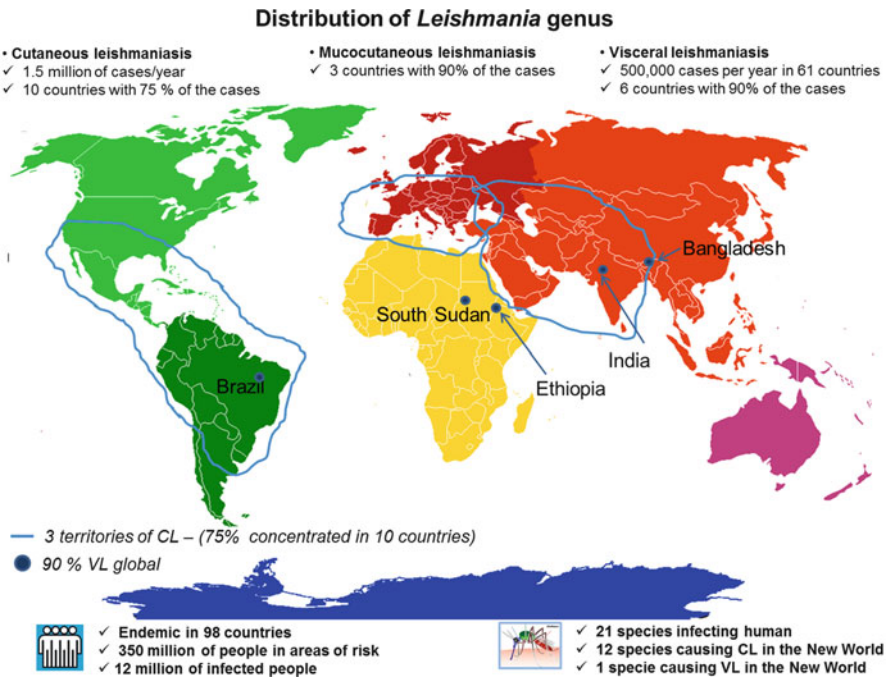

Fig. 1.1 World distribution of the different clinical manifestations of leishmaniasis

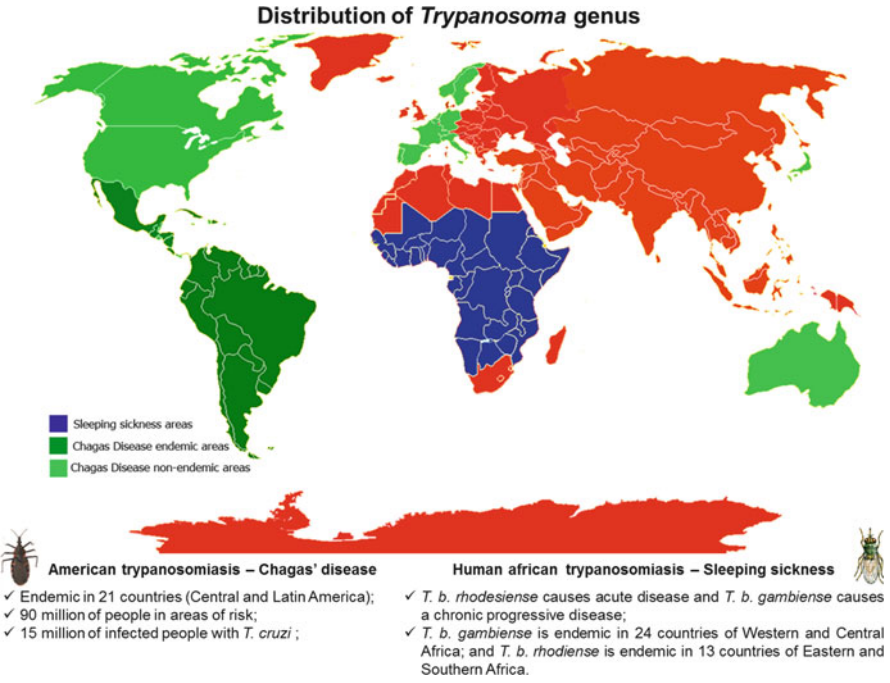

Fig. 1.2 World distribution of Chagas disease and human Africa trypanosomiasis (HAT)

2 Epidemiology, Clinical Manifestations and Chemotherapy

2.1 Leishmania *and Leishmaniasis*

The illness known as leishmaniasis refers to a complex of important NTDs caused by protozoan parasites of the *Leishmania* genus and affects people in both the poorest regions of the world and developing countries, being their distribution quite amazing for the twenty-first century. More specifically, new studies about the epidemiology of this disease revealed that 350 million people are considered at risk for contracting leishmaniasis, and 12 million people were infected worldwide with 2 million new cases yearly (WHO 2010a). Moreover, a recent detailed study based on a comprehensive literature review revealed that a total of 98 countries in five continents have endemic issues related to leishmaniasis transmission (Fig. 1.1) (Alvar et al. 2012). An estimated 0.2–0.4 and 0.7–1.2 million cases of visceral leishmaniasis (VL) and cutaneous leishmaniasis (CL), respectively, occur per year. More than 90 % of the global cases of VL are concentrated in the following six countries: India, Bangladesh, Sudan, South Sudan, Ethiopia, and Brazil. However, the distribution of the CL cases is broader; i.e., one-third of these cases are concentrated in the following three epidemiological regions: the Americas, the Mediterranean basin, and Western Asia (i.e., from the Middle East to Central Asia). More specifically, the following ten countries have highest incidence rate of CL: Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, North Sudan, Costa Rica, and Peru. These countries together are responsible for 70–75 % of the estimated global CL cases (Alvar et al. 2012).

At least 21 species of *Leishmania* can cause disease transmitted to humans by 1 of 30 species of sandflies from the genera *Phlebotomus* or *Lutzomyia*. These species are divided in two subgenera: *Leishmania* and *Viannia*. Together they are responsible for five main clinical manifestations: VL or kala-azar, CL, mucocutaneous leishmaniasis (MCL), diffuse cutaneous leishmaniasis (DCL), and post-kala-azar dermal leishmaniasis (PKDL). Table 1.1 summarizes these clinical manifestations.

In VL, the parasites display a marked tropism for visceral organs, such as the liver, spleen, bone marrow, and lymphatic system. Indeed, VL is considered the most severe form of the disease characterized by undulating fever, weight loss, splenomegaly, hepatomegaly, lymphadenopathy, and anemia. After treatment, the patients may develop a chronic form of cutaneous leishmaniasis that has been called PKDL, which requires long-term treatment. PKDL is a recurrence of VL and can appear up to 20 years after treatment has ended. Sometimes, PKDL appears as a co-infection with human immunodeficiency virus (HIV), which is another important feature of leishmaniasis (Zijlstra et al. 2003). Two specific species are responsible for VL around the world, i.e., *L. donovani* and *L. infantum* (syn *L. chagasi*) in Africa, Asia, Europe and in the Americas (Kuhls et al. 2011).

CL, MCL, and DCL are part of a larger group of diseases also called American tegumentary leishmaniasis, which are widespread with most cases occurring in Brazil. In the case of CL, localized lesions that form can be self-healing; however, when the lesions are multiple and disabling due to the formation of disfiguring

Table 1.1 Summary of the different clinical manifestations of leishmaniasis

Clinical manifestations		Species
Cutaneous leishmaniasis	Red lesions develop at the site of bite that can ulcerate	*L. braziliensis*[a] *L. amazonensis*[a] *L. major*[b]
Mucocutaneous leishmaniasis	The lesions can partially or totally destroy the mucous membranes of the nose, mouth, and throat cavities and surrounding tissues	*L. braziliensis*[a] *L. guyanensis*[a] *L. aethiopica*[b]
Diffuse cutaneous leishmaniasis	Presence of disseminated and chronic skin lesions resembling those of lepromatous leprosy. In this situation, the patients are anergic, and no treatment is available	*L. amazonensis*[a] *L. mexicana*[a] *L. aethiopica*[b]
Visceral leishmaniasis	Also known as kala-azar and characterized by the tropism of the parasites to the visceral tissues and presentation of high fever, substantial weight loss, swelling of the spleen and liver, and anemia	*L. infantum* (syn. *L. chagasi*)[c] *L. donovani*[b]
Post-Kala-azar dermal leishmaniasis	PKDL is a unique eruption that develops after treatment and apparent cure of visceral leishmaniasis	*L. infantum* (syn. *L. chagasi*)[c] *L. donovani*[b] *L. infantum*[b]

[a]Species found in the Americas
[b]Species found in Africa, Asia, and Europe
[c]Specie found in the Africa, Asia, Europe and Americas

scars, it is very difficult to treat, leading to a significant disfigurement and social stigmatization. In general, ulcerative lesions are most common in the Americas. CL can be caused by different species of *Leishmania* such as *L. mexicana*, *L. amazonensis*, *L. braziliensis*, or *L. panamensis* in the Americas, and *L. tropica* and *L. major* in other countries. DCL is rare even in leishmaniasis-endemic regions and is caused by an infection with *L. amazonensis* in Brazil or *L. aethiopica* in the Mediterranean basin, Middle East, and Africa. In general, DCL occurs when the immune system fails to react against *Leishmania* antigens in individuals with a defective cell-mediated immune response. Multiple non-ulcerative lesions form around the body, sometimes resembling those found in lepromatous leprosy. These lesions never spontaneously heal, and unfortunately no treatment is currently available for it. In the case of MCL, the parasites have a marked tropism for the oral-nasal and pharyngeal cavities, often causing extensive destruction that involves mutilation of the face and great suffering for the infected patients. In the Americas, it is associated with an infection by *L. braziliensis* and *L. guyanensis*. In contrast, MCL is rare in Africa, Asia, and Europe; however, when it occurs, it is frequently associated with an infection by *L. donovani* or *L. major* or in immunosuppressed patients infected with *L. infantum* (Desjeux 2004; WHO 2010a).

Nowadays, the following two pentavalent antimonials are available as the first line of treatment for leishmaniasis: meglumine antimoniate and sodium stibogluconate (i.e., sold as Glucantime and Pentostan, respectively). In general, these drugs can be administered intramuscularly or intravenously; however, they can also be administrated intralesionally for the treatment of CL. Several common side effects

are possible during treatment, including loss of appetite, vomiting, nausea, abdominal pain, myalgia, arthralgia, headache, metallic taste, and lethargy.

Due a high number of cases of resistance, other treatments have also become available, including amphotericin B deoxycholate, its liposomal formulation, and pentamidine. Amphotericin B is a polyene antibiotic that is administrated by intravenous infusion and has several toxic side effects mainly related to nephrotoxicity, hypokalemia, and myocarditis. Some studies have demonstrated the efficacy of a single dose of liposomal amphotericin B in the treatment of VL (Sundar et al. 2008, 2010). Moreover, miltefosine, an oral drug, is an alkylphosphocholine analog that was originally developed as an anti-cancer drug; however, it was shown to be active against *Leishmania* at the end of 1980 and licensed in India as the first oral treatment for VL in 2002. Besides, it has been tested on several continents in many clinical trials in the treatment of various forms of leishmaniasis, including CL and MCL (Dorlo et al. 2012). Their main side effects are related to gastrointestinal systems, including loss of appetite, nausea, vomiting, and diarrhea. In addition, it is teratogenic in high doses; thus, it is not recommended for use by pregnant women or women who may become pregnant (Ganguly 2002; Oliveira et al. 2011). Given the longer term use of this chemotherapeutic agent in this area, cases of resistance to miltefosine have been reported mainly in India.

Another interesting treatment is based on the intramuscular administration of paromomycin, an aminoglycoside antibiotic that can also be used in a topical lipid formulation (Thaku et al. 2000; Alavi-Naini et al. 2012). In addition to all of the monotherapies mentioned above, combination therapies have been used in some studies and could offer new solutions for the treatment of leishmaniasis (WHO 2010a). Based on their efficacy and function as a monotherapy, different combinations between pentavalent antimonials, amphotericin B, paromomycin and miltefosine have been proposed.

2.2 T. cruzi *and Chagas Disease*

T. cruzi is the etiologic agent of Chagas disease, which is otherwise known as American trypanosomiasis, and it was discovered in 1909 by the Brazilian medical doctor Carlos Chagas. Nowadays, Chagas disease is considered endemic in 21 countries mainly across Latin America and parts of North America (i.e., Mexico and Southern United States) (Fig. 1.2). Around 90 million people are exposed to the parasite, and current estimates indicate that 12 million people are infected with *T. cruzi* (WHO 2010b; Rassi Jr. et al. 2010, 2012). Historically, the disease (i.e., both transmission and morbidity) was restricted to this region; however, due the intense migration activity of Latin American immigrants, the number of cases in non-endemic developed countries (e.g., Australia, Canada, Japan, Spain, and the United States) has significantly increased, making Chagas disease an important public health problem of global concern (Fig. 1.2). Its chronic phase results in significant disability, which ultimately creates significant negative social and economic impacts on countries with high incidence of this disease. For example, Brazil has suffered the loss of around US $1.2 billion in salary and untold losses of industrial and rural productivity due to the number of infected workers (WHO 2010c).

The parasite *T. cruzi* is primarily transmitted to humans by the feces of blood-sucking reduviid insects widely known as "the kissing bugs;" however, non-vectorial mechanisms of infection can occur through such means as blood transfusions, organ transplantations, congenital and oral transmission (i.e., ingestion of contaminated food, especially fruit juices). The disease has the following three characteristic clinical phases: *the acute phase*, where around 5 % of children die but can spontaneously resolve itself in 4–6 weeks; *asymptomatic or indeterminate phase*, where the patients do not present any clinical symptoms of the disease, but they can transmit the parasite to other humans; and the *chronic symptomatic phase* occurring in 10–30 % of the infected patients, where the heart or the gastrointestinal tracts are affected. Note that Chagas disease is one of the most important causes of cardiomyopathy worldwide. In general, acute Chagas disease is asymptomatic. Nevertheless, symptoms can develop at around 8–10 days after infection by vector-borne transmission; in the case of symptom development, an edema known as "chagoma" or Romaña signal appears in the palpebral and periocular regions. In addition, most of the deaths at this stage are due to heart failure. The chronic stage begins 2–3 months after initial infection and after resolution of the acute disease. About 60–70 % of the infected patients with the acute symptoms eventually present the indeterminate form of the disease without any further clinical symptoms except the presence of antibodies against *T. cruzi* in the serum. The other 30–40 % of infected patients with acute symptoms develop the chronic symptomatic disease, which can affect the cardiac muscle, the digestive system (i.e., mainly as megaesophagus and megacolon) or the both. These manifestations can occur usually 10–30 years after the initial infection (Rassi Jr. et al. 2012).

Only two drugs are officially recommended for the treatment of Chagas disease, i.e., benznidazole and nifurtimox. Several clinical studies have demonstrated that benznidazole, a nitroimidazole derivative, is more safe and efficacious than nifurtimox, thereby making it the first-line treatment. Interestingly, both drugs act against the acute phase of Chagas disease, reducing the severity of the symptoms and shortening the clinical course and duration of the detectable parasitemia (Le Loup et al. 2011). However, neither is effective against the chronic phase of the disease. Both benznidazole and nifurtimox present several toxic side effects, such as the formation of a localized allergic dermatitis and gastrointestinal symptoms, respectively (Rassi Jr. et al. 2012). At this moment, clinical trials are taking place in Spain and Argentina using a new agent in the treatment of Chagas disease, i.e., posaconazole, alone or in combination with benznidazole (Loup et al. 2011; Urbina 2010; www.clinicaltrials.gov, accessed on January 10th, 2013).

2.3 T. brucei *and Sleeping Sickness*

T. brucei is the etiologic agent of HAT, which is otherwise known as sleeping sickness; this illness is transmitted by tsetse flies of the *Glossina* genus. Historically, HAT has occurred in the poorest rural areas of Africa, where weak health systems and political instability make disease surveillance and management difficult. Two subspecies are responsible for the transmission of HAT in rural parts of sub-Saharan

Africa. The majority of cases (i.e., >90 %) are caused by *T. b. gambiense*, which is endemic in 24 countries of Western and Central Africa (i.e., mainly Angola, Congo, Guinea, Southern Sudan, and Northwestern Uganda), whereas *T. b. rhodesiense* is endemic in 13 countries of Eastern and Southern Africa (i.e., mainly Malawi, Tanzania, and Southeastern and Central Uganda) (Fig. 1.2). However, the Democratic Republic of the Congo accounts for two thirds of the reported cases. More than 205 discrete, active HAT foci are recognized, most of which are in poor and remote rural areas where health systems are often weak as previously indicated. However, sleeping sickness has also been reported in peri-urban areas (Malvy and Chappuis 2011). As previously mentioned, HAT, leishmaniasis, and Chagas disease represent the most important NTDs and affect mainly the poorest, rural regions. Nevertheless, thanks to important work by the Medicins Sans Frontieres (MSF) and several control and intervention programs from the World Health Organization (WHO), the number of reported cases of HAT have declined from 37,385 in 1998 to 9,589 in 2009 (WHO 2006; Simarro et al. 2012; Blum et al. 2012).

The clinical manifestations that characterize sleeping sickness (i.e., HAT) are generally the same for both causative sub-species. However, *T. b. rhodesiense* causes a more acute disease with overt clinical manifestations developing within days following infection that can lead to death if untreated, while *T. b. gambiense* is characterized by a chronic progressive course of the disease (Malvy and Chappuis 2011). Signs and symptoms of HAT are classified according to the clinical progression of the disease and can be divided into two distinct stages. The first is *Stage 1*, i.e., the hemolymphatic phase, where the trypanosomes are restricted to the blood and the lymphatic systems. In general, this stage involves non-specific symptoms, like headaches, fever, and joint pain, which are difficult to diagnose correctly due the failure of the surveillance systems. *Stage 2*, i.e., the meningoencephalitic phase, is characterized by the active invasion of the central nervous system by the parasite. In this case, the trypanosomes cross the blood-brain barrier and can lead to serious sleep cycle disruptions, paralysis, and progressive mental deterioration, all of which can result in the death of the infected patients in the absence of an effective treatment. Clearly, the sleep disorder inspired the common name of the disease, i.e., *sleeping sickness*. The two stages involved in HAT caused by *T. b. gambiense* have an average duration of around 3 years, which is very different from that of HAT caused by *T. b. rhodesiense*, which is classically described as an acute disease progressing to stage 2 within a few weeks and death within 6 months (Odiit et al. 1997). One important difference between the two infections is the presence of the trypanosomal chancres that can appear after the tsetse bite. In infections of *T. b. gambiense*, the chancre is rarely seen; however, it occurs and can be numerous in approximately 26 % of patients infected with *T. b. rhodesiense* (Boatin et al. 1986; Blum et al. 2012; MacLean et al. 2010). The chancre is defined as the initial lesion at the bite site and is characterized by local erythema, edema, heat, tenderness, and a lack of any suppuration. In the cases where the chancre is present, it can be used as a clinically diagnostic indicator.

Five drugs are available for the treatment of HAT, and they are prescribed according to the stage of the disease and the infecting protozoan sub-species; these five drugs are pentamidine, eflornithine, nifurtimox, melarsoprol, and suramin. For cases of

HAT caused by *T. b. gambiense*, the treatment for stage 1 involves the intravenous administration of pentamidine by slow infusion for 7 days, which is repeated over the course of several decades. The most frequent adverse side effects are pain at the injection site, hypoglycemia, and hypotension. For stage 2 of HAT caused by *T. b. gambiense*, melarsoprol has been the only available effective drug for over 50 years. This drug is poorly tolerated due to a wide range of side effects, including encephalopathic syndrome, peripheral neuropathy, hepatic toxicity, skin rash, acute phlebitis, and vein sclerosis. In addition, a large number of cases of resistance to melarsoprol have been detected in endemic areas. More recently, eflornithine monotherapy has gradually replaced melarsoprol as the first-line treatment in these cases; however, its universal use is complicated due the associated difficulties in logistics and requirements for nursing care. In order to be efficacious, 56 intravenous infusions, each lasting 30 min, must be administered over at least 14 days. Several efforts have been made to attempt to shorten and simplify the eflornithine therapy. As a result, a large clinical trial demonstrated that nifurtimox-eflornithine combination therapy (NECT) has the same efficacy rate as the eflornithine monotherapy (Priotto et al. 2009; Yun et al. 2010). After this study, WHO and Drugs for Neglected Diseases initiative (DNDi) conducted a phase IIIb trial in May of 2009 involving 600 patients from the Democratic Republic of the Congo. Since 2010, MSF has adopted NECT as the first-line treatment in several endemic countries (Simarro et al. 2012). In the case of HAT caused by *T. b. rhodesiense*, the only treatment available is suramin in a complex dose regimen of more than 30 days for stage 1 and melarsoprol for stage 2. Unfortunately, *T. b. rhodesiense* is resistant to eflornithine (Malvy and Chappuis 2011). Table 1.2 summarizes all the treatments presently available for leishmaniasis, Chagas disease, and HAT (i.e., sleeping sickness).

3 Lifecycle

Leishmania, *T. cruzi*, and *T. brucei* are heteroxenic protozoan parasites; i.e., their lifecycles involve two hosts – the insect vector and the mammalian hosts, including sylvatic and domestic reservoirs and humans. During their lifecycles, different developmental stages essential for progression of the infection occur in both hosts. Figure 1.3 summarizes all the main developmental stages for all species as they occur in the insect and mammalian hosts. Each species has unique aspects in its lifecycle, which are described in the subsequent sections of this text.

3.1 Leishmania *spp.*

During the complex digenetic or heteroxenic lifecycle of *Leishmania* sp., the parasites alternate between elongated promastigotes that have adapted to live in the extracellular space of intestinal cavities of the insect vector and ovoid amastigotes that have adapted to survive in an intracellular habitat inside the mammalian macrophages

Table 1.2 Summary of the currently available treatments and their regimens adopted by the World Health Organization in different countries

Disease	Drugs available	Therapeutic regimen
HAT by *T. gambiense* – stage 1	Pentamidine isethionate	4 mg/kg/day IM or IV (diluted in saline and given in 2 h infusions) × 7 days
HAT by *T. gambiense* – stage 2	NECT (Eflornithine + Nifurtimox)	400 mg/kg/day eflornithine IV in 2 infusions (1 h each) × 7 days + 15 mg/kg/day nifurtimox PO in 3 doses × 10 days
	Eflornithine alone	400 mg/kg/day eflornithine IV in 4 infusions × 14 days
	Melarsoprol	2.2 mg/kg/day melasorprol IV × 10 days (second line treatment)
HAT by *T. rhodesiense* – stage 1	Suramin	Test dose of 4–5 mg/kg suramin (day 1), then 20 mg/kg IV weekly × 5 weeks (maximal dose injection: 1 g)
HAT by *T. rhodesiense* – stage 2	Melarsoprol	2.2 mg/kg/day melarsoprol IV × 10 days or three series of 3.6 mg/kg/day IV × 3 days spaced by intervals of 7 days
Chagas disease – acute phase	Benznidazole	5–7 mg/kg/day orally in two divided doses daily for 60 days for adults; 5–10 mg/kg/day in two divided doses daily for 60 days for infants and children up to 12 years old
	Nifurtimox	8–10 mg/kg/day orally in three divided daily doses ranging from 60 to 90 days for adults; 15–20 mg/kg/day orally in four divided daily doses for 90 days for children
Chagas disease – chronic phase		Specific treatment of the signals and symptoms form the organism in the affected systems
Cutaneous leishmaniasis	Paromomycin	*Worldwide*: Local therapy – 15 % paromomycin/12 % methylbenzethonium chloride ointment twice daily for 20 days; intralesional antimonials, 1–5 ml per session plus cryotherapy (liquid nitrogen: – 195 °C), both every 3–7 days (1–5 sessions); thermotherapy, 1–2 sessions with localized heat (50 °C for 30 s); intralesional antimonials or cryotherapy independently
	Pentavalent antimonials	System therapy: pentavalent antimonials – 20 mg Sb^{5+}/kg/day IM or IV for 20 days; pentamidine isethionate,– IM injections or brief infusions of 4 mg salt/kg/day for 3 doses; amphotericin B deoxycholate – 0.7 mg/kg per day, by infusion, for 25–30 doses; miltefosine – 2.5 mg/kg per day orally for 28 days
	Cryotherapy	
	Thermotherapy	
	Pentamidine isethionate	
	Amphotericin B deoxycholate	
	Miltefosine	

(continued)

Table 1.2 (continued)

Disease	Drugs available	Therapeutic regimen
Mucocutaneous leishmaniasis	Pentavalent antimonials Amphotericin B deoxycholate Liposomal amphotericin B Miltefosine	*The Americas*: pentavalent antimonials – 20 mg/kg/day IM or IV for 30 days; amphotericin B deoxycholate – 0.7–1 mg/kg by infusion every other day up to 25–45 doses; liposomal amphotericin B – 2–3 mg/kg daily by infusion up to a total dose of 40–60 mg/kg; in Bolivia, miltefosine – 2.5–3.3 mg/kg per day orally for 28 days
Diffuse cutaneous leishmaniasis	Pentavalent antimonials	*Africa*, *Asia*, *and Europe*: pentavalent antimonials – 20 mg Sb5+/kg/day IM or IV plus paromomycin, 15 mg (11 mg base)/kg/day intramuscularly for 60 days
	Paromomycin Ketoconazole Miltefosine	*The Americas*: pentavalent antimonials – 20 mg Sb^{5+}/kg/day IM or IV for 20 days; ketoconazole – adult dose, 600 mg oral daily for 28 days; miltefosine – 2.5 mg/kg per day orally for 28 days (B)
Visceral leishmaniasis	Pentavalent antimonials	*Africa*, *Asia*, *and Europe*: Pentavalent antimonials – 20 mg Sb^{5+}/kg/day IM or IV for 30 days; amphotericin B deoxycholate – 0.75–1 mg/kg/day by infusion, daily, or on alternate days, for 15–20 doses; liposomal amphotericin B – 3–5 mg/kg/day by infusion given over 6–10 days up to a total dose of 30 mg/kg
	Amphotericin B deoxycholate	Miltefosine – for children aged 2–11 years, 2.5 mg/kg/day; for people aged ≥12 years and < 25 kg, 50 mg/day; for people 25–50 kg, 100 mg/day; for people > 50 kg, 150 mg/day; orally for 28 days
	Liposomal amphotericin B	Combination: pentavalent antimonials (20 mg Sb^{5+}/kg/day IM or IV) plus paromomycin (15 mg [11 mg base]/kg/day IM) for 17 days
	Miltefosine Pentavalent antimonials + paromomycin	*Worldwide*: Pentavalent antimonials – 20 mg Sb^{5+}/kg/day IM or IV for 30 days; amphotericin B deoxycholate – 1 mg/kg/day by infusion on alternate days for 15–20 doses; liposomal amphotericin B – 1–1.5 mg/kg/day by infusion given over 21 days or 3 mg/kg/day during 10 days
PKDL	Pentavalent antimonials	*Africa*, *Asia*, *and Europe*: East Africa: pentavalent antimonials – 20 mg Sb^{5+}/kg/day IM or IV for 30–60 days; liposomal amphotericin B – 2.5 mg/kg/day by infusion for 20 days
	Liposomal amphotericin B Amphotericin B deoxycholate Miltefosine	*Bangladesh, India, Nepal*: amphotericin B deoxycholate – 1 mg/kg/day by infusion, up to 60–80 doses over 4 months; miltefosine – orally for 12 weeks at dosage

HAT human African trypanosomiasis, *PKDL* post-kala-azar dermal leishmaniasis, *IM* intramuscular, *IV* intravenous, *NECT* Nifurtimox-eflornithine therapy, *PO* per os, *Sb^{5+}* pentavalent antimonial

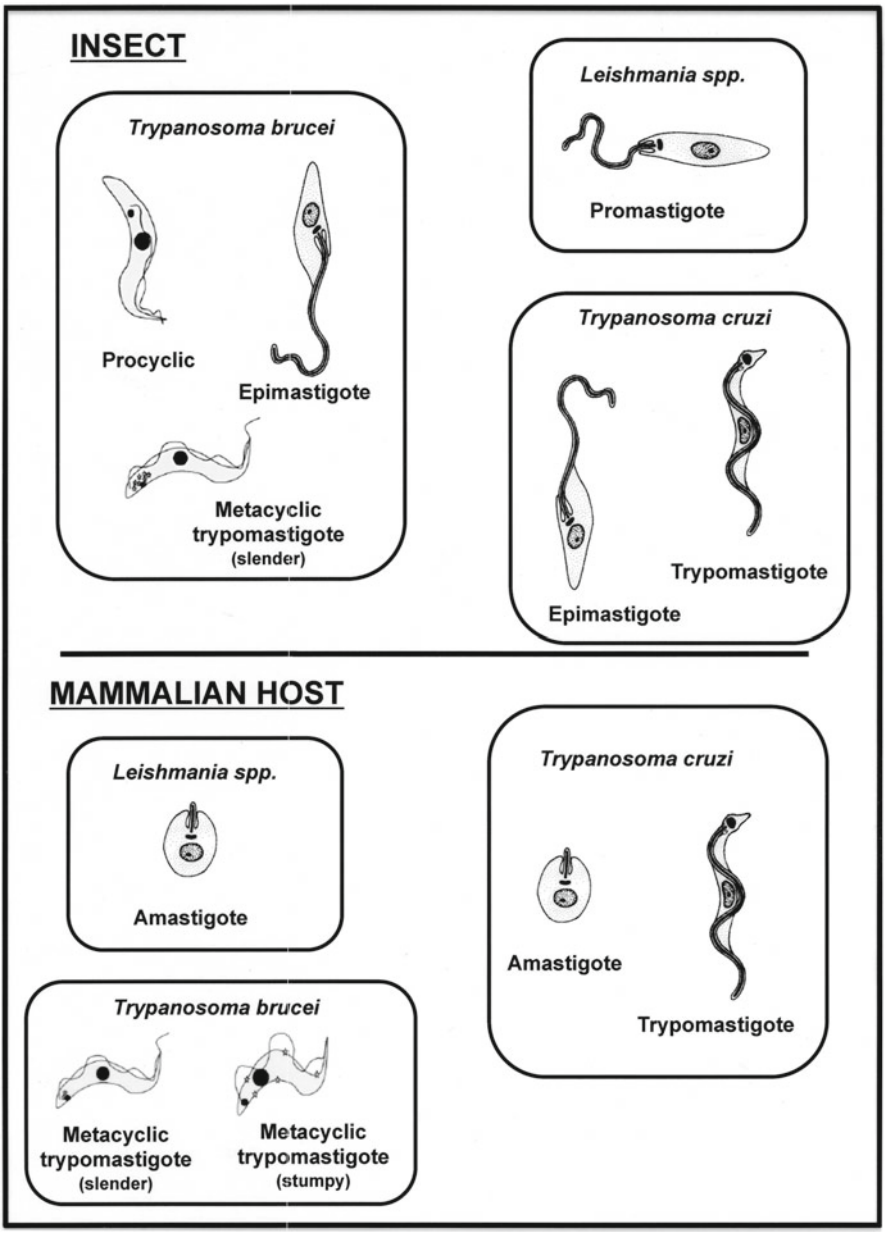

Fig. 1.3 Summary of all developmental stages found in the insect vector and mammalian hosts for the protozoan parasites *Leishmania* sp., *Trypanosoma cruzi*, and *Trypanosoma brucei*

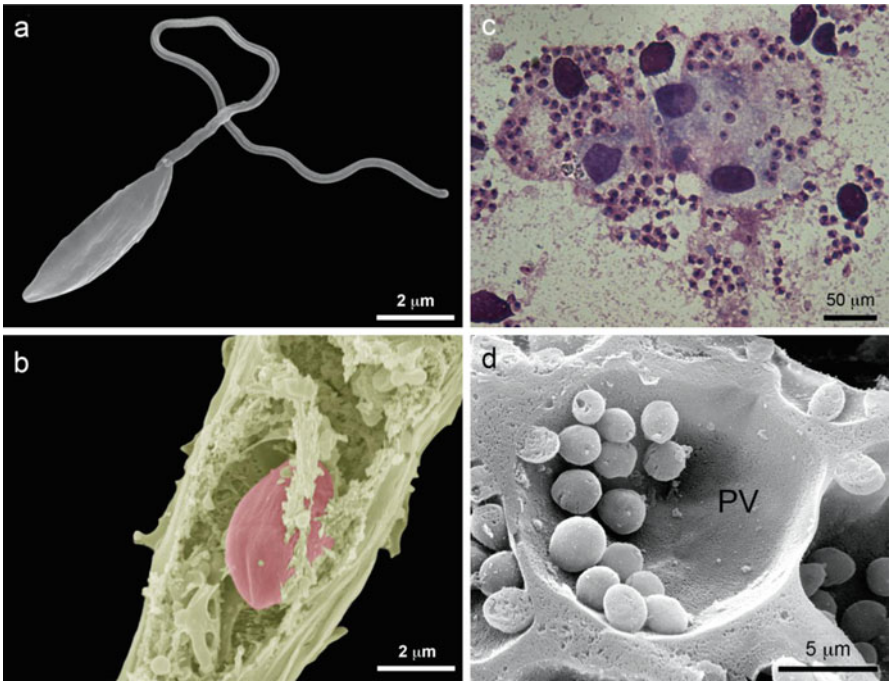

Fig. 1.4 (**a**–**d**) Micrographs of the developmental stages of *L. amazonensis*. (**a**) Extracellular promastigotes; (**b**) murine macrophage infected with intracellular amastigote by scanning electron microscopy; (**c**) light microscopy analysis of skin smears of BALB/c infected tissue stained with Giemsa showing several amastigotes; (**d**) scanning electron microscopy analysis of infected tissue after cleavage in liquid nitrogen to expose the intracellular environment. Note the interaction of amastigotes with the membrane of the parasitophorous vacuole (PV)

(Figs. 1.3 and 1.4). The metacyclic promastigotes, i.e., the infective stage, are inoculated into the host by the sandflies from their proboscis during their blood meals. Due the presence of several glycoconjugates on their surface, these promastigotes adhere to the plasma membrane of macrophages and trigger a phagocytic process (Lodge and Descoteaux 2008). The promastigotes are phagocytized and remain inside the parasitophorous vacuole, a vacuole found in the host cells where most of the protozoan parasites reside and develop during its life cycle. Then, the promastigotes differentiate into amastigotes that live in an acidic pH habitat where they divide by binary fission. After multiple divisions, a large number of amastigotes (Fig. 1.4) leads to the lyses of the macrophages, releasing parasites that can infect new macrophages or be ingested by sandflies during new blood meals. In sandflies, amastigotes transform into procyclic promastigotes in the gut that divide quickly to enter into a differentiation process known as metacyclogenesis. In this process, non-infective forms differentiate into infective metacyclic promastigotes that migrate to the proboscis, thereby starting a new round of infection again (Bates 1994). Note that in the *Viannia* subgenus, the promastigotes develop in the hindgut, while in the *Leishmania* subgenus the metacyclogenesis occurs in the midgut.

3.2 T. cruzi

Several developmental stages make up the digenetic lifecycle of *T. cruzi*; moreover, these stages alternate between blood-sucking triatome insect and hosts (Figs. 1.3 and 1.5). In the peripheral blood of the mammalian host, *T. cruzi* exists as trypomastigotes that exhibit two basic morphologies that are generally described as slender or stumpy. During its blood meal, the insect vector ingests the trypomastigotes, which go to the stomach and undergo differentiation to the spheromastigote form. These non-motile forms are around 3–5 μm in diameter, and, in this habitat, they transform into epimastigotes (Fig. 1.5) that migrate to the midgut; these epimastigotes are the replicative form. Elongated epimastigotes attach to the epithelial intestinal cells as well as to the wax cuticle of the rectum through their long flagella prior to differentiating into the metacyclic trypomastigotes. This differentiation is also called metacyclogenesis. Once differentiated, metacyclic trypomastigotes detach from the intestinal epithelia and migrate to insect rectum to be excreted with feces. This developmental stage is highly infective to a wide range of nucleated mammalian cells. Once in the vertebrate host, the metacyclic trypomastigotes can invade different cells, such as fibroblasts, macrophages, and epithelial cells, through an interaction between the surface glycoconjugates on the parasite and several receptors present on the plasma membrane of the host cell (Tyler and Engman 2001). This stage of mammalian host infection triggers the internalization process by the host cells, starting with the intracellular cycle of *T. cruzi*. Several steps occur in this cycle, including the following: (1) formation of an endocytic compartment known as the parasitophorous vacuole; (2) differentiation of the long and thin trypomastigotes into amastigotes, which have an ovoid shape and a short flagellum (Fig. 1.5); (3) lysis of the parasitophorous vacuole membrane by parasite-secreted enzymes, thus allowing the amastigotes to be released into the cytoplasm that remains in contact with host cells organelles; and (4) transformation of the amastigotes into trypomastigotes that are released into the extracellular space and may thus infect other cells or reach the bloodstream (de Souza et al. 2010).

3.3 T. brucei

The lifecycle of *T. brucei* has four main developmental stages that occur in the tsetse fly of the *Glossina* genus and in the mammalian host, i.e., epimastigotes, procyclic forms, slender metacyclic trypomastigotes, and stumpy metacyclic trypomastigotes (Fig. 1.3). In both *T. b. rhodesiense* and *T. b. gambiense* infections, the lifecycle can start with an infected tsetse fly injecting stumpy metacyclic trypomastigotes in a mammalian host during the blood meal. These parasites enter the lymphatic system and pass to the bloodstream. Inside the host, they transform into proliferative, slender, bloodstream trypomastigotes that go to other parts of the body, thereby reaching other fluids, such as lymph and cerebrospinal fluid, where they multiply

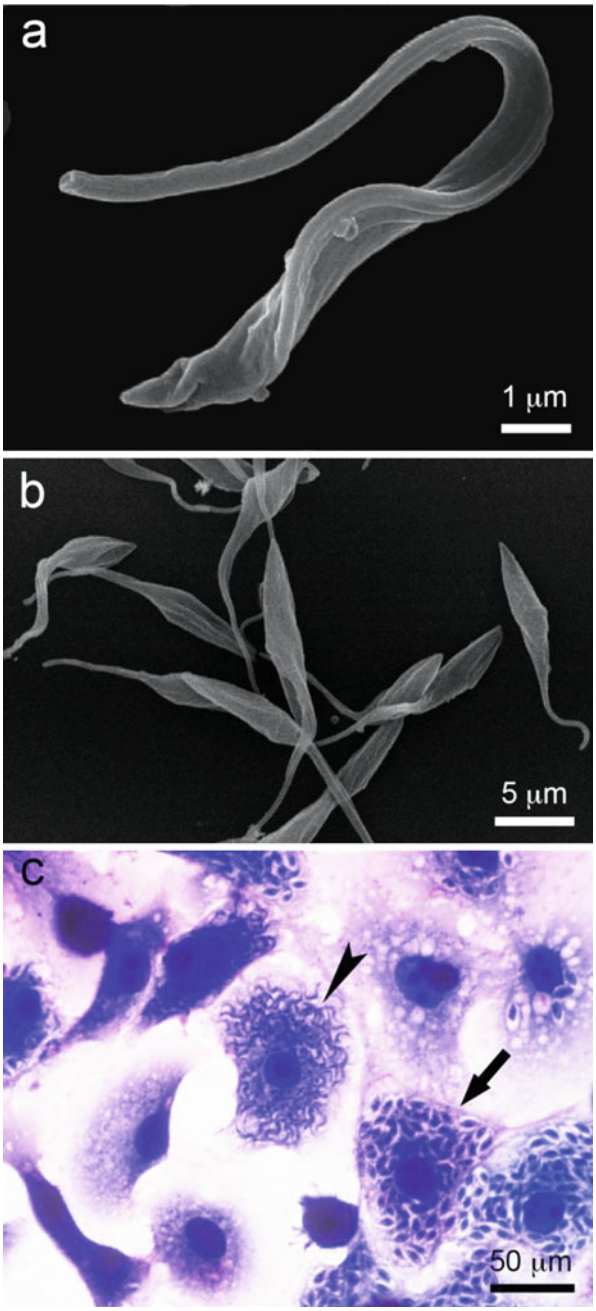

Fig. 1.5 (**a**–**c**) Micrographs of the developmental stages of *T. cruzi*. (**a**, **b**) Scanning electron microscopy analysis of a trypomastigote (**a**) and epimastigotes (**b**). (**c**) Light microscopy analysis of LLC-MK2 infected with amastigotes (*arrow*) and trypomastigotes (*arrowhead*) stained with Giemsa (The images are courtesy of Thiago Luiz de Barros Moreira)

via binary fission. In contrast to other trypanosomatids, the entire life cycle of *T. brucei* in the mammalian hosts occurs in the extracellular space, completing the parasite cycle when tsetse fly ingest bloodstream trypomastigotes from the infected mammal. In the fly's midgut, they transform into proliferative, procyclic trypomastigotes that also multiply via binary fission, leave the midgut, and transform into epimastigotes. In the salivary gland, epimastigotes also continue to multiply and eventually transform into non-proliferative, stumpy, metacyclic trypomastigotes, which are able to infect a new human host and thus continue the cycle. In the tsetse fly, parasite development takes 20–30 days to reach maturation because trypanosomes undergo multiple essential changes in their biology in order to be infective to the mammalian host (Aksoy et al. 2003; Fenn and Mathews 2007). There are some important biological differences between the slender and stumpy forms of the bloodstream trypomastigotes that are essential for the functions of the trypanosomes during the lifecycle. One of these differences occurs in the exposed surface antigens (Barry and McCulloch 2001). These antigenic changes are associated with alterations in the variant surface glycoprotein (VSG) composition with the ultimate loss of VSG. The expression of VSGs in the bloodstream trypomastigotes in the mammalian hosts allows trypanosomes to evade destruction by the host's immune system (Hajduk 1984).

4 Ultrastructure

The protozoan parasites of the Trypanosomatidae family are eukaryotic cells that have a classical ultrastructural organization with some of the same main organelles found in the mammalian host cells. However, they also have special organelles that are absent in other eukaryotic organisms or have features exclusively found in trypanosomatids. These different features also are of interest from the cell biology perspective. Several studies have attempted to better understand their functions and thus provide new information about their general biological significance. These studies have shown that several metabolic pathways take place in these organelles, some of which are completely different from those found in mammalian cells, thereby pointing to the possibility of new chemotherapeutic targets for the development of new drugs that are more selective and less toxic to the infected patients. In this section, we will briefly review the ultrastructural organization of *Leishmania*, *T. cruzi*, and *T. brucei*.

4.1 Plasma Membrane and Cell Surface

In the trypanosomatid parasites, the cell surface can be characterized by the presence of the following three components: the plasma membrane, the glycocalyx, and the subpellicular microtubules. The glycocalyx serves an important function mainly

associated with the interaction between the different developmental stages and the surface of the mammalian host cells and/or the intestinal epithelial cells of the insect vectors.

From a biochemical point of view, the glycocalyx is made up of several integral and peripheral proteins, glycoproteins, and glycolipids; some are glycosylphosphatidylinositol (GPI)-anchored glycoproteins, such as the VSGs of *T. brucei*, the surface mucins of *T. cruzi*, and the complex glycophospholipids (e.g., the lypophosphoglycans (LPG) and glycoinositolphospholipids) of *Leishmania* sp. (de Souza 1989; Ferguson 1997).

Conventional transmission electron microscopy of ultrathin sections is often used to analyze the structural organization of the cell surface (de Souza 2008). High resolution scanning electron microscopy can also be used to better observe the cell surface. Both techniques give a general idea of the thickness and density of the glycocalyx. However, more detailed information of the structural organization of the cell surface can be obtained using the conventional freeze-fracture technique, which allows the exploration of the inner portion of the membranes. In this technique, the hydrophobic portion of the membrane bilayer is cleaved, thereby exposing the inner portions of both the cytoplasmic and the extracellular leaflets; these can then be replicated, and the replicas can be examined in a transmission electron microscope or even in scanning electron microscopy. Generally, a smooth area corresponds with the tail of the phospholipids making up the membrane, and globular structures are designated as intramembranous particles that correspond mainly to integral proteins of the lipid bilayer. For example, replicas of *T. cruzi* epimastigotes showed that the plasma membrane is not homogenous in terms of the density and distribution of intramembranous particles (de Souza 2008).

The plasma membrane of the trypanosomatids is divided into the following three major macrodomains: the cell body, the flagellum, and the flagellar pocket. On the other hand, each macrodomain also contains some specialized regions, such as: (a) the flagellar necklace localized at the basal portion of the flagellum, (b) the attachment zone of the flagellum to the cell body, and (c) the cytostome-cytopharynx complex that is present only in epimastigote and amastigote forms of *T. cruzi* and absent in *Leishmania* and *T. brucei*. The cytostome is a deep invagination of the plasma membrane with a few special microtubules that reach the nuclear region of the parasite. The opening of this complex has a diameter of up to 0.3 μm, which is significantly smaller in the deeper portion called the cytopharynx, thereby adopting the appearance of a funnel (see Sect. 4.8 for more details). Therefore, a specialized region of the membrane lining the parasite starts in the opening of the cytostome and projects towards the flagellar pocket. Via the freeze-fracture technique, we can observe that this region is delimited by a palisade-like array of closely associated particles that correspond to transmembrane proteins, which currently remain unidentified (de Souza et al. 2009). Note that the plasma membrane domain that surrounds the cell body is strongly associated with the subpellicular microtubules via short filaments (see Sect. 4.2 for more details). These filaments also connect the microtubules to one another, thereby creating regular space between them (de Souza 2002).

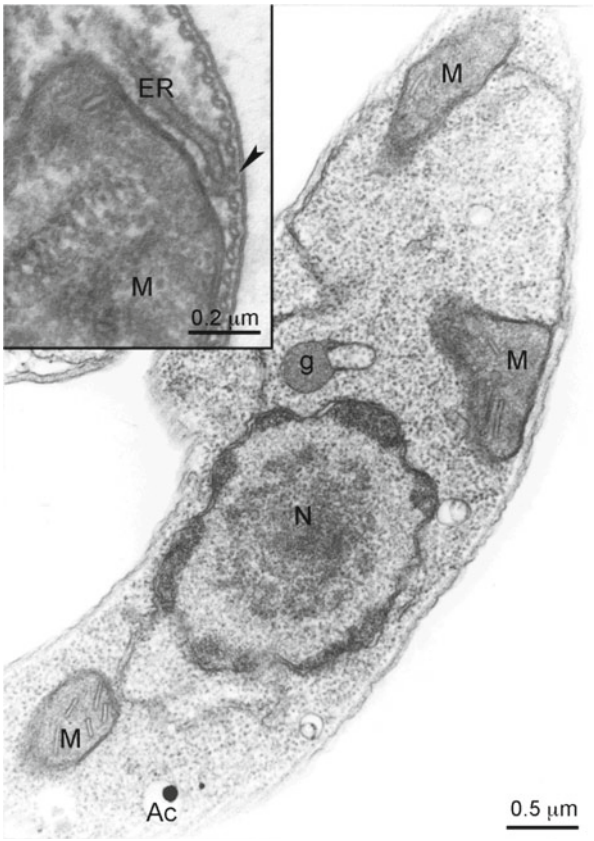

Fig. 1.6 Ultrathin sections of *L. amazonensis* promastigotes showing the close association between the mitochondrion, endoplasmic reticulum, and plasma membrane. At a higher magnification, we can better observe this association, the connection between the microtubules, and their interaction with the endoplasmic reticulum (*arrowhead*). The presence of an acidocalcisome (Ac) can also be observed in this figure. *Ac* acidocalcisome, *ER* endoplasmic reticulum, *g* glycosome, *M* mitochondrion, *N* nucleus (After Lorente et al. 2004)

4.2 Cytoskeleton

Trypanosomatids have special cytoplasmic cytoskeleton structures that maintain the shape of each developmental stage and participate in the motility and attachment of the parasites. Several microscopy techniques have been used to identify the cytoskeleton structure of protozoan parasites, thereby revealing new details of its organization (de Souza and Attias 2010).

The subpellicular microtubules of the trypanosomatids are associated with each other, the plasma membrane of the cell body, and other organelles, especially the endoplasmic reticulum, via thin filaments (i.e., 6 nm in thickness) as shown in Fig. 1.6 depicting *L. amazonensis* promastigotes. These filaments also appear near

the mitochondrion profiles (Fig. 1.6, see arrowhead in the inset). The composition of these filaments has not yet been established. The microtubules contain tyrosinated and acetylated tubulin monomers. Interestingly, they are not present in the domain of the flagellar pocket. Note that several drugs, such as taxol and nocodazole, do not depolymerize them at concentrations that typically depolymerize mammalian microtubules, which indicates that they are very stable.

Biochemical and molecular analyses have shown that actin, myosin, and other actin-related proteins, such as actin-depolymerizing factor (ADF)/cofilin, are present in the different members of Trypanosomatidae family, including *Leishmania* (Sahasrabuddhe et al. 2004; Nayak et al. 2005; Tammana et al. 2008; Batters et al. 2012; Kumar et al. 2012), *T. brucei* (Spitznagel et al. 2010; Dai et al. 2011), and *T. cruzi* (Melo et al. 2008; Cevallos et al. 2011). In addition, investigations of the genome of these protozoan parasites have confirmed these observations; however, typical filaments, i.e., those found in higher eukaryotic organisms, have not been observed. In *Leishmania*, actin appears to be associated with the flagellar structure, the flagellar pocket, the nucleus, the kinetoplast, and the plasma membrane and co-localizes with the subpellicular microtubules (Sahasrabuddhe et al. 2004). In *T. cruzi*, immunofluorescence assays with antibodies against TcActin revealed that actin is located in rounded and punctuated structures that are distributed throughout the cytoplasm (Melo et al. 2008). Another study in *T. cruzi* confirmed this location; however, they also observed labeling along the flagellum with high intensity at the base (i.e., probably where the flagellar pocket is located) and at the tip of the flagellum (Cevallos et al. 2011).

All eukaryotes possess at least one member of the ADF/cofilin family, i.e., a family of actin binding proteins that regulate actin dynamics by increasing the rate of actin treadmilling (Ono 2007) due to the high affinity of actin for ADP over ATP. In addition, ADF is a more efficient in terms of potent actin depolymerization than cofilin, mainly due its ability to sequester actin monomers (Yeoh et al. 2002). ADF/cofilin was recently described in *L. donovani*, and ADF/cofilin mutants failed to depolymerize F-actin, weakly bound to G-actin, and inhibited the exchange of G-actin that was bound to the nucleotide. On the other hand, the overexpression of ADF/cofilin in promastigotes impaired flagellum assembly and consequently hindered cell motility (Kumar et al. 2012).

In *Leishmania*, the presence of coronin that co-localized with actin filaments was seen via immunostaining (Nayak et al. 2005). Different isoforms of the motor proteins of the myosin family were also observed in trypanosomatids. Myosin is part of a superfamily of ATPase mechanoenzymes that are important for several cellular processes related mainly with movement, vesicular transport, contraction and cell division. The genome of *Leishmania* revealed the presence of only two myosin genes, i.e., one class IB myosin and one class XXI myosin (Odronitz and Kollmar 2007). The latter was cloned and showed to promote the gliding of the actin filaments; however, this myosin must bind to a calmodulin protein for this action to take place (Batters et al. 2012). In contrast, *T. brucei* expresses an unusual class I myosin in all developmental stages. In the bloodstream forms, this myosin localizes to the polarized endocytic pathway in the bloodstream forms, however it is distributed throughout the cytoplasm in the procyclic forms (Spitznagel et al. 2010).

4.3 Flagellum

In almost all members and developmental stages of the Trypanosomatidae family (i.e., except intracellular amastigotes) (Gluenz et al. 2010), the flagellum consists of a canonical 9+2 axoneme. In most of the developmental stages, the classical axoneme is connected to the paraflagellar rod (PFR), i.e., a complex paracrystalline array of filaments that is arranged parallel to the axoneme (Vickerman 1962; de Souza and Souto-Padron 1980; Portman and Gull 2010). Interestingly, the PFR seems to be essential for cell motility and viability in some developmental stages. However, species that contain a symbiotic bacterium typically have a rudimentary PFR that is restricted to the initial portion of the flagellum; yet, their motility and function are not altered (Freymuller and Camargo 1981; Sugrue et al. 1988; Fampa et al. 2003). The axoneme and the PFR are connected by a group of filaments with varying thicknesses, and the PFR is composed of a large number of proteins, most of which have not yet been characterized. Structural studies have revealed that the PFR consists of three distinct regions, i.e., the proximal, intermediate, and distal domains, and it is linked to the axonemal doublets numbered 4–7 and the flagellar attachment zone in *T. cruzi* (de Souza and Souto-Padron 1980; Farina et al. 1986). In *Leishmania mexicana*, previous studies revealed that mutations in the PFR1 and/or PFR2 proteins impaired the swimming ability of the promastigotes (Santrich et al. 1997; Maga et al. 1999). In *T. cruzi*, the angle of the distal region changes when the flagellum is bent versus when it is straight (Rocha et al. 2010). Cryo-electron tomography of the intact flagellum in *T. brucei* revealed new features of the flagellum and PFR structures (Höög et al. 2012). Indeed, the flagellum is connected to the cell body via electron-dense extracellular structures that span the flagellar and the cellular membranes; probably, these electron-dense structures are multi-domain proteins that extend to both sides and into the cytoplasm/flagellum. The flagellum originates from the basal body and passes through a large membrane invagination called the flagellar pocket. The basal body has a structure similar to that observed in eukaryotes with nine microtubule triplets. However, in *T. brucei*, electron-dense structures are found inside its triplet microtubules. Moreover, a microtubule quartet was also observed near the flagellar pocket, while a new and undescribed microtubule appears located between the mitochondrion and the basal body. Additionally, the electron tomography revealed that the distal region of the PFR is organized like an orthorhombic crystal (Höög et al. 2012). On the other hand, a recent study showed that the overexpression of ADF/cofilin in *L. donovani* promastigotes interferes with the assembly of the flagellum and the PFR without significantly affecting vesicular trafficking or cell growth (Kumar et al. 2012), thereby indicating that the actin cytoskeleton is also important in the maintenance of the flagellar structure. Furthermore, a proteomic study of *L. donovani* detected the presence of a protein that is a component of the outer dynein arm docking complex called as *Ld*DC (Bente et al. 2003). Dyneim-2 isoforms were also observed in *L. mexicana* (Adhiambo et al. 2005). Mutations in these proteins led to a decreased growth rate, problems in the flagellar assembly, a reduction in the flagellum size, and a

missing PFR; moreover, the morphology of promastigotes appears to be like that of the amastigotes (Adhiambo et al. 2005; Harder et al. 2010).

The flagellum of the trypanosomatids is strongly attached to a significant portion of the cell body via the flagellum attachment zone (FAZ), which has mainly been characterized in *T. brucei* and *T. cruzi*. Microscopy techniques have revealed that the FAZ is a specialized domain of the cell surface (de Souza 1995, 2002). This structure is more evident in trypomastigotes and epimastigotes, where only the distal tip of the flagellum is not attached to the cell body. The FAZ has been well characterized in *T. brucei* and has been described as a complex system with membrane connections, filaments, and specialized microtubules (i.e., the so-called "microtubule quartet" [MQT] that are located in a gap opening into the subpellicular microtubule array) (Sherwin and Gull 1989; Bastin 2000). The MQT is a sub-set of the subpellicular microtubules that are nucleated at the base of the flagellar pocket and are anti-parallel to the other microtubules (Sherwin and Gull 1989; Höög et al. 2012). This gap also contains the "FAZ filament," i.e., an electron-dense fiber that runs parallel to the MQT, and macular structures that are 25 nm in diameter and form the junctional complexes between the cell body and the flagellum (Vickerman 1969; Martinez-Palomo et al. 1976). In *T. cruzi*, cytochemistry studies and the freeze-fracture of this region revealed the presence of many intramembranous particles organized in small patches on both the fracture faces of the trypomastigote flagellar membrane (Martinez-Palomo et al. 1976; de Souza et al. 1978). The FAZ also links the axoneme to the PFR and both of these to the junction of the flagellum and the plasma membrane (Sherwin and Gull 1989). Certain proteins found in the FAZ have been described, such as the surface glycoprotein GP72 described in *T. cruzi* (de Jesus et al. 1993; Rocha et al. 2006) and the homologue FLA1 in *T. brucei* (Nozaki et al. 1996). Silencing of the *fla1* gene by RNAi in *T. brucei* led to a detachment of the flagellum, thereby decreasing cellular viability and leading to failure in cytokinesis (LaCount et al. 2002), while this mutation in *T. cruzi* led to alterations in the location of some proteins of the FAZ that remain concentrated in the flagellar pocket and also in the normal position of organelles, such as the endoplasmic reticulum and mitochondrion (i.e., they normally appear in close association with the FAZ) (Rocha et al. 2006). In promastigotes of *L. mexicana*, the flagellar membrane only makes contact at the region of its emergence from the flagellar pocket, i.e., where the presence of intramembranous particles on the P face of the flagellar membrane is observed (Benchimol and de Souza 1980).

In amastigotes of *L. mexicana*, the flagellum probably has a different structure and axoneme organization (Gluenz et al. 2010). The amastigote flagellum is short (i.e., about 1.5 μm of length) and spans the flagellar pocket; moreover, only a small bulbous tip is exposed to the parasitophorous vacuole environment. In some situations, the tip of the flagellum of *Leishmania* amastigotes is intimately associated with macrophage vacuole membrane. The arrangement of the microtubules along the amastigote axoneme is different with the following organization: (1) nine triplet microtubules in the basal body, (2) a ring of nine doublet microtubules in the transition zone with associated projections connecting to the flagellar membrane, (3) nine doublet microtubules surrounding a basal plate, and (4) nine doublets surrounding a

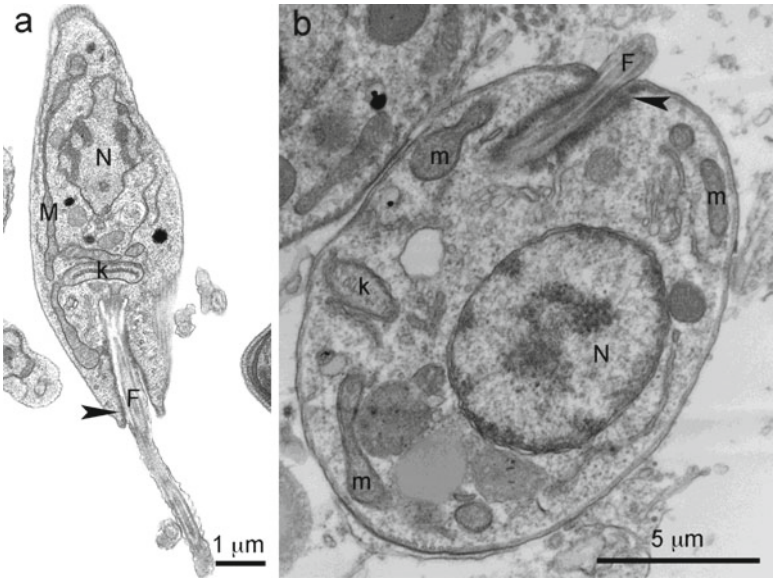

Fig. 1.7 (**a**–**b**) Electron micrographs of *L. amazonensis* promastigotes (**a**) and intracellular amastigotes (**b**) showing the different lengths of the flagellum in these two developmental stages. The promastigote has a long flagellum, whereas the amastigote has a short flagellum. Both stages have a zone of close association between the flagellar and plasma membranes (*arrowhead*) in the anterior region of the cell body. *F* flagellum, *k* kinetoplast, *M* mitochondrion, *N* nucleus ((**a**) After Rodrigues et al. 2008)

central electron-dense core with one or two central singlet microtubules occasionally observed before the neck region. This basal structure is connected to a 9+0 doublet ring by outer dynein arm-like structures. In the distal region of the axoneme, the nine-fold symmetry is broken by the presence of microtubule doublets occupying a more central position; this occurs far from the basal body but near the flagellar pocket neck and its exit. No PFR has been observed in amastigotes. These features indicate that the axoneme structure (9+0) in amastigotes varies significantly and is completely different from those observed in promastigotes (9+2), thus suggesting a structure similar to the primary cilium of vertebrates; indeed, they probably act as sensory organelles serving important functions within the host-parasite interactions and for signaling in the intracellular amastigotes of *Leishmania* (Gluenz et al. 2010).

Figure 1.7a and b presents the flagellar structure of *L. amazonensis* promastigotes (i.e., Fig. 1.7a) and amastigotes (i.e., Fig. 1.7b). These images highlight the differences in length, while Fig. 1.7b allows us to observe a region of close association between the membranes of the flagellum and cell body, probably indicating the presence of a structure similar to the FAZ found in other trypanosomatids as described previously.

4.4 Mitochondrion and Kinetoplast

Electron microscopy and 3D-reconstruction revealed that the trypanosomatid's mitochondrion is unique and ramified throughout the parasite cytoplasm (Paulin 1975). Mitochondria are among the most important organelles in eukaryotic cells and appear in widely varying distributions, numbers, and sizes and different levels of complexity in several organisms (Kakkar and Singh 2007). They are involved in energy production through oxidative phosphorylation, synthesis of key metabolites, and iron-sulfur clusters. In higher eukaryotes, mitochondrial respiration occurs via the electron transport chain (ETC) using the following five integral enzyme complexes present in the inner mitochondrial membrane: *Complex I* – NADH-ubiquinone:oxidoreductase, *Complex II* – succinate-ubiquinone dehydrogenase, *Complex III* – ubiquinol:cytochrome *c* oxidoreductase (or cytochrome *bc*1 complex), *Complex IV* – cytochrome *c* oxidase (COX or cytochrome *a*3 complex), and *Complex V* – F_0F_1-ATP synthase. Moreover, ubiquinone (coenzyme Q) and cytochrome *c* work as electron carriers between the complexes (Chance and Williams 1956). Complexes I, III, and IV work as H^+ pumps, thereby generating a proton-electron chemical gradient that drives the ATP synthesis by complex V (i.e., ATP synthase) and thus couple the processes of respiration and phosphorylation (Schagger 2001). Mitochondria also have several proteins that are involved in programmed cell death, i.e., apoptosis (Kakkar and Singh 2007).

In parasitic protists, mitochondria exhibit wide variations in terms of development (de Souza et al. 2009). The extension of the mitochondrion and the inner mitochondrial membrane organization varies according to the protozoan species and the developmental stages. The mitochondrial metabolism also varies according to the parasites and stages; in fact, some of them do not have a functional mitochondrion, and in these cases, the major source of ATP production is from the glycolitic pathway in the glycosomes (for more detail, see Sect. 4.5). An interesting example of this variation in the mitochondrial metabolism occurs in the ETC of *T. brucei*. Procyclic forms have a complete citric acid cycle and a fully functional respiratory chain (Durieux et al. 1991) with some divergences in the proteins of all complexes as recently observed in a proteomic study of the *T. brucei* mitochondrial respiratome (Acestor et al. 2011). However, the bloodstream trypomastigote is completely different, and its metabolism is based mainly on the glycolitic pathway; indeed, the mitochondrion houses only one complex of the respiratory chain (i.e., glycerol-3-phosphate dehydrogenase) and the salicylhydroxamic acid (SHAM)-sensitive alternative oxidase, also known as Trypanosome Alternative Oxidase (TAO) (Chaudhuri et al. 2006). TAO produces two molecules of ATP from breakdown of 1 molecule of glucose under aerobic conditions; under anaerobic conditions, TAO is inhibited, and the mitochondrial ATP production is reduced by 50 %, thus making the metabolic functions of the glycosome predominant (Clarkson et al. 1989; Sen and Majumder 2008). In *T. cruzi*, the mitochondrial metabolism is also different between epimastigotes and bloodstream trypomastigotes, where the last one exhibits lower oxygen consumption rates and increased H_20_2 production. The trypomastigotes of *T. cruzi*

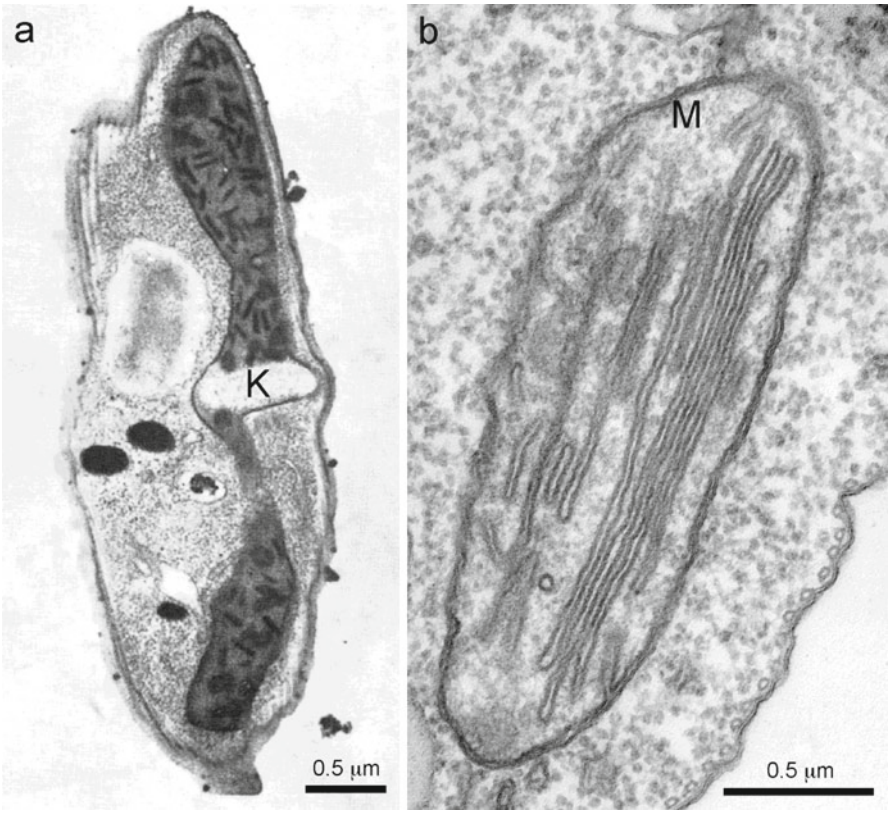

Fig. 1.8 (**a**–**b**) Transmission electron microscopy of *Herpetomonas*, a monoxenic trypanosomatid genus (**a**) and *L. amazonensis* (**b**) showing the ultrastructure of the mitochondrion and their cristae. **Panel a** represents the enzyme cytochemistry to localize the cytochrome oxidase in the mitochondrion, while **Panel b** shows the presence of many cristae. *K* kinetoplast, *M* mitochondrion ((**a**) After de Souza et al. 1977; (**b**) After de Souza et al. 2009)

also have an increased activity of complex II-III and reduced activity of complex IV (Gonçalves et al. 2011). These features of the trypomastigotes could be related with a protection against the burst oxidative induced by the host immune system activation. In trypanosomatids, the mitochondrion is unique and located near the subpellicular microtubules (Figs. 1.6 and 1.7); moreover, it is dilated at the region where the kinetoplast DNA (kDNA) is located (Fig. 1.7a). Depending on the genus and species, the ultrastructure of the mitochondrion can vary; nevertheless, it generally has a dense matrix and thin tubular cristae that are irregularly distributed (Fig. 1.8a). In different situations, a large number of cristae have been observed (Fig. 1.8b). The shape, number of cristae, and volume occupied by the mitochondrion in the cytoplasm can vary depending of the environmental and nutritional resources available. An interesting example of this mitochondrial plasticity can be found in *T. brucei*. Depending on the developmental stage and the environment

(i.e., low or high glucose availability or housed in the insect vector or in the blood of the mammalian host), trypomastigotes (i.e., stumpy or slender) can have mitochondria with different morphologies, volume, number of cristae, and activity (Böhringer and Hecker 1974, 1975). In the midgut and alimentary canal of the insect vector, i.e., where the glucose levels are low, the mitochondria of the stumpy trypomastigotes are large in volume and have many cristae. These features decrease in the metacyclic trypomastigotes located in the salivary gland and reach their lowest values in the slender trypomastigotes found in the blood of the mammalian host, i.e. where the glucose levels are higher. The glycolytic pathway predominates in this developmental stage, and the mitochondrial activity is quite low as compared to the procyclic forms. In protozoan parasites with low mitochondrial activity, the glycosomes predominate, thereby occupying a high volume of the cytoplasm, and this aspect is directly related to the available nutrition source (Clayton and Michels 1996; Tielens and Van Hellemond 1998; Faria-e-Silva et al. 2000).

The initial observation of protozoan parasites in an electron microscope revealed a special region on the mitochondrion that is dilated, where the kinetoplast DNA (kDNA) is located. This special region was named the kinetoplast, and the filamentous structure is the mitochondrial DNA, which is known as kDNA (Meyer 1968). Moreover, the kinetoplast is able to incorporate [^{3}H]-thymidine into the DNA (Steinert et al. 1958), thereby allowing the observation of the ultrastructure during its replication process (Burton and Dusanic 1968; Hill and Anderson 1969). However, the molecular configuration of the kDNA was not completely revealed until the 1970s (Riou and Delain 1979; Shapiro and Englund 1995; Lukes et al. 2002). Light microscopy allows the observation of kDNA using basic dyes that concentrate in the kDNA structure; this technique has been used for many years with the several developmental stages found in members of the Kinetoplastida order and to quantify parameters related to cell division. In the fine structure of the kinetoplast, the DNA strands are aligned parallel to the axis of the network, and the kDNA arrangement varies among species and developmental stages. The size of the entire kinetoplast also varies. For example, in *T. brucei*, the kinetoplast appears as a slightly concave disk that is about 0.6 μm in diameter and 0.1 μm thickness, while in *T. cruzi* epimastigotes, it is 1 μm in diameter with the same thickness and shape (Riou and Delain 1979). In bar-shaped kinetoplasts, the kDNA fibers are tightly packed, forming a compact structure; this type of structure is found in promastigotes, epimastigotes, amastigotes, and procyclic forms (Fig. 1.9b and c). In *T. cruzi* trypomastigotes, the kDNA is more loosely organized and fills the matrix, thereby making the kinetoplast appear rounded (Fig. 1.9a). The kDNA network is a planar structure with different sizes according to the species (Shapiro and Englund 1995). The kDNA represents about 30 % of the total cellular DNA, and it differs from nuclear DNA in several aspects, such as buoyant density, base ratio, and degree of renaturation. Basic proteins, topoisomerases, and histone-like proteins have been identified in the kinetoplast structure and contribute to the condensation and replication of the kDNA (Xu and Ray 1993; Cavalcanti et al. 2004; Motta 2008). Moreover, in contrast to the DNA of other eukaryotes, the kDNA is composed of circular molecules, i.e., minicircles and maxicircles that are

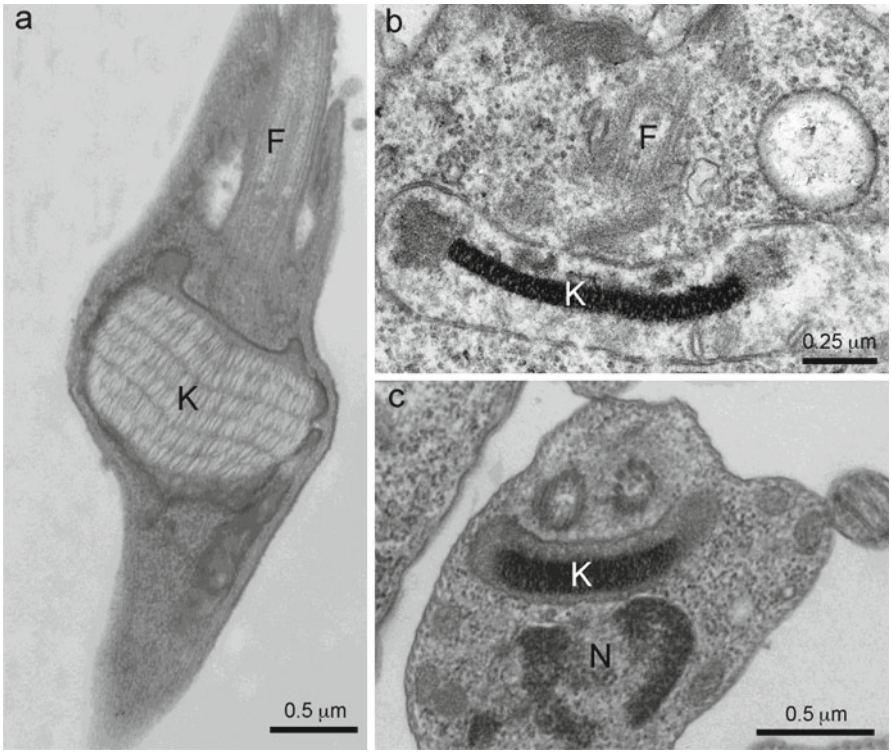

Fig. 1.9 (**a**–**c**) Ultrathin sections of the kinetoplast structure of a *T. cruzi* trypomastigote (**a**), a *L. amazonensis* promastigote (**b**), and a *T. cruzi* epimastigote (**c**). The images reveal the differences between the kDNA compaction in the developmental stages of *Trypanosoma* sp. and *Leishmania*. *F* flagellum, *K* kinetoplast, *N* nucleus ((**a**) Courtesy of Dr. Thais Cristina Souto Padron; (**b**) after de Souza et al. 2009; (**c**) courtesy of Juliana Vidal and Dr. Narcisa Leal da Cunha-e-Silva)

topologically relaxed and interlocked to form a single network. kDNA contains several thousand minicircles at around 0.5–2.5 kb each and a few dozen maxicircles, which usually vary from 20 to 40 kb (Shapiro and Englund 1995; Liu et al. 2005; Liu and Englund 2007). The maxicircles are similar to the mitochondrial DNA in higher eukaryotes, and they encode the rRNA and the subunits of the respiratory complexes. In contrast, the minicircles encode guide RNAs that act in the creation of maxicircle transcripts (Stuart and Panigrahi 2002). Furthermore, transmission electron microscopy revealed that the kDNA is connected to the mitochondrial membrane and to the basal body by cytoplasmic filaments forming a complex structure called tripartite attachment zone [TAC] (Ogbadoyi et al. 2003). This linkage is responsible for the positioning of the mitochondrial genome and its correct segregation during cell division.

4.5 Glycosomes

Glycosomes are membrane-bound cytoplasmic organelles found in protozoan parasites of the Kinetoplastida order. They are similar to organelles that were initially named microbodies and later peroxisomes in higher eukaryotes, and they have been described in the fine structure of trypanosomatids (Vickerman and Tetley 1977; Souto-Padron and de Souza 1982; de Souza 1984; Opperdoes and Coombs 2007). The first characterization of these glycosomes was done in 1977 when Opperdoes and Borst reported that the seven first enzymes of the glycolytic pathway of *T. brucei* were enclosed in an organelle similar to microbodies and suggested designating them as glycosomes. These enzymes are responsible for the conversion of glucose into 3-phosphoglycerate (Opperdoes and Borst 1977). Most of the glycosomes are spherical organelles with a dense granular matrix and involvement in several functions, such as the β-oxidation of fatty acids, fatty acid elongation, carbohydrate metabolism (i.e., glycolysis, the succinate production pathway, gluconeogenesis, and the glycerol pathway), reactions of the hexose-monophosphate pathway, ether-lipid biosynthesis, isoprenoid biosynthesis, purine salvage, pyrimidine and squalene biosynthesis, and reactive oxygen species metabolism (Opperdoes and Szikora 2006; Igoilho-Esteve et al. 2007; Gualdrón-López et al. 2012). The glycosomes do not contain nucleic acids. In addition, as with peroxisomes, some glycosomes contain catalase. Moreover, all enzymes identified to date are involved in energy, carbohydrate, and lipid metabolism as previously described, and they contain PTS1 and PTS2 sequences that are essential for the importation of proteins from the cytoplasm into the organelle (Opperdoes and Szikora 2006).

Glycosomes are spherical organelles with a diameter of about 0.7 μm. They are also characteristically bound by a single membrane and have a homogenous and slightly dense matrix (Fig. 1.10); however, a crystalloid core can be seen in some protozoa, such as *T. brucei* and *L. mexicana*. The number of glycosomes and the volume occupied in the cytoplasm varies according to the species and the developmental stage, and these features are directly related to the energy metabolism as previously mentioned in Sect. 4.4. This was first observed in *T. brucei*. Bloodstream forms have a large number of glycosomes and an active glycolitic pathway, which is different from that of the procyclic forms where the number of glycosomes is smaller and the mitochondrial metabolism is very active (Vickerman and Tetley 1977). Around 230 glycosomes were found in bloodstream forms of *T. brucei* (Opperdoes 1987), whereas approximately 50 glycosomes were found in the other trypanosomatids (Soares and de Souza 1988). A comparison of the area occupied in the cytoplasm by the glycosome versus that of the mitochondrion makes understanding the differences in the energy metabolism easy. Morphometric analysis allows the quantification of the area occupied by glycosomes and mitochondrion in *T. brucei*, thus improving our understanding on the distribution of these organelles in the developmental stages that have two different stages. Glycosomes occupy an

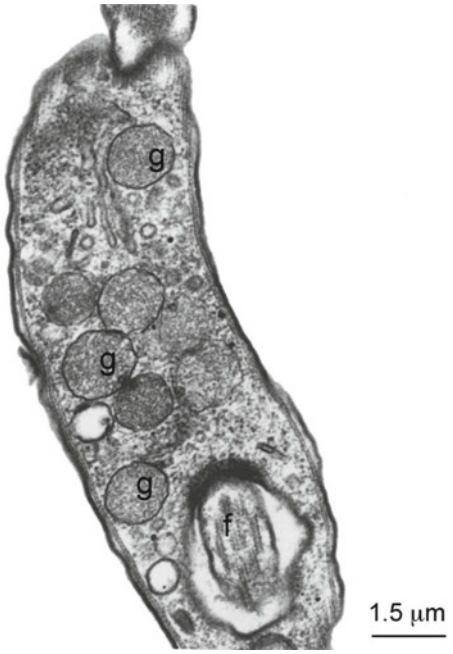

Fig. 1.10 Electron micrograph of glycosomes present in the bloodstream form of *T. brucei*. In this developmental stage, the glycolitic pathway predominates; thus, many glycosomes are frequently observed. The image shows a granular matrix and a single membrane for this round organelle. *f* flagellum, *g* glycosome (After de Souza et al. 2009)

area of about 9.0 % and 2.4 % in bloodstream and procyclic forms, respectively. On the other hand, the mitochondrion occupies an area of 3.3 % and 19.5 %, respectively (Bohringer and Hecker 1975). The differences in the volume occupied by the mitochondrion and the glycosomes have also been observed in cultured forms of *T. cruzi* and *Leishmania* and between symbiotic and aposymbiotic cells of the trypanosomatids harboring an endosymbiont (Brun and Krassner 1976; Bunn et al. 1977; Souto-Padrón et al. 1980; Soares and de Souza 1988).

Autophagy also has been described as an important cellular process in the generation of the different developmental stages during the lifecycle of such protozoan parasites as *Leishmania* and *Trypanosoma* (Brennand et al. 2011; Duszenko et al. 2011). The degradation of old glycosomes by pexophagy and the induction of the biogenesis of new glycosomes with important enzymes for the next stage in the lifecycle could play a crucial role in the survival and efficient adaptation of the parasites to a new host or a new habitat in the host (Gualdrón-López et al. 2012). A morphological analysis has shown the process of pexophagy in trypanosomatids, where sequestration of several glycosomes by the endoplasmic reticulum formed an organelle that resembles an autophagosome (Herman et al. 2008). Certainly, glycosomes and pexophagy play an essential role in the differentiation steps, thus directly linked to the success of the parasitism, mainly for *Leishmania* and *Trypanosoma*.

4.6 Acidocalcisomes

Acidocalcisomes are dense and acidic organelles that contain a high concentration of phosphorous in the form of pyrophosphate and polyphosphate (poly P), calcium, and other cations (e.g., Mg, Na, Zn, and Fe) (Docampo et al. 2005). Different pumps and channels that contribute significantly to its function have been described in the acidocalcisome membranes, such as Ca^{2+}-ATPase, V-H^{+}-ATPase, V-H^{+}-ATPase, Na^{+}-H^{+}-exchanger, Ca^{2+}-H^{+}-exchanger, chloride channels, and aquaporins. In addition, enzymes involved in the pyrophosphate and poly P metabolism are found in the acidocalcisome matrix (Docampo et al. 2005). This organelle was first identified at the beginning of the twentieth century and called metachromatic or volutin granules in different microorganisms. In protozoan parasites, it has received different monikers, including reservoir of metabolic products, pigment bodies, osmiophilic granules, and polyphosphate granules (Anderson and Ellis 1965). A very important study that defined the biochemical and physiological features of the acidocalcisomes took place in 1994; in this study, Vercesi et al. suggested that these organelles contained large amounts of Ca^{2+} due to the participation of the Ca^{2+}-H^{+}-ATPase in its transport and have acidic environments. These two features justify the name "acidocalcisome" for these cytoplasmic vacuoles (Docampo et al. 1995).

Transmission electron microscopy revealed that acidocalcisomes are bound by a single membrane and have an electron-dense matrix. The amount of dense materials varies according to the procedures used to prepare the samples for electron microscopy. In conventionally processed cells (i.e., glutaraldehyde fixation and osmium post-fixation), the matrix content may be removed, thereby leaving a thin dense ring below the membrane (Fig. 1.6). The whole matrix content is better preserved when the samples are fixed using a high-pressure freezing method followed by freeze-substitution, where the luminal content of the acidocalcisomes generally appears completely filled by an electron-dense material (Miranda et al. 2000). However, the best way to observe the acidocalcisomes under electron microscope is via a special preparation where the whole cell is allowed to dry on carbon formvar-coated grids for transmission electron microscopy and then observed using an energy filter in the microscope to obtain the electron microscopic images (Fig. 1.11a) (Miranda et al. 2000). In general, the acidocalcisomes appear to be spherical organelles with an average diameter of 0.2 μm; however, this feature can change according to the species, the developmental stage, and the culture media in which the parasite grows. In *Leishmania*, different culture media modulate the morphology and the chemical composition of the acidocalcisomes (Miranda et al. 2004b). The same feature was also observed for other members of the Trypanosomatidae family where the conditions of cultivation are essential for the modulation of the organelle; i.e., they changed not only the morphology but also the chemical composition (Miranda et al. 2004a). Although typically randomly distributed in some cells, acidocalcisomes are preferentially located near the cell periphery and vary in terms of their proportions of the cell body, i.e., a feature that depends on the species and the developmental stage. However, they sometimes appear in contact with the

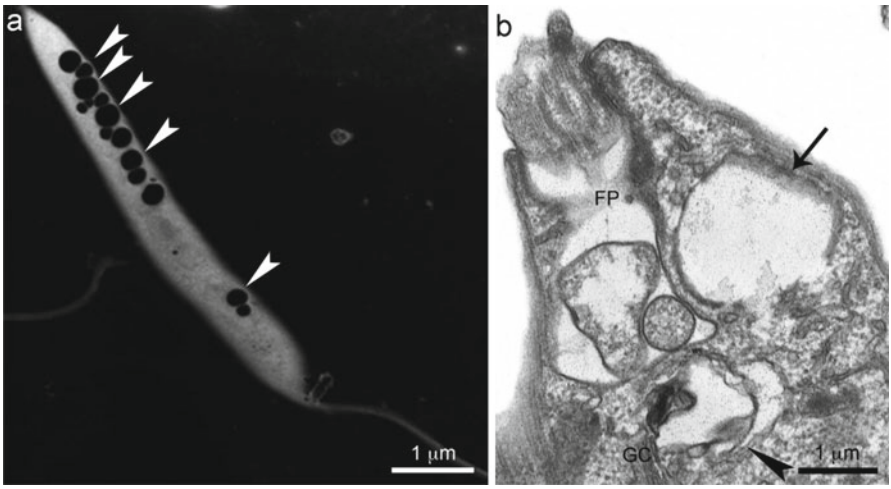

Fig. 1.11 (**a**, **b**) Acidocalcisomes in *L. major* promastigotes (**Panel a**, *arrowheads*) can be visualized using an energy filter with electron microscopy to obtain a spectroscopy image that allows us to observe the matrix without any extraction of its chemical content. **Panel b** shows the presence of a contractile vacuole in the anterior region of the cell body of *L. amazonensis* promastigotes after treatment with ergosterol biosynthesis inhibitors (*arrow*). In this image, it is possible to observe alterations in the Golgi complex induced by the treatment (*arrowhead*). *FP* flagellar pocket, *GC* Golgi complex

subpellicular microtubules, lipid bodies, mitochondrion, nucleus, and contractile vacuole (de Souza 2008). A morphometric study revealed that *T. cruzi* amastigotes have more acidocalcisomes that occupy a larger volume of the cytoplasm than trypomastigotes and epimastigotes in the same species (Miranda et al. 2000).

The function of acidocalcisomes is mainly related to the storage of several cations and phosphorous; however, they are also very important for pyrophosphate and poly P metabolism, calcium homeostasis, maintenance of the intracellular pH homeostasis, and osmoregulation. In fact, they probably collaborate with the contractile vacuole, a structure that will be described in Sect. 4.7 (Docampo et al. 2005).

4.7 Contractile Vacuole

Protozoan parasites of the Trypanosomatidae family contain a contractile vacuole (CV) localized in close association with the flagellar pocket (Fig. 1.11b). Furthermore, a rudimentary spongiome formed by tubules has also been observed in connection with the CV (Girard-Dias et al. 2012). Some data suggest that acidocalcisomes fuse to the CV, thus contributing to the osmoregulation in *T. cruzi* and other protozoan parasites. Aquaporin, a water transporter channel, was also found in acidocalcisomes (Montalvetti et al. 2004). A proteomic analysis of isolated CVs has revealed the presence of several proteins that are involved in many cellular

processes, such as protein and amino acid metabolism, energy metabolism, cell structure and organization, transport of proteins and intracellular traffic, and others. Some proteins, such as V-H^+-ATPase, Rab11, Rab32, phosphate transporter, AP180 (i.e., clathrin coat assembly protein), and vesicle-associated membrane protein (VAMP), are located in the bladder of the CV, while others, such as SNARE2.1, SNARE2.2, and calmodulin, are located on the spongiome; calmodulin was also found in the cytoplasm (Ulrich et al. 2011). These results indicate that the CV is also involved in phosphate metabolism, and the presence of adaptor protein complexes may provide insights into its biogenesis. The majority of the biochemical and physiology studies on the CV have concentrated on *T. cruzi* and CV's response to changes in its environment during its lifecycle (Kollien et al. 2001). Thus, the presence of the CV is essential to the survival and rapid adaptation to differences in osmotic pressure and the ability to tolerate osmotic stress. The CV in *T. cruzi* and other parasites contributes to regulatory volume decreases under hyposmotic stress (Rohloff et al. 2004; Montalvetti et al. 2004; Rohloff and Docampo 2008). The CV also contributes to the regulation of Ca^{2+} homeostasis (Moniakis et al. 1999; Malchow et al. 2006) and the transport of proteins to the plasma membrane (Sesaki et al. 1997). In *T. cruzi*, the CV also has a polyamine transporter that is probably responsible for the transference of polyamines to the plasma membrane as evidenced in the study in which parasites were incubated in a culture medium deficient in polyamines (Hasne et al. 2010). Several studies have proposed a theoretical mechanism for the regulatory volume decrease in hyposmotic environments based on certain observations (Rohloff et al. 2004; Docampo et al. 2005; Rohloff and Docampo 2008; Schoijet et al. 2011). During stress, *T. cruzi* undergoes a significant increase in cAMP concentration that stimulates the translocation of TcAQP1 to the CV after fusion with acidocalcisomes (Rohloff et al. 2004). The hydrolysis of polyphosphate osmotically drives water from the cytosol into the CV, and this process finishes with the hydrolysis of cAMP by the CV-localized TcPDEC (cyclicAMP phosphodiesterease C) (Rohloff and Docampo 2008; Schoijet et al. 2011). On the other hand, when *T. cruzi* was exposed to hyperosmotic stress, the CV was also identified as an important structure essential for recovery from cell shrinking. In this condition, the function of aquaporin, amino acid accumulation, polyphosphate synthesis, and the up-regulation of genes encoding *trans*-sialidase-like and ribosomal proteins are essential for the process of volume recovery (Li et al. 2011).

4.8 Endocytic Pathway

Like other eukaryotic cells, protozoan parasites of the Trypanosomatidae family are able to ingest macromolecules from the medium through the endocytic pathway, which is essential for the survival of all cells. In general, *Leishmania*, *T. brucei*, and *T. cruzi* use the flagellar pocket for endocytosis and exocytosis (Fig. 1.11); however, *T. cruzi* also has the cytostome-cytopharynx complex (Fig. 1.12a) as described in Sect. 4.1. These protozoan parasites have specific receptors that are involved in the

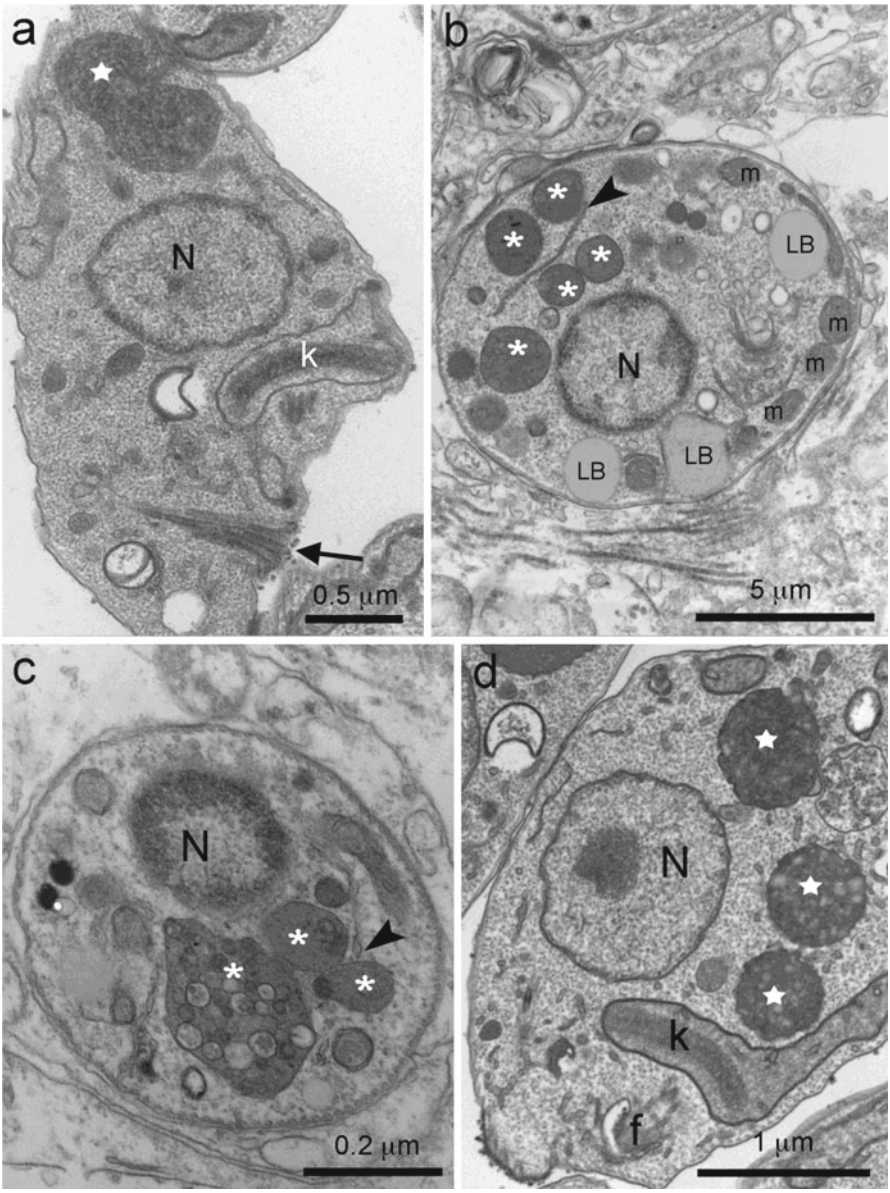

Fig. 1.12 (**a**–**d**) Ultrathin sections of *T. cruzi* epimastigotes (**a**, **d**) and *L. amazonensis* intracellular amastigotes (**b**, **c**) showing the presence of structures and organelles involved in endocytosis. **Panel a** shows the presence of the cytostome-cytopharynx complex (*arrow*) and a huge reservosome in the posterior region of the cell body (*white star*). In **Panels b and c**, it can be seen that amastigotes of *L. amazonensis* present an organelle bounded by a single membrane called megasome (*asterisks*). The size of the megasomes can vary significantly; i.e., sometimes they appear smaller (**b**) or as huge organelles containing internal vesicles (**c**). In **Panel b**, we can observe the presence of lipid bodies in the cytoplasm. **Panel d** shows three reservosomes found in epimastigotes of *T. cruzi* (*white stars*). *k* kinetoplast, *LB* lipid body, *m* mitochondrion, *N* nucleus ((**a**, **d**) Courtesy of Juliana Vida and Dr. Narcisa Leal da Cunha-e-Silva)

endocytosis of such molecules as transferrin and low-density lipoproteins (LDL) (McConville et al. 2002; Field and Carrington 2004). *T. cruzi* is also able to carry out fluid-phase pinocytosis (Figueiredo and Soares 2000). After ingestion, the cargo molecule is delivered to early endosomes, late endosomes, and lysosomes following the same patterns that occur in mammalian cells.

Interestingly, several similarities can be identified in these protozoan parasites; however, a divergence in their structural organization has been described (de Souza et al. 2009). In *T. brucei*, all the endocytic and exocytic machinery are concentrated in the posterior region of the cell body; in contrast, the flagellar pocket and cytostome are located at the anterior region of *T. cruzi*, and the organelles involved in the digestion of the cargo molecules are situated in the posterior region. *Leishmania* does not have a conventional system that acts in the endocytic pathway; instead, it has a long endosomal/lysosomal, multivesicular tubule that spans from the anterior region of the cell body, i.e., where the flagellar pocket is located, to the posterior region (Weise et al. 2000; Waller and McConville 2002).

The promastigotes of *Leishmania* contain unique structures, including (i) multivesicular bodies, which are electron lucent and membrane-bound organelles with a diameter of approximately 0.3 μm and filled with several vesicles (45 nm); (ii) tubular structures, which are clusters of regular tubules with a diameter of 0.6 μm located near the Golgi complex and flagellar pocket; and (iii) a multivesicular tubule, which is a large membrane-bound tubule containing several inner vesicles of heterogeneous sizes and morphology and has a diameter of around 0.2 μm (de Souza et al. 2009). On the other hand, amastigotes of *Leishmania* have an interesting lysosome-like organelle known as a megasome, which is the last organelle participating in the endocytic route. The megasomes were first described in the *L. mexicana* complex (Alexander and Vickerman 1975), and they have also been observed in *L. chagasi* (Alberio et al. 2004). They are membrane-bound, electron-dense organelles that vary in size and morphology (Fig. 1.12b and c). Sometimes they are similar in size to the nucleus; thus, they are gigantic, thereby justifying this name. Another interesting feature of the megasome is the presence of small vesicles in its matrix (Fig. 1.12c) and a high expression of cysteine protease, which is considered the molecular marker of the megasomes.

Much information about endocytosis in *T. brucei* has been gathered. The endocytic pathway in *T. brucei* involves a complex of structures that are highly polarized; moreover, all of the endocytic and secretory machinery is confined in the posterior region of the cell body, and this location is probably related to the necessity of rapid degradation and recycling for parasite survival in the host blood (de Souza et al. 2009). Endocytosis begins in the flagellar pocket that is located in the anterior region of the cell body and the cargo is completely internalized in 2 min. The cargo molecule is delivered to the early endosomes, which is a system of cisternae and tubules. Clathrin-coated vesicles (i.e., class I and class II) have been identified by electron microscopy (Grunfelder et al. 2003), and ablation of clathrin is lethal for the parasite; thus, these clathrin-coated vesicles are essential for parasite survival (Allen et al. 2003). Five minutes after uptake of the cargo, the material can be observed in the vesicles, cisternal structures, and lysosomes (Webster 1989). The major surface protein of *T. brucei*, i.e., the GPI-anchored VSG, has been extensively used to investigate endocytosis and the recycling route (Overath and

Engstler 2004). The VSG is rapidly endocytosed through the flagellar pocket and accumulates in the class I clathrin-coated vesicles. These vesicles bud off from recycling endosomes and fuse with late endosomes. Finally, they can fuse with lysosomes or can enter via a distinct and slower route toward the recycling endosomes, which can give rise to exocytic vesicles that fuse with the flagellar pocket, thereby returning VSGs to the parasite's surface. Many members of the superfamily of Rab small GTPases are involved in these processes (de Souza et al. 2009). The *T. brucei* lysosomes are unique rounded organelles in the posterior region of the cell body and sometimes present as a cluster of organelles (Webster 1989). Like other lysosomes, they also have a molecular marker, i.e., type I glycoprotein p67, which is structurally similar to mammalian lysosome-associated membrane proteins (LAMPs, the lysosomal markers for mammalian lysosomes). Ablation of p67 using the RNAi technique induces significant alterations in the lysosome morphology (Peck et al. 2008).

In *T. cruzi*, the endocytosis pathway has only been described in epimastigotes and appears to be low or absent in trypomastigotes and intracellular amastigote forms. However, more recently, Sant'Anna et al. (2008a) found the presence of lysosome-related organelles in trypomastigotes and intracellular amastigotes. These organelles concentrated cruzipain, chagasin, and a serine carboxypeptidase and are acidic compartments. On the other hand, *T. cruzi* epimastigotes, which typically demonstrate intense endocytic activity, have several reservosomes in the cytoplasm that act as big compartments with similar function to that of mammalian lysosomes (de Souza et al. 2009). Reservosomes are spherical organelles surrounded by a single membrane with a mean diameter of 0.7 μm, and their morphology can vary according to growth conditions and strains (Fig. 1.12a and d). The reservosome matrix is more electron-dense than the cytosol and mainly consists of proteins and electron lucent lipid inclusions (Soares and de Souza 1988). They were first described as multivesicular bodies due to the presence of small vesicles revealed by incubation with peroxidase (de Souza et al. 1978). The uptake of cargo molecules occurs mainly throughout the cytostome but also in the flagellar pocket of epimastigotes. The cargo molecules are then delivered via early tubular endosomes to the reservosomes, which are located in the posterior region of the cell body (Soares and de Souza 1991; Porto-Carreiro et al. 2000). 3D- reconstruction of *T. cruzi* epimastigotes revealed that before reaching the reservosomes, the cargo molecules travel along an intricated and branched network formed by vesicles and tubules that are interconnected; additionally, this vesicular-tubular network is also an acidic compartment (Porto-Carreiro et al. 2000). Note that cruzipain, i.e., a major cysteine protease synthesized in the endoplasmic reticulum and processed in the Golgi complex, is also transported via cytoplasmic vesicles to the reservosomes (Souto-Padron et al. 1990). In addition, chagasin, a natural inhibitor of cruzipain, was also found in the reservosomes (Santos et al. 2005). The acidic nature of these compartments was demonstrated using the DAMP [N-(3[2,4-dinitrophenyl amino] propyl)-N-(3-aminopropyl)methylamine] technique, and the pH was determined to be 6.0, which is similar to that found in the late endosomes of mammalian cells. This acidic pH is maintained by the presence of a P-type H^+-ATPase (Vieira et al. 2005). Reservosomes also have an arsenal of proteins from the Rab GTPase superfamily (Cunha-e-Silva

et al. 2006; de Souza et al. 2009). A recent study on the morphology of *T. cruzi* reservosomes revealed new insights about its ultrastructure (Sant'Anna et al. 2008b). The researchers observed the presence of internal small vesicles and the presence of inner membranes that are similar to the boundary membrane. In addition, the endocytosed cargo does not appear associated with the internal vesicles and reaches the reservosomes in vesicles that fuse with its boundary membrane. Presence of electron lucent bodies with a saturated core surrounded by a monolayer membrane that has an unusual rectangular shape was also found (Sant'Anna et al. 2008b). A proteomic analysis of isolated reservosomes revealed the presence of 456 proteins with predicted function and 253 hypothetical proteins (Sant'Anna et al. 2009). This protein collection is related to various cellular processes, such as protein metabolism, endosomal/lysosomal membrane proteins, vesicular traffic, pumps and channel proteins, cell surface proteins, lipid metabolism, carbohydrate metabolism, cell signaling, cytoskeleton-associated proteins, and others (Sant'Anna et al. 2009). On the other hand, the lipid composition of the isolated reservosomes was also examined via mass spectrometry. The obtained results revealed that the lipid inclusions inside the organelle are mainly composed of cholesterol and cholesterol esters, and this composition can be modulated according to the fetal bovine serum concentration used in the culture medium (Pereira et al. 2011).

5 Conclusions

Knowledge of the epidemiology, lifecycle, and ultrastructure of the organelles of *Leishmania*, *T. brucei*, and *T. cruzi* is essential for a better understanding of the metabolism, physiology, host cell-interaction, behavior inside the vertebrate host, and other features of these parasites. This knowledge is important for the identification of potential enzymes or metabolic pathways that could be the target for the development of new chemotherapeutic drugs.

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Chapter 2
Selection of Molecular Targets for Drug Development Against Trypanosomatids

Despina Smirlis and Milena Botelho Pereira Soares

Abstract Trypanosomatid parasites are a group of flagellated protozoa that includes the genera *Leishmania* and *Trypanosoma*, which are the causative agents of diseases (leishmaniases, sleeping sickness and Chagas disease) that cause considerable morbidity and mortality, affecting more than 27 million people worldwide. Today no effective vaccines for the prevention of these diseases exist, whereas current chemotherapy is ineffective, mainly due to toxic side effects of current drugs and to the emergence of drug resistance and lack of cost effectiveness. For these reasons, rational drug design and the search of good candidate drug targets is of prime importance. The search for drug targets requires a multidisciplinary approach. To this end, the completion of the genome project of many trypanosomatid species gives a vast amount of new information that can be exploited for the identification of good drug candidates with a prediction of "druggability" and divergence from mammalian host proteins. In addition, an important aspect in the search for good drug targets is the "target identification" and evaluation in a biological pathway, as well as the essentiality of the gene in the mammalian stage of the parasite, which is provided by basic research and genetic and proteomic approaches. In this chapter we will discuss how these bioinformatic tools and experimental evaluations can be integrated for the selection of candidate drug targets, and give examples of metabolic and signaling pathways in the parasitic protozoa that can be exploited for rational drug design.

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Abbreviations

AK	Adenosine kinase
ALD	Fructose 1,6 aldolase
APRT	Adenine phopsphoribosyltransferase
CatB	Cathepsin B
CDK	Cyclin dependent kinase
CNS	Central Nervous System
CPA	Cysteine proteinase A
CPB	Cysteine proteinase B
CRK	cdc2 related kinase
CYC	Cyclin
CYP51	Cytochrome P-450 51
DHFR	Dihydrofolate reductase
ECK1	ERK-like, CRK-like Kinase-1
ENO	Enolase
G3DPH	Glycerol-3-phosphate dehydrogenase
GD3DPH	Glyceraldehyde 3-phosphate dehydrogenase
GK	Glycerol kinase
GSH	Glutathione
GSK-3	Glycogen synthase kinase 3
GSpS	Glutathionylspermidine
HAT	Human African trypanosomiasis
HGPRT	Hypoxanthine guanine phopsphoribosyltransferase
HK	Hexokinase
kDNA	Kinetoplast DNA
MPK	Mitogen activated kinase
PFK	Phosphofructose kinase
PGI	Phosphoglucose isomerase
PGK	Phosphoglycerate kinase
PGM	Phosphoglycerate mutase
PK	Protein kinase
POS	Posaconazole
PTR	Pteridine reductase
PYK	Pyruvate kinase
SMT	$\Delta^{24(15)}$-sterol methyltransferase
SQS	Squalene synthatase
STE7	Signaling terminal 7 extension
TPI	Triose phosphate isomerase
TryR	Trypanothione reductase
TryS	Trypanothione synthatase
TS	Thymidylate synthase
TXN	Tryparedoxin
WHO	World Health Organization
XPRT	Xanthine phopsphoribosyltransferase

1 Introduction

Trypanosomatid protozoan parasites of the genera *Trypanosoma* and *Leshmania* are infectious agents of important diseases, such as human African trypanosomiasis (HAT or sleeping sickness), Chagas disease and leishmaniases (cutaneous, mucocutaneous and visceral). According to the World Health Organization (WHO), the leishmaniases are endemic in 88 countries, with 350 million people being at risk of acquiring the infection, sleeping sickness occurs in sub-Saharan Africa and threatens more than 60 million people and Chagas disease is prevalent in Latin America and more than 25 million people are at risk of acquiring the infection. Together, these three parasitic diseases represent a huge social and economic burden since approximately 0.5 million people are infected with *T. brucei*, ten million with *T. cruzi* and an estimated 12 million with different species of *Leishmania* (Teixeira et al. 2012), and cause approximately 150,000 deaths annually (Nussbaum et al. 2010).

Since there are no human vaccines yet, the only treatment for these diseases relies solely on chemotherapeutic drugs. For HAT, the first-line treatment for second-stage cases is melarsoprol, a toxic drug in use since 1949. Increase in drug resistance and high therapeutic failure rates have been reported recently in several foci (Robays et al. 2008). Eflornithine is an alternative treatment that is better tolerated, but difficult to administer (Burri and Brun 2003). Nifurtimox, an orally administered cheap drug, has many side effects and is not yet fully validated for use in HAT (Legros et al. 2002). For the treatment of Chagas disease there are two drugs available, benznidazole and nifurtimox, which are highly toxic and ineffective in the chronic phase of infection (Castro and Diaz de Toranzo 1988). For the treatment of leishmaniases, the mostly widely prescribed drug discovered, the pentavalent antimony, was discovered almost a century ago and has various limitations, including serious side effects, prolonged course of treatment and emergence of drug resistance (Croft et al. 2006). Although newer treatments for leishmaniases exist, they are not optimal (Singh et al. 2012). Overall, current chemotherapy for trypanosomatid parasitic diseases has the pre-mentioned serious limitations, including high toxicity, low efficacy, high cost and increasing parasitic resistance. Therefore there is an urgent need to discover new drugs for the treatment of these devastating diseases.

There are several strategies for expanding the repertoire of new antiparasitic agents. Amongst these, one of the most attractive is targeted drug discovery, because in general it requires less investment to develop a molecule against the target. In addition, targeted drug discovery allows lead optimization, which is often essential to make the drug more effective and safer. In this context, identifying a suitable potential target is the first and most important step towards targeted drug discovery.

2 Essential and Desirable Criteria for the Selection of a Trypanosomatid-Specific Drug Target

A good drug target has to meet different criteria. Amongst these criteria some are essential, whereas others are desirable (Fig. 2.1). One of the first essential criteria for drug target selection is its ability to be disease-modifying. For trypanosomatid parasites this means that the target must be essential for the viability of the parasite or its inhibition affects significantly the virulence of the parasite. In this context, it is necessary to select a target that participates in these processes in the "mammalian" forms of the parasite (intracellular amastigote for *Leishmania*, bloodstream form for *T. brucei* and trypomastigotes and amastigotes for *T. cruzi*). Another criterion that is essential for drug target selection is its "druggability", i.e., the likelihood of small molecule drugs being able to modulate the target. In addition, ideally the target must be either found solely in the parasite, or have differences in the drug binding region/active site that can be exploited for inhibiting more potently the parasitic enzyme, thus allowing the identification of compounds with selective toxicity. The identification of targets for achieving parasite/host selectivity is a less difficult task, as

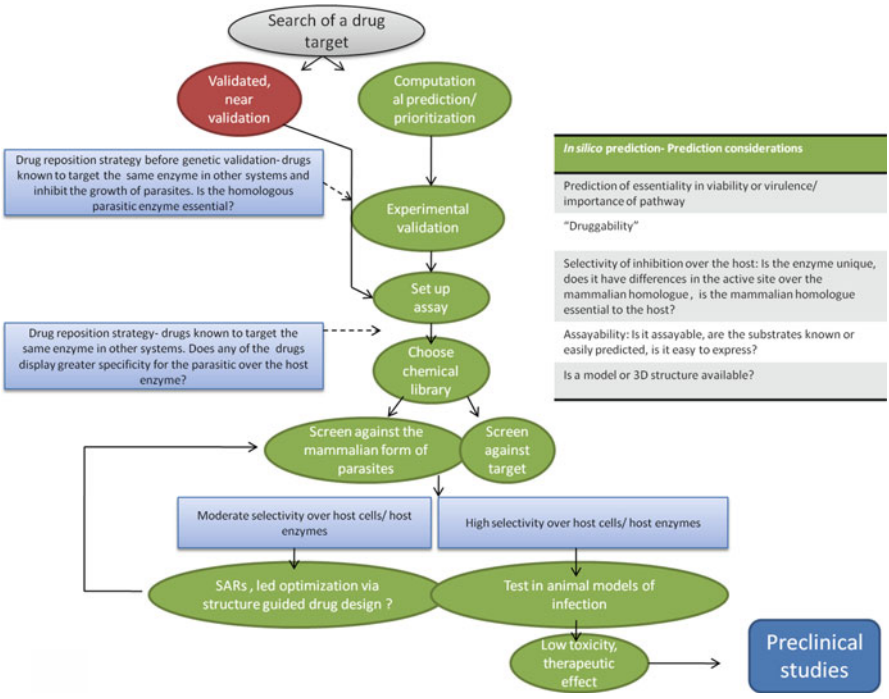

Fig. 2.1 Considerations for the selection of a trypanosomatid-specific molecular target for drug development and representation of procedures required until a drug reaches preclinical trials

potential molecular targets that are essential for the parasite could be redundant and thus not essential in mammalian cells. Moreover, an assayable target is required for performing drug screening. A simple assay that allows high throughput screening is a desirable criterion for drug target selection, facilitating the screening of a large number of compounds. The necessity of an active recombinant protein to perform screening assays has to be taken into consideration and a desirable criterion would be the feasibility to express the protein in its active form. High molecular weight proteins, or proteins that have transmembrane domains might prove difficult to express in an active form. Furthermore, it is useful if a drug target is found in more than one trypanosomatid species and also shares a function that can be disease-modifying in all trypanosomatid pathogens. Finally the presence of an available three-dimensional structure or a model is useful for drug target selection, as it opens possibilities for the development of structure-activity relationship studies (SARs) for lead optimization and structure-guided drug design.

3 Computational Approaches that Aid Drug Target Prioritization

Prerequisite for the identification of a new drug target is its experimental validation. This process is expensive and time consuming. Thus for the selection of a new drug target, an *in silico* approach is often essential. To this end, the completion of the genome project of trypanosomatid pathogens (http://www.genedb.org), including *Leishmania* (*L. infantum, L. major* and *L. braziliensis*) and *Trypanosoma* (*T. brucei* and *T. cruzi*) (Berriman et al. 2005; El-Sayed et al. 2005a, b; Ivens et al. 2005) has opened new possibilities for guided drug discovery. These parasites encode from 8,300 to 12,000 protein genes, of which 6,500 are common to *L. major*, *T. brucei* and *T. cruzi* genomes. In addition, another database, the TriTrypDB (Aslett et al. 2010) (http://tritrypdb.org/tritrypdb/), allows the integration of datasets from *L. braziliensis*, *L. infantum*, *L. major*, *L. tarentolae*, *T. brucei* and *T. cruzi* and enables a user to construct complex queries combining multiple data types. The TriTrypDB database gives information on individual genes or chromosomal spans in their genomic context, including syntenic alignments with other kinetoplastid organisms. The TriTrypDB database has allowed *in silico* metabolic pathway analysis using genome information (Alves-Ferreira et al. 2009). An inventory of predicted metabolic components and integrated metabolic networks of TriTryp is collected in publicly available databases, and a comprehensive review of many of these databases is provided in detail by Myler (Myler 2008). Amongst these, the database BRENDA (Braunschweig Enzyme Database, http://www.brenda-enzymes.org/), contains a plethora of metabolic enzymes that belong to more than 10,000 different organisms, including entries from *T. brucei*, *T. cruzi* and *L. major*. This database includes Enzyme Commission classification, function (including pathways, inhibitors, reaction types and substrates) and structure (including 3D structures), isolation and stability and links to bibliographic references. Another database,

the KEGG (Kyoto Encyclopedia of Genes and genomes, http://www.genome.jp/kegg/), is dedicated to the understanding of high-level function utilities and integrates information and maps of metabolic pathways and associated human diseases, including infectious, and prescription drugs. In addition, it contains enzymatic families and reactions from 2,185 organisms including five trypanosomatids (*L. infantum*, *L. major*, *L. braziliensis*, *T. cruzi* and *T. brucei*). LeishCyc is a pathway/genome database (http://biocyc.org/LEISH/organism-summary?object=LEISH) that captures information about *Leishmania* metabolic pathways from genome annotation and literature resources and organizes this information into a structured database supported by a publicly available ontology. It contains 1,027 enzymes, 573 compounds and 143 metabolic pathways and allows the analysis and visualization of *Leishmania* high-throughput (metabolic 'omics' data), including metabolomics and proteomics (Doyle et al. 2009). In addition, pathway tools available in this database allow the identification of reactions that consume unique substrates or participate in unique reactions, the so-called network "chokepoints" (Doyle et al. 2009), which can be particularly useful for drug target selection.

In silico drug target prioritization for major tropical disease pathogens, including the trypanosomatids *L. major*, *T. brucei* and *T. cruzi*, is now possible and can be implemented by TDRtargets.org (http://tdrtargets.org, Crowther et al. 2010). In TDRtargets.org each criterion is assigned a subjective value and targets earn points of each criterion they meet, in a flexible ranking system that allows BOOLEAN intersection of criteria. The criteria used for drug target selection include the availability of structure (PDB structures or ModBased models), phylogeny (for the selection of unique proteins not present in the host), essentiality, druggability, assayabilty (information regarding the availability of recombinant protein) and specific criteria that are applicable in some species, like location, pathway, expression in distinct morphological stages, phenotype and others (Crowther et al. 2010). In addition, the database is not only restricted to drug targets, but also allows the search for compounds (Crowther et al. 2010). Although computational searches may save money and time for drug target identification, experimental validation is always required. The knowledge of important parasitic pathways for virulence or survival will aid this step. Several of these pathways and drug targets are already validated and herein some examples of these are listed.

4 Examples of Putative or Validated Drug Targets in Parasitic Metabolic and Signaling Pathways

Metabolic pathways in trypanosomatids have peculiarities that may be exploited for targeted drug discovery. Since excellent descriptions of these pathways are present (Lakhdar-Ghazal et al. 2002; de Souza and Rodriguez, 2009; Flohe, 2012), herein we will focus only specific points and data that describe validated or potential trypanosomatid drug targets.

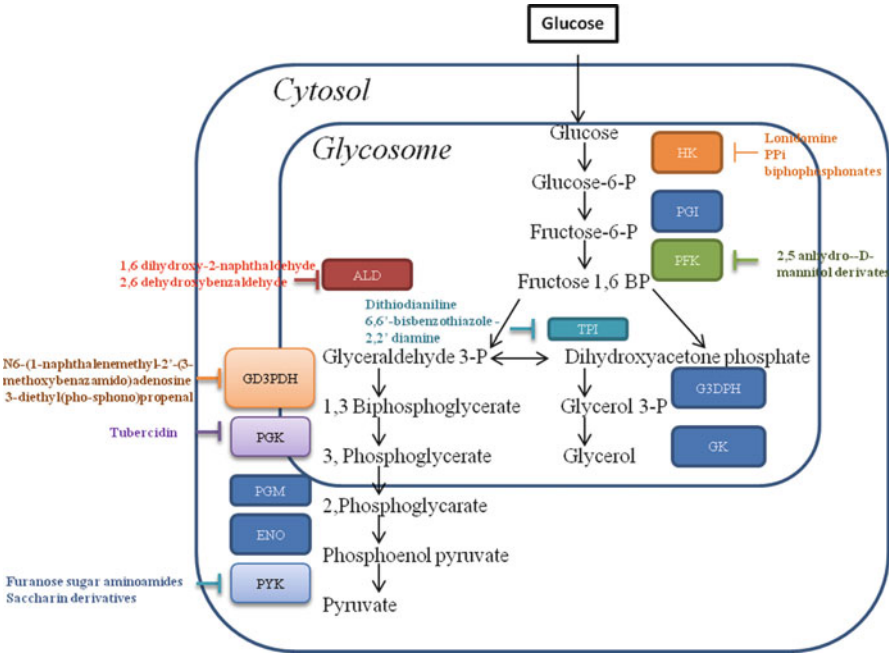

Fig. 2.2 Drug inhibition of the glycolytic pathway in trypanosomatids. The figure shows the glycolytic pathway in trypanosomatids, and drugs known to inhibit the parasitic glycolytic enzymes

4.1 Glycolysis

Glycolysis is an important pathway for drug development in trypanosomatid parasites, not only because it has a critical role for ATP production (the only way for bloodstream *T. brucei*), but also because it has many unique features reflecting both the peculiar glycosomal compartmentalization and differences in structure-activity relationships (Verlinde et al. 2001) (Fig. 2.2). To this end, the available crystal structures of many trypanosomatid glycolytic enzymes have been resolved, revealing significant differences compared to the corresponding mammalian enzymes. These differences point out the importance of glycolytic enzymes as good drug target candidates (Fig. 2.2).

In this respect, the first enzyme of glycolysis, hexokinase (HK), has been evaluated as a potential drug target. HK is encoded by two very similar enzymes in *Leishmania* and *T. brucei* (HK1 and HK2) and one in *T. cruzi*. The hexokinases from *T. brucei* (*Tb*HK1 and *Tb*HK2) have distinct C-terminal domains that result in distinct oligomer formation (Morris et al. 2006). The knocking down of *Tb*HK1 by RNA interference (RNAi) revealed that this protein was essential for viability (Chambers et al. 2008a), and this enzyme was inhibited by lonidamine, a drug that killed effectively *T. brucei* parasites (Chambers et al. 2008b). The hexokinase

from *T. cruzi* (*Tc*HK) displayed significant kinetic differences from its mammalian orthologue, as this enzyme was not inhibited by mammalian HK regulators such as D-glucose-6-phosphate, fructose 1,6 diphosphate, phosphoenolpyruvate, lactate or citrate (Racagni and Machado de Domenech 1983; Urbina and Crespo 1984). In addition, inorganic pyrophosphate (PPi) inhibited this enzyme (Caceres et al. 2003). The synthesis of a series of 42 bisphosphonates confirmed that these compounds could inhibit *Tc*HK, whereby the most potent of which displayed an IC_{50} of 2.2 μM against intracellular amastigotes (Hudock et al. 2006). Another series of non-competitive or mixed *Tc*HK inhibitors, the aromatic arinomethylene biphosphonates, also displayed antiparasitic activity against intracellular amastigotes (Sanz-Rodriguez et al. 2007).

Two other enzymes of the glycolytic pathway, phosphoglucose isomerase (PGI) and phosphofructose kinase (PFK), are promising molecular targets for drug discovery. The crystal structures of PGI from *T. brucei* (Arsenieva et al. 2009) and *L. mexicana* (Cordeiro et al. 2004) and the *Tb*PFK (Martinez-Oyanedel et al. 2007; McNae et al. 2009) have been determined and reveal unique features compared to mammalian orthologues. Moreover, the evaluation of a series of 2,5anhydro-D-mannitol derivatives have been described and determined as inhibitors of the *T. brucei* and *L. mexicana* PFK (Nowicki et al. 2008).

The crystal structure of *L. mexicana* and *T. brucei* fructose 1,6 aldolase (ALD) is also available, and allows the design of specific inhibitors (Chudzik et al. 2000). In this context, selective inhibitors of the trypanosomal over the corresponding mammalian enzyme that belong to the series of 1,6-dihydroxy-2-naphthaldehyde and 2,5 dihydroxybenzaldehyde were designed. However, they displayed low antiparasitic activity, possibly due to their poor potential to cross membranes (Dax et al. 2006).

The trypanosomatid triose-phosphate isomerase (TPI) from *T. cruzi* (Maldonado et al. 1998), *T. brucei* (Wierenga et al. 1991a, b) and *L. mexicana* (Williams et al. 1999) is another glycolytic enzyme with available structure that predicts important differences in the parasitic enzymes compared to the mammalian enzyme. Thus, specific inhibitors of the trypansomatid TPI that are also potent antiparasitics have been developed. More specifically, dithiodianiline and 6,6′-bisbenzothiazole-2,2′ diamine were potent inhibitors of *Tc*TPI and trypanosomatid TPIs respectively, without significantly affecting human TPI (Olivares-Illana et al. 2006, 2007).

The next enzyme in the glycolytic pathway is glyceraldehyde-3-phosphate dehydrogenase (GD3PDH). Trypanosomatid GD3PDH has been also evaluated as a potential drug target. The structures of GD3PDH from *L. mexicana* (Kim et al. 1995), *T. cruzi* (Souza et al. 1998) and *T. brucei* (Vellieux et al. 1993) are resolved and specific inhibitors based on its structure have been designed. A series of adenosine competitive inhibitors that compete for NAD^+ binding with substitutions at the 2′positions of the ribose and N^6 position of adenine inhibited the leishmanial enzyme. In particular, one of the analogs [N6-(1-naphthalenemethyl-2′-(3-methoxybenazamido) adenosine] displayed potent antiparasitic activity at the nanomolar range (Suresh et al. 2001). One of the best inhibitors of *Tb*GD3PDH is 3-diethyl (phosphono) propenal, which kills cultured trypanosomes with an LD_{100} of 300 nM (Willson et al. 1994).

The crystal structure of *T. brucei* phosphoglycerate kinase (PGK) is also available (Bernstein et al. 1997, 1998), and the comparison with the porcine PGK suggested that the ATP/ADP binding pockets display significant differences. The resolved crystal structures suggest that the ATP/ADP binding pockets between porcine and trypanosomatid PGKs display significant differences. Thus, the adenosine analogue tubercidin, which displays antitrypanosomal activity, has been shown to block the *Tb*PGK (Drew et al. 2003). The trypanosomatid enolase (ENO) crystal structure is available, and the enzyme has a more flexible active site from its mammalian counterpart that might allow the design of specific and potent inhibitors (de AS Navarro et al. 2007).

Pyruvate kinase (PYK) catalyzes the last reaction of glycolysis and the enzyme from *L. mexicana* has an available crystal structure (Morgan et al. 2010; Tulloch et al. 2008). From this information, unique features of the effector binding site become apparent that could be used for drug design. Stepwise library synthesis and inhibitor design from a rational starting point identified furanose sugar amino amides (Nowicki et al. 2008) and saccharin derivatives as novel inhibitors of trypanosomatid PYK (Morgan et al. 2012). Overall, these data suggest that the glycolytic pathway enzymes are promising drug target candidates for future studies.

4.2 Purine Salvage Pathway

Trypanosomatid parasites are totally deficient in the *de novo* biosynthesis of purines, and rely on the scavenging from the host (Boitz et al. 2012). Research on purine transport (nucleobase/nucleoside) has focused on the use of purine antimetabolites or specific inhibition of the host nucleoside transporters (de Koning et al. 2005). Moreover the purine transport system can be exploited for the selective transfer of antiparasitic drugs, as in the case of melaminophenyl arsenicals that are efficiently accumulated through the *T. b. brucei* (De Koning 2008). Other genes involved in the purine salvage pathway include: (i) three phosphoribosyltransferases, hypoxanthine guanine phosphoribosyltransferase (HGPRT), xanthine phosphoribosyltransferase (XPRT), and adenine phosphoribosyltransferase (APRT), which catalyzes purine phosphoribosylation, (ii) adenosine kinase (AK) that phosphorylates adenosine (Datta et al. 1987; Iovannisci and Ullman 1984), and a multitude of interconversion enzymes (LaFon et al. 1982; Looker et al. 1983; Marr et al. 1978) (Fig. 2.3). Although none of the enzymes that convert host purine nucleobases or nucleosides to nucleotides are essential, genes in this pathway display properties that allow their exploitation for targeted drug design.

One such example is HPGPRT, which displays differences from the mammalian homologue with respect to substrate specificity (Monzani et al. 2007). Allopurinol, an effective drug against visceral and cutaneous leishmaniasis (Kager et al. 1981; Martinez and Marr 1992) and Chagas disease, is metabolized more efficiently by parasitic HGPRT than the mammalian homologue (Eakin et al. 1997) and therefore incorporated into RNA during transcription, resulting in its degradation and

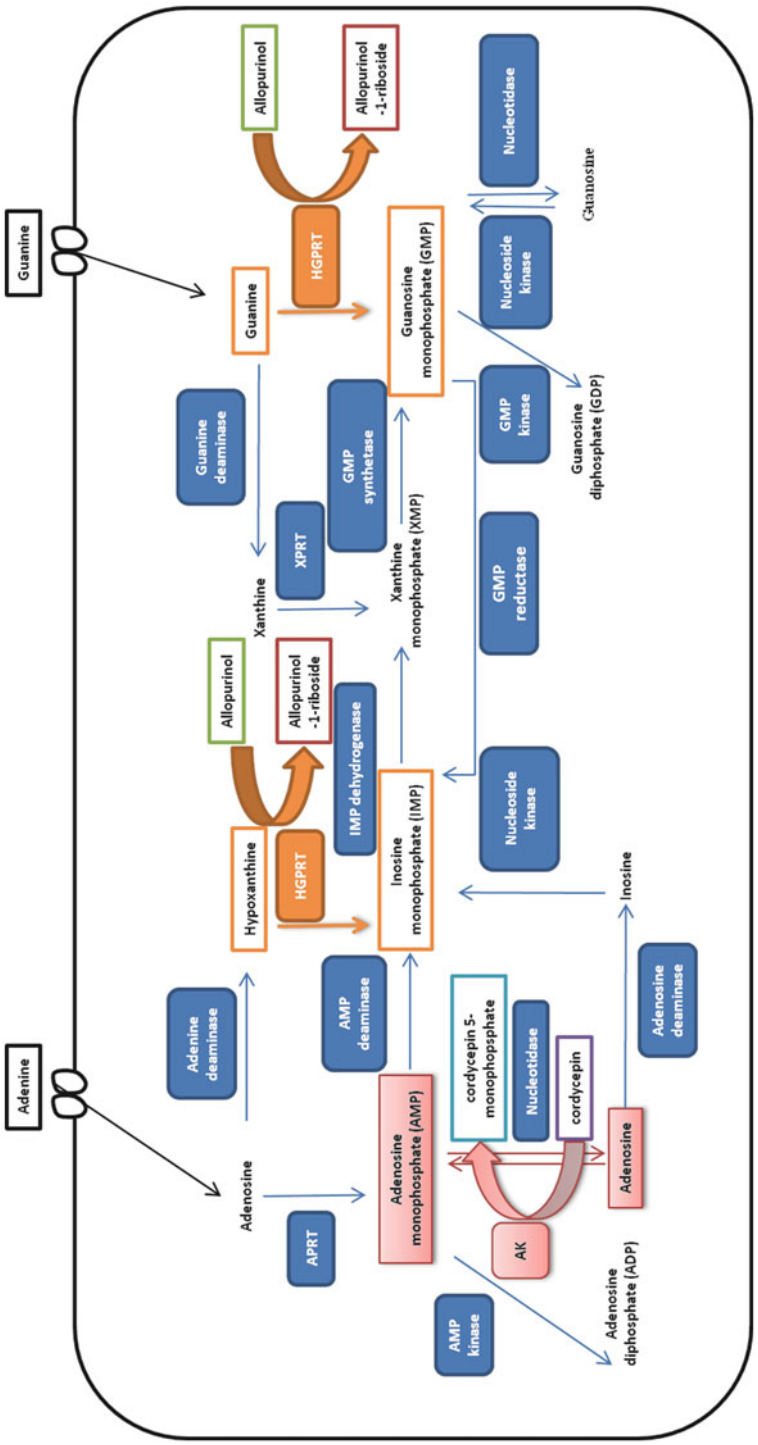

Fig. 2.3 Simplified scheme of the purine salvage pathway in trypanosomatids. The scheme includes drugs which are converted to toxic metabolites by enzymes of the purine salvage pathway

inhibition of protein synthesis (Marr and Berens 1983). Freymann et al., using a structure-based docking method based on the crystal structure of HGPRT from *T. cruzi* (*Tc*HGPRT) provided a remarkably efficient path for the identification of HGPRT inhibitors with potent antiparasitic activity against *T. cruzi* trypomastigotes (Freymann et al. 2000).

Another example involves *T. brucei* AK. This enzyme activates adenosine antimetabolites, like cordycepin (3′-deoxyadenosine), and consequently contributes to their incorporation into RNA, ceasing protein synthesis (Luscher et al. 2007). *In vitro* incubation with cordycepin reduced not only the growth of *T. brucei* but also of *T. cruzi*, as well as *L. major* and *L. amazonensis*, suggesting that cordyceptin is activated by all the corresponding parasitic AKs (Rottenberg et al. 2005). In addition, this drug was active in mouse models of Chagas disease and African trypanosomiasis, when co-administered with deaminase inhibitors (Rottenberg et al. 2005). Thus the purine salvage pathway and the enzymes therein can be "druggable" targets with good potential.

4.3 The Trypanothione System

Trypanothione is a low-molecular mass thiol, unique in trypanosomatids, that adopts the metabolic roles of glutathione (GSH) from other systems. It is implicated in the defense against oxidants, xenobiotics and regulatory proteins and is essential for parasitic survival (Flohe 2012). The biosynthesis and use of trypanothione relies on enzymes that are unique in trypanosomatids and absent from the mammalian host. Trypanothione is known to be synthesized by trypanothione synthetase (TryS) from GSH and spermidine or glutathionylspermidine synthesized by glutathionylspermidine synthase (GSpS). Reduction of oxidized trypanothione is NADPH-dependent and occurs by trypanothione reductase (TryR). Reduced trypanothione then participates in the reduction of glutaredoxin (Ceylan et al. 2010), and dehydroascorbate (Krauth-Siegel and Comini 2008) and tryparedoxin (TXN) (Flohe 2012). The latter mediates the reduction of other regulatory proteins (Flohe 2012). Most of the components of the trypanothione system are essential for parasite viability and allow selective inhibition over the mammalian corresponding enzymes. In addition, the structure of most of the enzymes in this pathway has been determined, making this pathway attractive for therapeutic interventions (Urbina 2010). One of the most attractive targets for drug design is TryR. Numerous reports in the literature exist on the inhibition of TryR, including tricyclic antidepressants and congeners, 2-aminodiphenylsulfides, quaternary alkylammonium, polyamine derivatives and others [reviewed in (Khan 2007)]. However, despite the multitude of TryR inhibitors, only a few were efficacious in animal models of leishmaniasis and trypanosomiasis (Flohe 2012). The differences observed between *in vitro* and *in vivo* antiparasitic effects of TryR inhibitors can be attributed to the pleiotropic effects of these inhibitors (Urbina 2010), to their metabolic instability (Khan 2007) and finally to the fact that redox metabolism of trypanosomatids is normal unless the residual activity of TryR

is below 5 % of wild-type activity (Krieger et al. 2000). Apart from TryR, TryS, the enzyme that catalyzes the synthesis of trypanothione, has been validated as a drug target. Interestingly, compounds that inhibit this enzyme proved to effectively kill *T. brucei* parasites (Torrie et al. 2009). Thus, it is clear that the unique trypanothione system provides opportunities for the development of novel inhibitors with limited "off-target" activity to the host.

4.4 Sterol Biosynthesis

The abundant supply of cholesterol present in their mammalian hosts cannot be used by trypanosomatid protozoa. Instead, *Trypanosoma* and *Leishmania* parasites have a strict requirement for specific endogenous sterols (ergosterol and other sterols) for survival and growth. The main sterols of trypanosomatids belong to the C28-ergostane or C29-stigmastane [reviewed in (de Souza and Rodrigues 2009)]. An exception to this rule is the membrane composition of bloodstream *T. brucei* parasites, which contains predominantly cholesterol, incorporated through a receptor-mediated endocytic process by suppressing the *de novo* ergosterol biosynthesis (Coppens et al. 1988). The sterol biosynthesis pathway is a promising target for the development of new anti-trypanosomatid drugs. Several drugs available today are known to interfere with the sterol biosynthesis pathway (Fig. 2.4).

Amongst these are quinuclidines that target squalene synthase (SQS, E.C. 2.5.1.21), the enzyme that catalyzes the first committed step in sterol biosynthesis (Fig. 2.4). This enzyme is a very attractive target and has been validated not only for treating hypercholesterolhaemia in humans but also for treating trypanosomatid diseases (Suckling 2006; Urbina et al. 2002). Two quinuclidines, ER-119884 and E5700, had potent *in vitro* anti-*Leishmania* (Fernandes Rodrigues et al. 2008) and anti-*T. cruzi* activity and the latter compound provided full protection against death in an *in vivo* murine model of Chagas disease (Urbina et al. 2004). Despite the fact that most SQS inhibitors tested in the parasite also block the mammalian host enzyme, this inhibition is tolerable in mammals. In addition, it has been possible to develop specific antiparasitic SQS inhibitors (Orenes Lorente et al. 2005; Sealey-Cardona et al. 2007), such as aryloxylethyl thiocyanates (WC-9) (Elhalem et al. 2002; Urbina et al. 2003) and 2-alkylaminoethyl-1,1-bisphosphonic acids (Rodrigues-Poveda et al. 2012). Another enzyme in the parasitic sterol biosynthesis pathway is squalene epoxidase (EC1.4.99.7) that converts squalene to 2,3 oxidosqualene. The antifungal drug terbinafine displayed potent antileishmanial activity against promastigotes and intracellular amastigotes by inhibiting this enzyme (Goad et al. 1985; Vannier-Santos et al. 1995). This drug displayed a synergistic effect with ketoconazole, another antifungal drug that also interferes with ergosterol biosynthesis (Vannier-Santos et al. 1995). The same drug combination also displayed a synergistic antiproliferative effect against epimastigotes and amastigotes of *T. cruzi* (Urbina et al. 1988). Lanosterol synthase

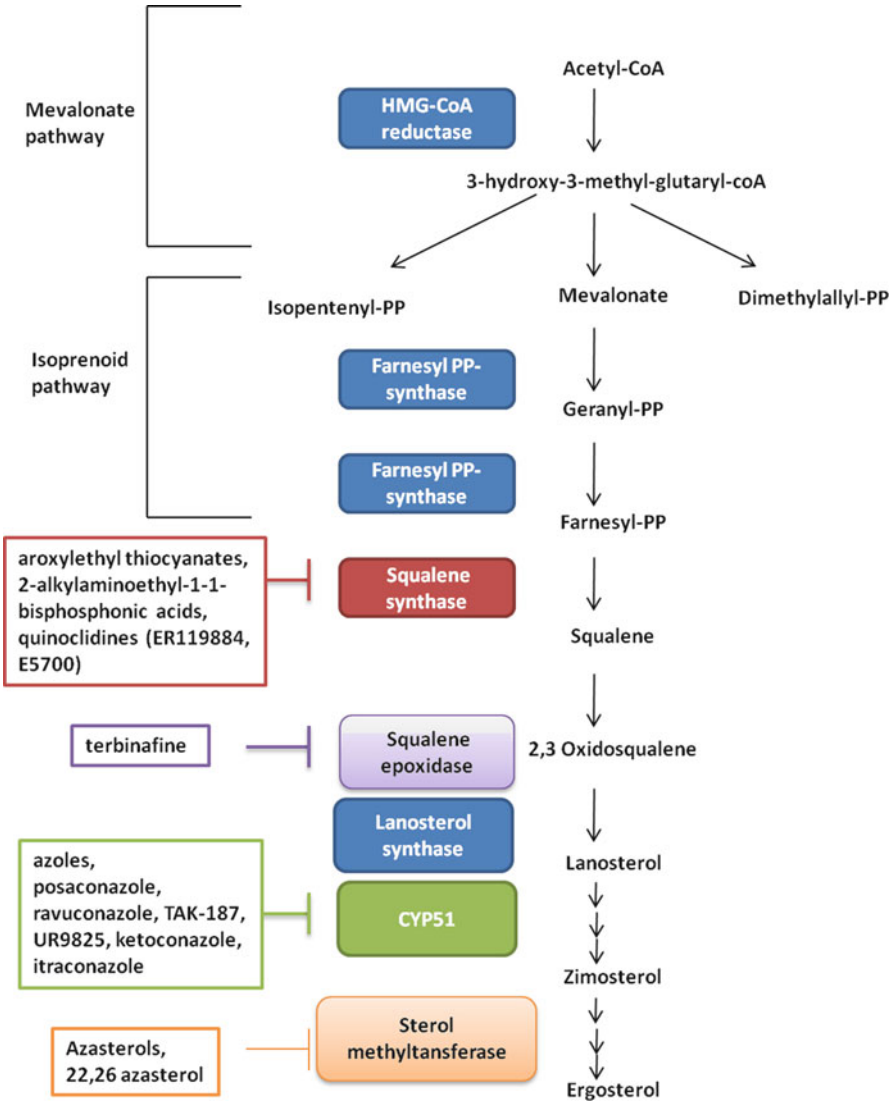

Fig. 2.4 Simplified scheme of sterol biosynthesis in trypanosomatids and known inhibitors of the pathway

(or oxidosqualene cyclase) is a key enzyme in sterol biosynthesis as it catalyzes the cyclization of 2,3 oxidosqualene (Fig. 2.4). This enzyme has already been validated as a chemotherapeutic target against *T. brucei* and *T. cruzi* (Buckner et al. 2001, 2000; Urbina and Docampo 2003).

Another reaction in sterol biosynthesis is the formation of zimosterol from lanosterol. This reaction is catalyzed by C14-demethylase (CYP51), a very diverse enzyme amongst kingdoms that is known to be inhibited by azoles (Urbina 2010). Several studies have shown that commercial inhibitors of yeast CYP51 available for the treatment of fungal infections (like ketoconazole, itraconazole) have a suppressive effect against Chagas disease in humans or in animals (Urbina 2002; Urbina and Docampo 2003). More potent and specific fungal and parasitic CYP51 inhibitors such as D0870 and posaconazole (POS) (Molina et al. 2000), a structural analogue of itraconazole, could completely cure experimental acute and chronic Chagas disease (Urbina et al. 1996a). Posoconazole is now poised to enter clinical trials to evaluate the effectiveness in treatment of Chagas disease (Robertson and Renslo 2011). POS has been tested in leishmaniasis in experimental murine models of leishmaniasis, showing a good efficacy against cutaneous leishmaniasis and to a lesser extent against visceral leishmaniasis due to *L. donovani* infection (Al-Abdely et al. 1999). Other triazoles like TAK-187, UR-9825 and ravuconazole displayed potent antiparasitic activity (Urbina 2010).

Finally, another important enzyme that catalyzes the C24 transmethylation reaction in the sterol biosynthesis is $\Delta^{24(25)}$-sterol methyltransferase (SMT) (EC 2.1.41), which is unique in trypanosomatids, but absent in the mammalian host. This enzyme has been shown to be inhibited by various azasterols, leading to *in vitro* antiproliferative effects against *L. amazonensis* and *T. cruzi* parasites (Lorente et al. 2004; Magaraci et al. 2003; Rodrigues et al. 2002; Urbina et al. 1995). One of the most potent inhibitors of SMT, 22,26 azasterol, displays selective antiparasitic activity in a murine model of acute Chagas disease (Urbina et al. 1996b). Overall, the data presented here suggests that the enzymes within the sterol biosynthesis pathway may be utilized for the design of potent and specific antitrypanosomatid inhibitors.

4.5 Pteridine Metabolism

Trypanosomatid parasites lack a *de novo* pathway for the synthesis of pteridines (folate and pteridines) and rely on salvage from the host (Beck and Ullman 1990). Among the key proteins of the pteridine salvage network that mediates the accumulation and reduction of pteridines, there is a bifunctional enzyme (DHFR-TS) that has activities of dihydrofolate reductase (DHFR) and thymidylate synthase (TS) -unlike the monofunctional enzyme of mammalian hosts-, and pteridine reductase (PTR1) that reduces both folate and biopterin (Fig. 2.5). These two enzymes have been proposed as drug targets in trypanosomatids, since they participate in essential pathways for the parasite metabolism, like thymidylate production by folates and oxidant resistance by the reduction of pterins (Moreira et al. 2009; Nare et al. 2009). Drugs that have been used to inhibit DHFR have proven ineffective in *Leishmania*

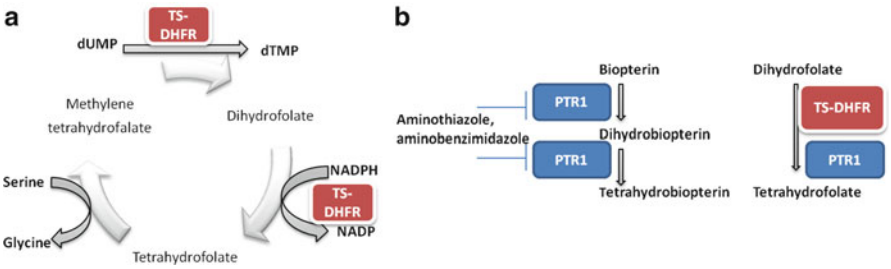

Fig. 2.5 Pteridine metabolism in trypanosomatids. (**a**) Thymidylate synthesis. The figure shows the thymidilate cycle in which thymidilate is synthesized by DHFR-TS, with the use of methylene tetrahydrofolate and its convertion to dihydrofolate. The same enzyme converts dihydrofolate to tetrahydrofolate. (**b**) Contribution of PTR1 to the reduction of pterins and folates and known inhibitors of this enzyme

(Neal and Croft 1984). This observation can be explained by the emergence of drug resistance. As PTR1 may reduce both, pterins and folates, this enzyme can act to override the inhibition of DHFR-TS (Nare et al. 1997). Thus, in *L. major* resistant strains, the amplification of PTR1 was observed (Nare et al. 1997). On the other hand, DHFR-TS is a validated drug target in *T. brucei* (Sienkiewicz et al. 2008), as null mutants had an absolute requirement for thymidine. Removal of thymidine from the medium resulted in growth arrest, followed by cell death and PTR1 was not able to compensate for loss of DHFR activity (Sienkiewicz et al. 2008). Mice infected with DHFR-TS null mutants were incapable of establishing infections (Sienkiewicz et al. 2008). The above suggests that both DHFR and TS activities in *T. brucei* are required for thymidylate synthesis and that thymidine concentrations in the host are limiting. Thus, the targeting of this enzyme could prove fruitful for improving/curing HAT.

On the other hand, research on the inhibition of trypanosomatid PTR1 has been more intense. PTR1 is an essential enzyme for viability, as PTR1 null mutants in *T. brucei* are not viable (Sienkiewicz et al. 2008). Virtual screening identified aminothiazole and aminobenzimidazole inhibitors as potent and selective *Tb*PTR1 inhibitors (Fig. 2.5). Despite the good potency against PTR1 and favorable physicochemical properties, representative members of the scaffolds were not trypanocidal (Mpamhanga et al. 2009). The reason for this lack of correlation between enzymatic inhibition and antitrypanosomal activity is currently under investigation (Mpamhanga et al. 2009). As both PTR1 and DHFR-TS catalyze the same reactions, an attempt to inhibit both of these enzymes would be desirable. A screen against *L. major* PTR1 identified a number of compounds inhibiting both PTR1 and DHFR-TS. Four of these compounds displayed antiparasitic activity, suggesting that the inhibition of both of these enzymes is required for the efficacy of new drug candidates (Hardy et al. 1997).

4.6 Drug Targets in Signaling and Other Pathways Essential for Parasitic Survival

4.6.1 Topoisomerases

Topoisomerases use DNA strand scission, manipulation and rejoining activities to deal with DNA torsional stress, which makes them potential targets for treating parasitic diseases. As topoisomerases are involved in replication, transcription, chromosomal condensation and segregation, inhibitors of these enzymes have a drastic inhibitory effect on the growth of trypanosomatid parasites (Balana-Fouce et al. 2006; Das et al. 2006a; De Sousa et al. 2003; Deterding et al. 2005; Douc-Rasy et al. 1988). These enzymes are grouped into type I and II, depending on their ability to cleave single- and double-stranded DNA, respectively. Trypanosomatid parasites possess both types of topoisomerases (Bakshi and Shapiro 2004; Balana-Fouce et al. 2006; Das et al. 2006b; De Sousa et al. 2003; Douc-Rasy et al. 1988; Strauss and Wang 1990). Type I topoisomerases include type IA and IB subclasses, which are grouped on the basis of differences in their aminoacid sequences and mechanism of action (Champoux and Dulbecco 1972). Topoisomerase IB in trypanosomatids acts as an unusual bi-subunit enzyme, and it is localized in both the nucleus and the kinetoplast (Bakshi and Shapiro 2004; Das et al. 2004).

Topoisomerase inhibitors are often used as anticancer drugs in mammalian cells. These drugs may inhibit topoisomerases via two distinct mechanisms. The first type of inhibition includes the covalent stabilization of DNA-topoisomerase complexes, and the drugs that have this action are referred to as topoisomerase poisons. The second type of inhibition is mediated by drugs that interfere with the active site of the enzyme, and are termed topoisomerase inhibitors (Steverding and Wang 2009). Camptothecins, anthracyclins, epipodophyllotoxins and quinolones are classified as topoisomerase poisons, whereas coumarin antibiotics and forstriecin analogues are topoisomerase inhibitors (Steverding and Wang 2009). Topoisomerases are essential proteins for parasite viability. Thus, topoisomerase inhibitors or poisons have been shown to have activity against trypanosomatid protozoa, mediating apoptosis-like death (Smirlis et al. 2010). Moreover, the antileishmanial compounds sodium stibogluconate and urea stibamine have been shown to act via the inhibition of type I topoisomerase (Chawla and Madhubala 2010). Camptothecin, a drug that acts as a poison against leishmanial topoisomerase I (Das et al. 2006a), has been found to block *L. donovani*, *T. cruzi* and *T. brucei* proliferation, whereas analogues have been screened against *T. brucei* bloodstream parasites and different substitutions have been identified (9,10,11-methylenedioxy analogs) that display a selectivity over the *T. brucei* parasites (Bodley et al. 1995). In addition, the flavones baicalein, quercetin and luteolinhave been shown to stabilize the DNA- *L. donovani* bi-subunit topoisomerase I cleavage complex by a differential mechanism, providing in this way additional insights into the ligand-binding properties of *L. donovani* topoisomerase I (Das et al. 2006b). Besides the abovementioned flavones, three isoflavanoids, 8-prenylmucronulatol, lyasperin H and smiranicin display antileishmanial activity

correlated with topoisomerase II inhibition and kinetoplast DNA (kDNA) linearization (Salem and Werbovetz 2005). Acridine compounds, such as 9-anilinoacridine derivatives, display antiparasitic activity against *Leishmania* and *Trypanosoma* and their mode of action is via the inhibition of topoisomerase II (Figgitt et al. 1992). The topoisomerase II poisons, belonging to the family of anthracycline antibiotics daunomycin and its hydroxyl derivative doxorubicin, had activity against *T. b. rhodesiense* affecting parasite motility and infectivity to mice (Williamson and Scott-Finnigan 1978). Moreover, doxorubicin has been shown to be highly active in an *in vivo* model of visceral leishmaniasis (Sett et al. 1992), its mechanism of action being the inhibition of leishmanial topoisomerase II (Singh and Dey 2007). Another set of topoisomerase II poisons, the fluoroquinolones like KB5426, ofloxacin and ciprofloxacin have been shown to have activity against *T. brucei* (Nenortas et al. 1999, 2003). Finally, aclarubicin, an anthracenedionemotoxantrone derivative that acts as a catalytic inhibitor of topoisomerase II, exhibited potent antiparasitic activity against *T. brucei* bloodstream parasites and a 1,000 fold selectivity index, making this drug a good drug candidate for the treatment of trypanosomiasis (Deterding et al. 2005). Despite the fact that parasitic topoisomerases are essential for survival, and that a multitude of compounds that target these enzymes are available, up to date most of the known topoisomerase inhibitors lack selectivity over the host cells. To this end it is essential to exploit differences between parasite and mammalian topoisomerases.

4.6.2 Proteases

Proteases in trypanosomatid protozoa have been shown to be involved in many important signaling processes, including cell-cycle progression, stage differentiation, autophagy, apoptosis and others (Abdulla et al. 2008; Ambit et al. 2008; Boukai et al. 2000; Galvao-Quintao et al. 1990; Leon et al. 1994; Smirlis and Soteriadou 2011). Amongst the multitude of proteases in parasitic protozoan, cathepsin-like cysteine proteases have been the focus of attention. The cathepsin L-like proteases include CPA and CPB in *Leishmania*, and cruzipain and brucipain (or rhodesain) in *T. cruzi* and *T. brucei*, respectively. On the other hand, the cathepsin B-like proteases are termed CPC in *Leishmania* and *Tb*CatB in *T. brucei*. CPB deletion mutants in *L. mexicana* displayed reduced virulence in BALB/c mice, suggesting that the pharmacological inhibition of this protease may compromise the progression of leishmaniasis (Alexander et al. 1998). Indeed, a peptide that has been characterized as a natural inhibitor of cysteine proteases and a potent inhibitor of CPB led to a reduced virulence and a Th1 response (Bryson et al. 2009). In *L. major*, a class of cathepsin inhibitors, known as aziridine-2,3-dicarboxylate derivatives, induced cell death correlated with the presence of autophagy-related lysosome-like vacuoles (Schurigt et al. 2010).

In *T. brucei*, downregulation of brucipain by RNAi showed that this protease is not essential for growth. However, a vinyl-sulfone inhibitor (K11777, see below) of this protease limited the ability of *T. b. rhodesiense* to cross the blood brain barrier

in brain endothelial cells *in vitro*, suggesting its potential role ameliorating central nervous system (CNS) damage during *T. brucei* infection. Despite the fact that brucipain was not proven to be essential for *T. brucei* viability, *Tb*CatB was required for parasite growth, participating in iron acquisition by degrading host transferrin (Abdulla et al. 2008; O'Brien et al. 2008). Thus, a series of selective purine nitrile inhibitors with enhanced potency for *Tb*CatB were developed via structure-guided optimization. These compounds were trypanocidal, reaffirming the potential of *Tb*CatB as a therapeutic target, whereas a lead compound of the series significantly prolonged the life of infected mice with *T. brucei* (Mallari et al. 2009, 2010).

In *T. cruzi*, cruzipain is required for all the major proteolytic activities of the parasite life cycle (Cazzulo 2002; Urbina and Docampo 2003). Inhibition of this protease by K-11777 resulted in cessation of proliferation in epimastigotes and intracellular amastigotes and arrested metacyclogenesis. In murine models of acute and chronic Chagas disease, the inhibitor significantly reduced parasitemia and prolonged survival, conferring minimal toxicity to the host (Engel et al. 1998). In addition, the same compound displayed therapeutic activities in an immunodeficient mouse model of Chagas disease (Doyle et al. 2007). In a canine model of acute Chagas disease, K-11777 significantly reduced cardiac damage (Barr et al. 2005). Following these promising results in animal models of Chagas disease, the development of K-11777 as a new treatment for Chagas diseases was initiated. Despite the hepatotoxicity and other serious problems with this compound that resulted in terminating the project, this effort demonstrated that cruzipain inhibitors could prove promising drugs for the treatment of Chagas disease. Thus, attempts to identify potent and specific inhibitors of cruzipain were initiated (Brak et al. 2008; Caffrey et al. 2000; Du et al. 2002). Structure-activity relationship studies (SARs) demonstrated that thiosemicrbazone and semicarbazone scaffolds are potent and selective cruzipain inhibitors (Du et al. 2002; Guido et al. 2008). Moreover, several other classes of non-peptidic inhibitors have been identified, including vinyl-sulphone containing macrocycles (Brak et al. 2008), aryl oxymethylketone inhibitors (Brak et al. 2008) and quinoxaline-N-acyl hydrazone inhibitors (Romeiro et al. 2009). Thus, cruzipain is a confirmed drug target, and research for the identification of a good cruzipain inhibitor suitable for future drug development is ongoing.

Overall, parasitic proteases are enzymes with a potential to be good drug target candidates and thus further investigation on their role in the parasitic life-cycle, essentiality and druggability is required.

4.6.3 Kinases

In view of the success in targeting eukaryotic protein kinases (PKs) in other disease contexts (notably cancer), parasitic kinases are considered attractive drug targets. Moreover, additional reasons exist that justify the selection of parasitic kinases as ideal drug targets. These include the fundamental role of parasitic kinases and phosphorylation cascades in critical parasite pathways for survival and virulence, such

as cell-cycle progression, differentiation and others and the divergence between the kinomes of the parasites and of their human hosts, which allows the design of drugs that may specifically target the parasitic kinase (Naula et al. 2005). Despite the pharmaceutical interest on host protein kinases, protozoan PKs remain largely underexplored as targets for neglected diseases. Interestingly, tryapanosomatids have a significant number of PK genes, with 179 genes present in *L. major*, 156 in *T. brucei* and 171 in *T. cruzi* (Naula et al. 2005). Several of these kinases, notably members of the CMGC group of kinases, have already been validated as drug targets for the treatment of parasitic diseases. Amongst these are the cyclin-dependent kinases (CDKs), mitogen-activated protein kinases (MPK) and glycogen synthase kinase 3(GSK-3) (Grant et al. 1998; Hassan et al. 2001; Tu and Wang 2004; Wang et al. 1998, 2005; Wiese 1998, 2007; Ojo et al. 2008; Xingi et al. 2009).

The number of MPK homologues in *Leishmania* is rather large, with 15 putative mitogen activated protein kinases (MPK) identified, along with 13 in *T. brucei*, several of which are two-copy genes (Wiese 2007). Several of these kinases are characterized, including *Tb*ECK1 (the homologue of *Lmx*MPK6) (Ellis et al. 2004), *Lmx*MPK11 and *Lmx*MPK13 (Marshall and Rosenbaum 2001; Bengs et al. 2005), with no evidence up to date that they could serve as molecular targets for the treatment of trypanosomatid diseases. On the other hand, MPK5 is a validated drug target, since a null *Lmx*MPK5 mutant displayed an impaired ability to cause lesions in BALB/c mice infected with *L. mexicana* (Wiese 2007). The *Lmx*MPK5 homologue in *T. brucei*,*Tb*MPK5, has been found to be involved in the differentiation of bloodstream forms to stumpy forms, and a null mutant of this kinase displayed a massive reduction of parasitemia (16-fold lower) in immunosuppressed mice (Domenicali Pfister et al. 2006). Moreover, a null *Lmx*MPK5 mutant displayed an impaired ability to cause lesions in BALB/c mice infected with *L. mexicana*. Thus, these data suggest that trypanosomatid MPK5 might be exploitable as a target for chemotherapy against human sleeping sickness and leishmaniases. Another MPK, *Lmx*MPK1, has been shown to be essential for cell-cycle progression and survival in *L. mexicana* amastigotes in a murine model of leishmaniasis, representing thus a functionally validated drug target (Wiese 1998). In addition, the related kinase *Lmx*MPK2 also represents a putative drug target, as it is shown that it is required for the establishment of infection and in important processes in the mammalian amastigote stage (Wiese 2007). One of the most studied and validated target that belongs to this group of kinases is *Lmx*MPK4. This enzyme is essential for viability in *L. mexicana*, as *Lmx*MPK4 null mutants could not be generated (Wang et al. 2005). The same kinase displays enhanced activity upon exposure to pH 5.5 and 37°C in *L. major* promastigotes and *L. donovani* axenic amastigotes, the natural stress signals that occur during the differentiation of the parasite from the insect to the mammalian stage (Morales et al. 2007). In addition, a new *E. coli* based expression system was generated for *Lmx*MPKK5 that included a STE7-like protein kinase from *L. mexicana*, required for its activation. This effort is the basis for the development of drug screening assays of *Lmx*MPK4 (John von Freyend et al. 2010).

Despite the promising results that suggest that targeting MPKs in trypanosomatid parasites could provide a disease-modifying mechanism, another group of CMGC

kinases, namely the CDKs, has attracted more attention towards the validation of its members as potential drug targets. This family is well represented in trypanosomatids, with 11 members in *L. major* and *T. brucei* and 10 members in *T. cruzi* (Naula et al. 2005). Trypanosomatid CDKs contain cyclin-binding regions as anticipated (Naula et al. 2005), whereas the presence of a cyclin (CYC2, CYC3, CYC6 for *T. brucei*, and CYC1, CYCA and CYC6 for *Leishmania*) for the activity of the trypanosomatid homologue of mammalian CDK1 (CRK3) has been shown (Wang et al. 1998; Van Hellemond et al. 2000; Li and Wang 2003; Hammarton et al. 2003, 2004; Tu and Wang 2004; Banerjee et al. 2006; Gomes et al. 2010). The function of two kinases from *L. mexicana* and *T. brucei*, namely CRK1 and CRK3, have been analysed in detail. In *L. mexicana*, the activity of *Lmx*CRK1 was shown to be restricted to the promastigote stage, whereas its role in amastigotes has not been investigated in detail (Grant et al. 2004). In *T. brucei*, downregulation of CRK1 by RNAi in bloodstream and procyclic forms led to reduced growth and increase in the number of cells in the G1 phase of the cell-cycle, demonstrating the importance of this kinase in regulating the G1→S transition (Tu and Wang 2004). Despite these results, the validation of this kinase as a drug target in both *Leishmania* and *Trypanosoma* still remains an open issue.

On the other hand, CRK3 has been validated as a drug target in trypanosomatid parasites and has been studied in great detail compared to other parasitic CDK kinases. As mentioned above, CRK3 is considered to be the functional homologue of mammalian CDK1. Initially, the functional homology of the *L. major* CDK, CRK3 to *Schizosaccharomyces pombe* CDKs was demonstrated by the functional complementation of this kinase in a temperature-sensitive *S. pombe* cdc2/CDK1 null mutant (Wang et al. 1998). The gene for *L. mexicana* CRK3 was shown to be essential for viability, as it is a crucial regulator of cell division. CRK3 activity was found to peak in the G2/M phase of the cell cycle, and inhibition of CRK3 *in vivo* resulted in cell cycle arrest (Hassan et al. 2001). The same effect on cell-cycle progression was observed upon the downregulation of *Tb*CRK3 in *T. brucei* procyclics and bloodstream parasites (Tu and Wang 2004). Grant et al. previously described the screening of a diverse chemical library of antimitotic compounds for potential inhibitors of *Leishmania* CRK3 (Grant et al. 1998). However, despite the potent effect of these inhibitors on the leishmanial kinase, they failed to show selectivity over the mammalian CDK1–CYCB complex (Grant et al. 2004). Recent studies identified *Leishmania* CRK3–CYC6 inhibitors belonging to the class of azapurines, with micromolar potency, following a high-throughput screen with heterocyclic and kinase libraries (Walker et al. 2011). However, potent inhibitors of CRK3-CYC6 did not always display antiparasitic activity (Cleghorn et al. 2011). This suggests that CYC6 might not be the cyclin that *in vivo* is required for the function of CRK3 (Cleghorn et al. 2011). Further work is required to delineate the discrepancy between the drug inhibition of CRK3 and the lack of antiparasitic activity of certain inhibitors.

Apart from the MPK and CDKs of the CMGC kinase family, as mentioned above, GSK-3 has attracted attention, constituting a model candidate for drug target selection. GSK-3 is a multifunctional serine/threonine kinase of the CMGC family,

found in all eukaryotes. This enzyme is known to play a key role in many cellular and physiological events, including Wnt[Int and Wg (wingless) in *Drosophila*] signaling, transcription, cell-cycle and differentiation, neuronal functions and circadian rhythm (Phukan et al. 2010). These functions of GSK-3 and its implication in many human diseases, such as Alzheimer's disease, non-insulin-dependent diabetes mellitus and cancer, have stimulated an active search for potent and selective GSK-3 inhibitors (Phukan et al. 2010). Two GSK-3 genes, GSK-3s (short) and GSK-3l (long), are encoded in the *T. brucei* and the leishmanial genome. Of the two isoforms, the short isoform has been analysed in both *T. brucei* and *L. donovani* (Ojo et al. 2008; Xingi et al. 2009). Previous studies demonstrated that leishmanial GSK-3s was essential for parasitic viability, and its inhibition causes cell-cycle defects and apoptosis-like death (Xingi et al. 2009). Moreover, RNAi experiments showed that *Tb*GSK-3s is essential for viability in bloodstream forms of *T. brucei* (Ojo et al. 2008). These results validate this kinase as a drug target for the treatment of leishmaniasis and HAT. Apart from the essentiality in the life-cycle of trypanosomatid parasites, parasitic GSK-3s has many desirable characteristics that justify its selection as a drug target. More importantly, inhibitor scaffolds and inhibitors of parasitic GSK-3 have been identified that also show good antiparasitic activity (Oduor et al. 2011; Ojo et al. 2011, 2008; Xingi et al. 2009). Indirubins, a class of bis-indole compounds known for over a century as a minor constituent of plant, animal and microorganism-derived indigo, represent inhibitory scaffolds targeting *Ld*GSK-3s (Xingi et al. 2009). More specifically, a series of 6- and 5- halogen substituted indirubins, were tested against promastigotes and intracellular amastigotes (Xingi et al. 2009). Four of these compounds displayed antileishmanial activity against *L. donovani* promastigotes and intracellular amastigotes. All of the analogues with antiparasitic activity inhibited both CRK3 and *Ld*GSK-3s. Of these, 6-Br-5methylindirubin-3′oxime inhibited 7 times more potently *Ld*GSK-3s (IC_{50} 90 nM) and killed effectively promastigotes and intracellular amastigotes ($IC_{50} < 1.2$ μM). Interestingly, although 6 bromo-substituted indirubins are powerful ATP competitive inhibitors of mammalian GSK-3 (Polychronopoulos et al. 2004), they displayed a high selectivity index because the inhibition of host kinase is tolerable in adult mammals (Henriksen et al. 2003; Kaidanovich-Beilin and Eldar-Finkelman 2006). Furthermore, since GSK-3 has also been pursued as a human drug target, a large number of inhibitors are available for screening against parasites. To this end, a collaborative industrial/academic partnership facilitated by the World Health Organization Tropical Diseases Research division (WHO TDR) was initiated, to stimulate research aimed at identifying new drugs for treating HAT. In this context, a subset of 16,000 inhibitors that target human GSK-3β from the Pfizer compound collection were screened, and potent and selective inhibitors of *Tb*GSK-3s were identified (Oduor et al. 2011).

The continuation of a drug discovery program by the synthesis of more potent and selective inhibitors of parasitic GSK-3s is possible because critical differences exist in the ATP-binding pocket between parasite and human GSK-3 (Ojo et al. 2011; Xingi et al. 2009). More specifically, the two major differences in the leishmanial enzyme that are conserved in *T. brucei* are the replacement of Gln185*h*GSK-3β

by His155*Ld*GSK-3s in the sugar-binding region, and the replacement of the "gatekeeper"Leu132*h*GSK-3β by Met100*Ld*GSK-3s (Xingi et al. 2009). In addition, the crystal structure of *L. major* GSK-3s is now available that allows more accurate structure-activity relationship studies for future drug design.

Thus, with respect to parasite essentiality, assayability for high throughput screening, the availability of crystal structure, the existence of differences in the active site in comparison to its mammalian homologues, the redundancy of mammalian GSK-3β function in the mammalian host and the tolerance of its inhibition, and finally the presence of a multitude of compounds known to inhibit parasitic GSK-3s, make this enzyme an excellent "model for drug target selection". Research into parasitic GSK-3s kinases provides an excellent opportunity to develop a targeted drug therapy for leishmaniases and HAT.

5 Investigating Drug Repurposing Opportunities for Drug Target Identification and for Antiparasitic Drug Discovery

The process of finding new uses for existing drugs (marketed drugs and failed or idle compounds, or drugs whose mammalian targets are known) outside the scope of the original indication is variously referred to as repositioning, redirecting, repurposing, and reprofiling. Nowadays, as more and more pharmaceutical companies are exploring the existing arsenal of known drugs for repositioning candidates, the number of repositioning successful stories is steadily increasing (Padhy and Gupta 2011).

Thus, the exploitation of drugs whose targets have already been discovered in other systems can offer advantages for both drug target identification and antiparasitic drug discovery. For drug repositioning strategy, in order to facilitate the access of information concerning therapeutic targets, there are publicly accessible databases, such as DrugBank, Potential Drug Target Database, Therapeutic Target Database, and SuperTarget. These databases complement each other to provide target and drug profiles (Zhu et al. 2010). Moreover, it is now possible to reposition marketed drugs to novel targets and vice-versa. This can be achieved by a java-based software called IDMap (Ha et al. 2008). The next step is to gain access to compound libraries.

This step is challenging and the choice of a compound library is critical for the discovery of a potential drug target in protozoa. Despite the fact that genetic validation will be required for assessing the essentiality for viability or virulence of a potential drug target, this approach saves time and aids researchers to avoid laborious and high-cost techniques, like genetic manipulation (i.e. targeted gene deletion) over targets that are not essential for parasite viability (Fig. 2.1). This approach has been used to validate *Ld*GSK-3s as a drug target for the treatment of leishmaniasis by using known inhibitors of mammalian GSK-3. These inhibitors displayed both antiparasitic activity and activity against *Ld*GSK-3s. The inhibitory activity of *Ld*GSK-3s, as the leishmanicidal mechanism of action, was further validated by the over-expression of *Ld*GSK-3s in *L. donovani* promastigotes, and the demonstration

that *L. donovani* over-expressing *Ld*GSK-3s were protected from the leishmanicidal effects of indirubins (Xingi et al. 2009).

Drug repositioning can be also a good strategy once a target is selected (Fig. 2.1), for identifying compounds that represent potential attractive starting points for a drug discovery program. The *T. brucei* GSK-3s has provided an excellent example for this strategy and the screening of a compound library from the Pfizer compound collection as mentioned above, known to target mammalian GSK-3β,resulted in the identification of inhibitors that target better the parasitic *Tb*GSK-3s (Oduor et al. 2011).

Finally, drug repurposing offers an additional advantage for the choice of drugs that are marketed. For these drugs, information already exists concerning their clinical safety data, pharmacokinetics, and viable dose range are available at the start of a development project, and the risks associated with clinical development are significantly reduced with fewer failures in the later stages. One such example is amphotericin B, an antifungal agent that creates membrane leaks by binding to ergosterol used to treat thrush, and is used today as an antileishmanial agent (Hartsel and Bolard 1996). Commercial inhibitors of yeast CYP51, an enzyme that is also present in trypanosomatids, have proved to have antiparasitic effects. One such example is posaconazole (Noxafil) a broad spectrum antifungal, is also a prime candidate for clinical trials in patients with Chagas disease (Robertson and Renslo 2011).

6 Concluding Remarks

There are many opportunities for the selection and/or identification of novel drug targets for drug development against diseases caused by trypanosomatid parasites. These include methods, like systems biology and network analysis, which integrate biochemistry and cell biology with genetics, as well as bioinformatics and computational biology to obtain holistic descriptions at the organism level. A good drug target gives the opportunity to develop safer drugs at a lower cost. Today the process to predict and validate drug targets is faster than before. This is due to the advances in *in silico* drug target prediction, in the completion of the genome project of trypanosomatid pathogens, and in systems biology. Despite this, researchers that are new to the field have to have in mind that a multitude of trypanosomatid (potential) drug targets are already validated or close to validation. In addition to these molecular targets, a variety of attractive drugs that inhibit many of these enzymes exists, which could serve as promising scaffolds for the synthesis of more potent and selective antiparasitic agents. However, despite these advances, there seems to be little interest to make new drugs for the neglected diseases caused by trypanosomatid parasites. This cannot be attributed to a lack of scientific knowledge. It is more likely that the lack of interest to develop antiparasitic drugs is attributed to the low probability of pharmaceutical companies to have a profitable financial return. Thus, a targeted response is required for an adequately funded, needs-driven priority R&D agenda to combat these devastating diseases.

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Chapter 3
A2 and Other Visceralizing Proteins of *Leishmania*: Role in Pathogenesis and Application for Vaccine Development

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Abstract Visceral leishmaniasis is a re-emergent disease and a significant cause of morbidity worldwide. Amongst the more than 20 *Leishmania* species, *Leishmania donovani*, *Leishmania infantum* and more rarely *Leishmania amazonensis* are associated with visceral leishmaniasis. A major question in leishmaniasis research is how these species migrate to and infect visceral organs whereas other species such as *Leishmania major* and *Leishmania braziliensis* remain in the skin, causing tegumentary leishmaniasis. Here we present the more recent advances and approaches towards the identification of species-specific visceralizing factors of *Leishmania*, such as the A2 protein, leading to a better understanding of parasite biology. We also discuss their potential use for the development of a vaccine for visceral leishmaniasis.

Abbreviations

cGAPDH	Cytosolic GAPDH
CL	Cutaneous leishmaniasis
CTL	Cytolytic T lymphocytes
CVL	Canine visceral leishmaniasis
DALYs	Disability-adjusted life years
DCL	Diffuse cutaneous leishmaniasis

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DCs	Dendritic cells
DHFR-TS	Dihydrofolate reductase-thymidilate synthase
ELISA	Enzyme-linked immunosorbent assay
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
gp	Glycoprotein
hsp	Heat shock protein
IFN-γ	Gamma interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
MHC	Major histocompatibility complex
ML	Mucosal leishmaniasis
mo-DCs	Monocyte-derived dendritic cells
mRNA	Messenger ribonucleic acid
NK	Natural killer
PCR	Polimerase chain reaction
PKDL	Post-kala azar dermal leishmaniasis
RNI	Radical nitrogen intermediates
ROI	Radical oxygen intermediates
SIDER	Small interspersed degenerate retroposons
TAP	Transporter associated with antigen processing
TGF-β	Transforming growth factor-beta
Th	T helper
TNF-α	Tumor Necrosis factor-alfa
Treg	Regulatory T cells
UPR	Unfolded protein response
UTR	Untranslated region
VL	Visceral leishmaniasis

1 *Leishmania* Parasites

Leishmania (order Kinetoplastida, family Trypanosomatidae) are vector-borne protozoan parasites. Out of the approximately 30 *Leishmania* species, 20 are human pathogens, the causative agents of human leishmaniasis in the Old World (Europe, Asia and Africa) and the New World (Americas). *Leishmania* are divided into two subgenera, the subgenus *Leishmania*, found in both Old and New World, and the subgenus *Viannia*, exclusively found in South America. The separation between Old and New World *Leishmania* species occurred 40–80 million years ago and Old World species such as *L. major* and *L. donovani* then diverged approximately 14–24 million years ago (Lukes et al. 2007). A more recent development is the introduction of *L. infantum* into South America, 500 years ago by the conquerors (Leblois et al. 2011). New World *L. infantum* is often referred to as *L. chagasi*.

Leishmania parasites have a dimorphic lifecycle divided between the promastigote stage in the sandfly vector and the amastigote stage in mammalian hosts. The flagellated promastigote stage is transmitted to the mammalian host by the bite of an infected sandfly (genus *Phlebotomus*, in the Old World or *Lutzomyia*, in the New World) during a blood meal (Murray et al. 2005; WHO 2010). Neutrophils are the first cells recruited to the bite site and take up promastigotes by phagocytosis. Parasites are then taken up by dendritic cells and macrophages, either via phagocytosis of free parasites or of infected neutrophils (Ribeiro-Gomes et al. 2012). Macrophages represent the main host cell for *Leishmania* parasite replication. Within the macrophage phagolysosome, increased temperature and decreased pH induce promastigote into amastigote differentiation. Amastigotes can then infect other macrophages or be taken up by a feeding sandfly. In the sandfly gut, amastigotes differentiate into procyclic (non-infective) promastigotes. Promastigotes mature as they migrate to the anterior portion of the sandfly gut, leading to metacyclic (infective) promastigote accumulation at the junction of midgut and foregut and in the proboscis, thereby completing the parasite lifecycle (Sacks and Kamhawi 2001). Transmission can occur either via an anthroponotic cycle (*L. donovani* and *L. tropica*), where humans are the only reservoir, or via a zoonotic cycle (all other *Leishmania* species), where transmission occurs from an animal reservoir to humans (Murray et al. 2005; WHO 2010).

2 Human Leishmaniasis

Leishmaniasis in humans is associated with a spectrum of syndromes depending on the infecting species of *Leishmania* and the host immune response. The three main forms of disease are cutaneous, mucosal and visceral leishmaniasis. In cutaneous leishmaniasis (CL), parasite replication and formation of lesions occur at the site of sandfly bite days to months after the initial parasite inoculation. Lesions usually progress from papules to nodules and then to wet ulcers with a typical "volcano" appearance, but can also be non-ulcerative. CL usually self-heals, albeit slowly (3 months to years depending on the causative species); it is mainly caused by *L. major* and *L. tropica* in the Old World and by parasites from the *L. mexicana* and *L. braziliensis* species complexes in the New World. Rarer clinical manifestations include disseminated cutaneous leishmaniasis (multiple non-contiguous pleomorphic lesions), diffuse cutaneous leishmaniasis (disseminated non-ulcerative lesions) and leishmaniasis recidivans (centrally healing lesions with lateral spread) (Murray et al. 2005; WHO 2010). Diffuse cutaneous leishmaniasis (DCL) is an anergic form of leishmaniasis, since patients do not respond specifically to *Leishmania* antigens with cellular immune responses; it is not self-healing and is usually not responsive to treatment. DCL is caused mainly by species of the *L. mexicana* complex, including *L. amazonensis*, in the New World and *L. aethiopica* in the Old World (Goto and Lauletta Lindoso 2012). Abundance of parasites is seen in lesions, since immune responses fail to limit parasite replication. DCL is characterized by multiple nodules, papules, or tubercles with diffuse cutaneous infiltration and no ulceration.

The second main form of leishmaniasis is mucosal disease. Mucosal leishmaniasis (ML) usually develops years after an initial cutaneous infection and is caused by parasite metastasis to facial mucosal tissues. Clinical symptoms include ulceration of the nose and mouth, followed by perforation of the nasal septum, and destruction of mucosal and cartilage tissues in the nose, mouth and throat, leading to severe facial disfigurement. Death is rare and is most often due to secondary bacterial infections. *L. braziliensis* is the main causative agent of ML, but other members of the *Viannia* subgenus such as *L. panamensis* and *L. guyanensis* can also cause the disease (Murray et al. 2005; WHO 2010).

Finally, the most severe form of disease is the visceral leishmaniasis (VL), also known as kalazar, in which parasites disseminate to the liver, spleen and bone marrow (Murray et al. 2005; WHO 2010). Symptoms include high fever, hepatosplenomegaly, cachexia, hypergammaglobulinemia and pancytopenia, and the disease is fatal if untreated. VL is caused by *L. donovani*, *L. infantum* and rarely by *L. amazonensis* (Barral et al. 1991; Almeida et al. 1996). Successful treatment and resolution of VL may be followed by post-kalazar dermal leishmaniasis (PKDL) occurrences. PKDL is associated with non-ulcerative papules or nodules and is more common in East Africa than in India (Murray et al. 2005; WHO 2010).

Overall leishmaniasis disease burden exceeds two million disability-adjusted life years (DALYs) (Hotez et al. 2004). Disease prevalence is estimated at 10 million cases, with 350 million people at risk in 98 countries (Alvar et al. 2012). Incidence is 1.6 million new cases per year, with 0.7–1.2 million CL cases and 200,000–400,000 VL cases (Alvar et al. 2012). Mucosal incidence is estimated at 5 % of CL cases in the Americas (WHO 2010). Leishmaniasis ranks second in mortality that is estimated at 20,000–40,000 deaths per year (Alvar et al. 2012), fourth in morbidity among tropical diseases, and is the ninth-highest disease burden for infectious diseases (Hotez et al. 2004). However, due to significant underreporting, actual incidence and mortality values may be higher (Bern et al. 2008).

3 Canine Visceral Leishmaniasis and Control of Zoonotic Visceral Leishmaniasis

Dogs are highly susceptible to infection by *L. infantum*, and canine visceral leishmaniasis (CVL) is a potentially severe and fatal disease, although the outcome of the infection is highly variable and not all infected animals will develop the disease (Solano-Gallego et al. 2001). Some dogs are able to completely clear the infection, some remain asymptomatic, whereas others evolve to clinical disease of varying severity, from mild papular skin disease to severe generalized disease characterized by alopecia, cachexia, renal failure and death (Costa et al. 2003; Ordeix et al. 2005). Curiously, *L. amazonensis* has been also isolated from dogs with visceral leishmaniasis in Brazil and this situation is becoming more frequent than expected (Tolezano et al. 2007; Dias et al. 2011).

Wild mammals, such as foxes and marsupials (*Didelphis albiventris* and *D. marsupialis*), have been implicated as reservoirs for *L. infantum* in Brazil (Almeida et al. 2005; Santiago et al. 2007). The occurrence of these animals may favor the presence of phlebotomines and their infection by *Leishmania* spp. The transmission of infection that occurs in domiciliary, peri-domiciliary and extra-domiciliary areas reflects, however, not only the behavior of the vector, but also the characteristics of the host reservoir and the activities of man (Wijeyaratne et al. 1994).

The migration from rural to urban areas has led to a quick urbanization of VL in Brazil and many other countries of South America (Desjeux 2001). VL currently presents peri-urban and urban patterns of occurrence in many Brazilian cities (Brasil 2006a) with nearly 10 % positive of all CVL tests (Caiaffa et al. 2005) and 3,894 confirmed cases in 2011 in the whole country (Sinan et al. 2012). The fast-growing and/or partly unplanned urbanization encompass neighbored rural areas where the zoonotic cycle occurs. Although wildlife animals may also act as reservoirs, in peridomestic and domestic foci, transmission from dogs via sandflies is the main route for VL human infection in South America and Mediterranean regions of Europe. In these regions, VL is expanding to areas where it was not considered previously to be endemic, in spite of the adopted control measures (Miro et al. 2008).

Infected sandflies inject promastigotes into the dog skin during the blood meal. The parasites are phagocytized and then they proliferate into the host cells. In the early stages of infection, dogs with VL may develop cutaneous lesions or remain asymptomatic, though they may already transmit the parasite to another sandfly during its blood meal. The close relationship between dogs and human populations in the domiciliary and peri-domicialiary environments, besides the presence of competent vectors in urban areas, increases the risk of parasite transmission from dogs to sandflies and then to humans. Euthanasia of seropositive dogs is practiced in Brazil as part of a public health control program. However, its effectiveness in the control of infection is questionable (Shaw 2007; Miro et al. 2008). Treatment of infected domestic dogs has limited effectiveness and is not recommended in endemic regions, since dogs that respond to treatment may still be a source of parasites (Tesh 1995). Thus, the future for CVL control may require an integrated approach (Dye 1996). In this complex scenario, a CVL vaccine is certainly an important tool, but to be used in endemic areas it must fit some requirements, such as allowing the discrimination between vaccinated and infected dogs by means of inexpensive serological tests, including those that utilize native promastigote or recombinant antigens.

4 Immune Responses in Leishmaniasis

Infection outcomes in leishmaniasis depend on the parasite species and immune responses mounted by the host (McMahon-Pratt and Alexander 2004). In mice, susceptibility and resistance to *L. major* infection have been linked to the development of $CD4^+$ interleukin (IL)-4-dominated T helper (Th) 2 immune responses and gamma interferon (IFN-γ)-dominated Th 1 immune responses, respectively (McMahon-Pratt

and Alexander 2004; Alexander and Bryson 2005). The protective responses are believed to be mediated by the induction of inducible nitric oxide synthase (iNOS) in macrophages upon stimulation by Th1 cytokines such as IFN-γ (Kaye and Scott 2011). Although, it is generally agreed that type 1 immune responses are necessary to control *Leishmania* multiplication and dissemination, susceptibility to infection by other *Leishmania* species in murine models may not fit completely into this Th1-Th2 dichotomy (McMahon-Pratt and Alexander 2004; Alexander and Bryson 2005).

The Th1 response in C57BL/6 mice infected with *L. mexicana* is more limited and the lesions fail to resolve, resulting in chronic infection (Alexander and Kaye 1985). The chronic nature of *L. mexicana* infection in this model is most likely due to their inability to stimulate an effective Th1 response (Alexander and Kaye 1985; Buxbaum et al. 2003; Buxbaum and Scott 2005). Similarly, *L. amazonensis* fails to induce a strong Th1 response and leads to chronic lesions in C57BL/6 mice (Afonso and Scott 1993; Jones et al. 2000; Qi et al. 2001; Ji et al. 2003). However, the immune mechanisms limiting Th1 responses following either *L. mexicana* or *L. amazonensis* infection are not fully defined yet. It has been suggested that infection with *L. mexicana* suppresses IL-12 production by macrophages and dendritic cells (DCs) (Weinheber et al. 1998; Alexander et al. 1999; Bennett et al. 2001), which may limit the Th1 response (Satoskar et al. 1995; Stamm et al. 1998; Rodriguez-Sosa et al. 2001). Nevertheless, administration of IL-12 failed to promote disease resolution, suggesting that the inability of the infected mice to resolve their infection is not dependent only of IL-12 suppression (Buxbaum et al. 2002). Recently, researchers have demonstrated that fewer monocytes are recruited to lesion during infection with *L. mexicana* as compared to *L. major*. Moreover, fewer iNOS producing monocyte-derived dendritic cells (mo-DCs) are present in the lesions and fewer migrate to the draining lymph node during *L. mexicana* infection. When IL-10 receptors were blocked, an increased monocytes recruitment and more robust Th1 response during *L. mexicana* infection were observed, suggesting an important role for this immunoregulatory cytokine in susceptibility to leishmaniasis in this model. The injection of DCs into the ear at the time of infection with *L. mexicana* also increased levels of iNOS and IFN-γ (Petritus et al. 2012).

In murine VL, promastigotes interact with skin resident cells, including dermal macrophages and DCs, shortly after infection in resistant mice (Kaye and Scott 2011). These cells then secrete cytokines such as IL-12 or type I IFN that promote the polarization of naive T cells into Th1 effector lymphocytes (Pepe et al. 2006). Natural killer (NK) cells are also involved in the innate immune response and control of the parasites, as an early source of IFN-γ and contributing to the development of Th1 cells that produce high levels of IFN-γ and TNF-α (Tumor Necrosis Factor-alfa) (Prajeeth et al. 2011). IFN-γ and TNF-α activate macrophages to generate toxic molecules, radical nitrogen intermediates (RNI) or radical oxygen intermediates (ROI) that destroy *Leishmania* parasites inside macrophages (Kaye and Scott 2011). On the other hand, regulatory T (Treg) cells and suppressor $CD8^+$ T cells produce IL-10 and TGF-β (Transforming Growth Factor-beta) (Weiner 2001; Levings et al. 2002), that suppress IL-12 production by macrophages and DCs and modulate the

development of an adequate Th1 response (Nylen and Sacks 2007; Kaye and Scott 2011), preventing resolution of infection. Other sources of IL-10 have been described. A model of no healing *L. major* in conventionally resistant C57BL/6 mice showed the presence of Th1 cells that also produce IL-10 (Anderson et al. 2005, 2007). However, the factors that regulate IL-10 production by Th1 cells in this setting are unknown.

In human VL, it was originally believed that individuals display an impaired ability to produce IFN-γ, allowing parasite multiplication and progressive disease (Schriefer et al. 2008). Decreased numbers of $CD4^+$ and $CD8^+$ T cells are detected in peripheral blood of VL patients suggesting an impaired immune response during active disease (Clarêncio et al. 2009). However, more recently it was reported that VL patients do have an IFN-γ response but that high levels of IL-10 abrogate the IFN-γ response (Singh et al. 2012). Indeed, a growing body of evidence indicates an important role of IL-10 and TGF-β in regulating this response and in parasite persistence, in down modulation of type 1 responses during VL and DCL as well as in the development of PKDL (Nylen and Sacks 2007; Saha et al. 2007; Schriefer et al. 2008; Clarêncio et al. 2009; Singh et al. 2012).

In canine VL, dogs which remain asymptomatic after infection with *L. infantum* develop a predominantly Th1 profile response, whereas oligosymptomatic and symptomatic dogs present a Th2 profile (Barbieri 2006) and hypergamaglobulemia, although a clear dichotomous Th1/Th2 pattern is also lacking in this species (Alves et al. 2009). However, despite the fact that the situation is extremely complex, it is now also widely accepted that the protective canine immune response is mediated by a dominant $CD4^+$ Th1 influence in an overall mixed cellular response (Barbieri 2006; Alves et al. 2009). Among the immunological responses associated to CVL, those involving T cells and IL-10 and TGF-β production have also been correlated with progression to disease, whereas control of the infection in asymptomatic dogs has been associated with the production of IFN-γ (Barbieri 2006; Alves et al. 2009). Therefore, a loss or suppression of appropriate modulation of type 1 responses underlies the immunopathology in both human and canine visceral leishmaniasis.

$CD8^+$ cytolytic T lymphocytes (CTL) are also essential for the control of primary infections in mouse model of VL, since depletion of this cell subset renders mice unable to eliminate parasitized phagocytic cells (Stern et al. 1988). Besides, $CD8^+$ cells predominate in liver granuloma in the late stages of resolution of infection (McElrath et al. 1988). $CD8^+$ cells also correlate with protection following vaccination and cure upon immunotherapy (Schriefer et al. 2008). In dogs, increased levels of $CD8^+$ lymphocytes appear to be the major phenotypic feature of asymptomatic disease (Barbieri 2006). These cells have also been associated with cure in human VL patients (Clarêncio et al. 2009).

The counter acting immune responses (Th1/Th2) in leishmaniasis reflect a complex immunological scenario, suggesting that additional regulatory molecules may be important players in the disease pathogenesis, which may include Th17 responses. In different diseases and conditions, inflammatory responses favor the Th17 phenotype and Th17 cells are induced in mice by TGF-β, when combined with proinflammatory cytokines such as IL-6 (Bettelli et al. 2006; Veldhoen et al. 2006; Mangan et al. 2006).

In contrast, TGF-β alone drives expression of the lineage-specific transcription factor Foxp3, leading to a Treg cell phenotype (Bettelli et al. 2006). *In vivo*, $CD4^+$ T cells are a crucial source of TGF-β for the generation of Th17 cells (Li et al. 2007), although it remains to be proven that Treg cells drive Th17 cells *in vivo*. TGF-β may be produced by many cell types, and Treg cells may be one source of Th17 cell regulation. If the balance between proinflammatory and regulatory signals is shifted, such as through decreased numbers of Treg cells, IL-10 production may be lost concomitant with an upregulation of proinflammatory mediators. Such 'unrestrained' cells would then be 'armed' to establish the immune pathology that has now been attributed to the subset of T helper cells, Th17 (McGeachy et al. 2007).

Recently, the role of Th17 response is being unrevealed in leishmaniasis. Therefore, there is still limited information related to role of Th17 in VL. Recent reports documented the presence of Tregs cells and demonstrated their role in parasite persistence by establishing positive correlation with parasite load in PKDL tissue lesions (Ganguly et al. 2010; Katara et al. 2011). Moreover, Th17 responses are present during *L. donovani* infection in PKDL which possibly contributes to disease pathogenesis by inducing TNF-α and nitric oxide production. In contrast to PKDL, low abundance of IL-17 has been demonstrated recently in VL (Ansari et al. 2011), correlating with the diminished production of TNF-α by monocytes (Peruhype-Magalhaes et al. 2006).

5 Genes and Molecules Associated to Visceralization in Leishmaniasis

A major question in leishmaniasis research is why some species remain in the skin and others migrate to and infect visceral organs such as *L. infantum* and *L. donovani*. Since *Leishmania* species have diverged by 15–100 million years of evolution, differences in their genomes were expected to explain how they differ regarding tissue tropism. However, the publication of the *L. major*, *L. infantum* and *L. braziliensis* genomes revealed, surprisingly, that the *Leishmania* genomes are highly conserved and have less than 1 % species-specific genes. Comparison of these genomes identified over 8,000 genes of which there are about 200 differentially distributed genes (2.5 %) among these three species. Among these 200 differentially distributed genes, only 14 genes are *L. major*-specific, two genes are *L. mexicana*-specific, 19 genes are *L. infantum*-specific and 67 genes are *L. braziliensis*-specific (Rogers et al. 2011). These 19 *L. infantum*-specific genes are either absent or present as pseudogenes in *L. major* and *L. braziliensis* (Peacock et al. 2007).

Different approaches may be used to predict the function of these genes, including sequence similarity searches, although the majority of them encode hypothetical proteins with no known function. In addition, prediction of gene function needs to be confirmed by functional studies. An interesting functional approach to test if these genes are associated to survival in visceral organs is to transfect *L. major*

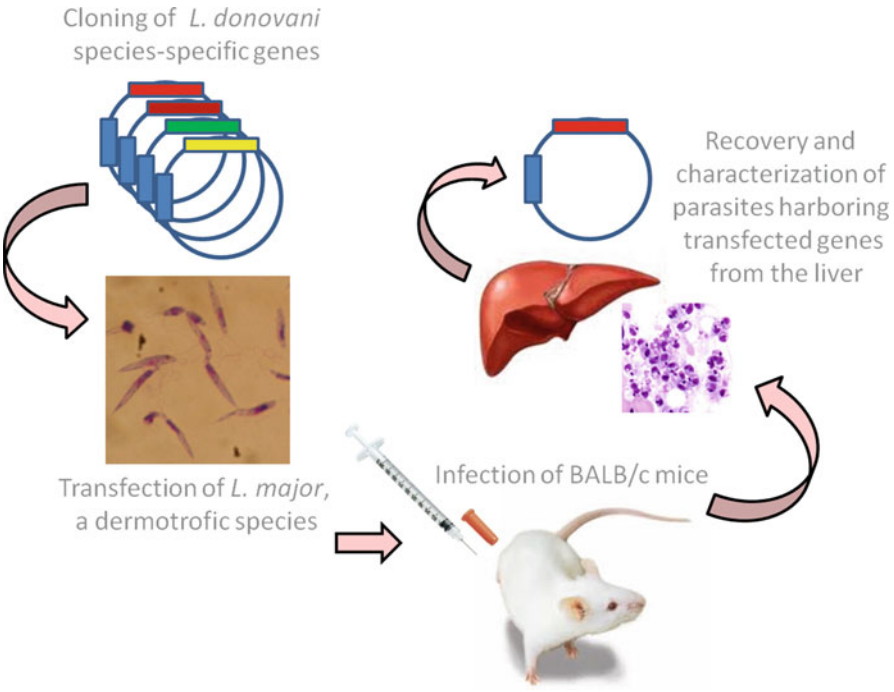

Fig. 3.1 Functional assay for identification of visceralizing molecules in *Leishmania* genomes. Comparisons of the sequenced *Leishmania* genomes available have led to the identification of species-specific gene sequences present in the viscerotropic *L. infantum* that are not shared or are pseudogenes in the other sequenced *Leishmania* genomes. These gene sequences were cloned into a shuttle vector for high expression in *L. major*, a cutaneous leishmaniasis species. Transgenic *L. major* parasites were then used to infect BALB/c mice. Infected BALB/c mice were examined for parasite burden in the liver and spleen. The amastigotes were then isolated from the liver and spleen of infected mice 4 weeks post-infection. The recovered amastigotes were cultured in promastigote culture medium, and the *Leishmania* parasite burdens were determined by limiting dilution assay

with species-specific genes from *L. donovani* and then infected BALB/c mice with the recombinant parasites (Zhang et al. 2008; Zhang and Matlashewski 2010). Since *L. major* has a decreased ability to survive within the visceral organs following intravenous injection in this mice model of infection, recovery of *L. major*-recombinant parasites from the liver and spleen should reveal *L. donovani* genes involved in visceralization (Fig. 3.1). In this manner, a number of species-specific genes have been identified by increasing the ability of *L. major* to survive in visceral organs (Table 3.1). Additional studies may be carried out to confirm the role of these genes in visceralization and virulence, for instance, by knocking out the species-specific genes in *L. donovani* and afterwards adding back and over expressing the knocked out gene (Zhang and Matlashewski 1997; Zhang et al. 2013).

Table 3.1 Species-specific genes and their impact on visceralization

					Impact on virulence		
L. infantum	*L. major*	*L. mexicana*	*L. braziliensis*	Function	Introduction into *L. major*	KO in *L. donovani*	References
LinJ.28.0340	(*LmjF.28.0420*)	(*LmxM.28.0420*)	(*LbrM.28.0430*)	Hypothetical	11–14× increase	200× decrease	Costa et al. (2003), Debrabant et al. (2004)
LinJ.15.0900	(*LmjF.15.0840*)	LmxM.15.0840		nucleotide sugar transporter, putative	18–20× increase	No effect	Debrabant et al. (2004), Mangan et al. (2006)
LinJ.22.0670		LmxM.22.0691 LmxM.22.0692		A2	4× increase	25× decrease	Dias et al. (2011), Fernandes et al. (2012)
LinJ.36.2480	(*LmjF.36.2350*)	LmxM.36.2350		Cytosolic glyceraldehyde 3-phosphate dehydrogenase	7–8× increase	4–10× decrease	Debrabant et al. (2004)

5.1 A2 as a Prototype Species-Specific Gene

This functional assay was first applied to study the role of A2 gene in visceralization (Zhang et al. 2013), since A2 is a species-specific gene that increases the survival of transfected *L. major* and *L. donovani* in visceral organs (Table 3.1). The A2 gene family was initially studied as an amastigote specific virulence factor (Zhang and Matlashewski 1997), but lately it has become a prototype *L. donovani* species-specific gene. Besides the *L. donovani* species complex, A2 is also expressed in the *L. mexicana* species complex, including in *L. amazonensis*, but not in the *L. tropica* or *L. braziliensis* species complexes (Ghedin et al. 1997; Carvalho et al. 2002).

5.1.1 *L. donovani* A2 Genomic Organization

A2 genes are present as a multi-gene family and are arranged in tandem arrays on chromosome 22, with up to four such arrays per *L. donovani* diploid genome (Zhang and Matlashewski 2001; Zhang et al. 2003). A2 proteins in *L. donovani* range from 42 to 100 kDa and are made up almost entirely of 40–90 copies of a repetitive ten amino acid sequence (Val-Gly-Pro-Leu-Ser-Val-Gly-Pro-Gln-Ser, VGPLSVGPQS), preceded by an N-terminal secretory leader sequence (Charest and Matlashewski 1994; Charest et al. 1996; Zhang et al. 1996; Zhang and Matlashewski 2001). The number of A2 isoforms varies between strains, with up to nine different-sized A2 proteins detected in the *L. donovani* 1S/Cl2D strain. In addition, sequencing of 17 *L. donovani* clinical isolates from India and Nepal showed significant sequence and structural variation at the A2 locus (Downing et al. 2011).

5.1.2 Regulation of A2 Expression

Regulation of A2 protein expression is complex. A2 protein expression is induced by promastigote into amastigote differentiation (Charest et al. 1996) and by a variety of stresses, including unfolded protein response (UPR) (Gosline et al. 2011) and misfolded protein stress (Barak et al. 2005; Harder et al. 2010) but not by thapsigargin-induced calcium depletion stress (Cloutier et al. 2012). The main site of regulation is located in the 3′UTR (untranslated region). Regulation is via increased mRNA (messenger ribonucleic acid) stability rather than by increased mRNA synthesis, with higher A2 mRNA levels in amastigotes compared to promastigotes. Increased mRNA stability requires a combination of increased temperature and decreased pH (Charest et al. 1996). However, heat shock alone is sufficient to induce A2 protein expression (McCall and Matlashewski 2010). Other mechanisms are therefore involved in the regulation of A2 protein levels when temperature alone is changed.

The A2 3′ UTR contains a 309 nucleotide small interspersed degenerate retroposons (SIDER) one regulatory element (Boucher et al. 2002; Rochette et al. 2008).

SIDER1 promote increased translation in response to heat shock (McNicoll et al. 2005) without affecting mRNA stability (Boucher et al. 2002). This is supported by microarray data showing no changes in A2 mRNA levels following heat shock alone (Alcolea et al. 2010). Indeed, increased A2 translation with more mRNA association with polysomes was observed following increased temperature alone or following a combination of increased temperature and decreased pH. Acidic pH alone was not sufficient to induce increased A2 translation and protein expression (Cloutier et al. 2012). Decreased A2 mRNA levels and decreased mRNA association with polysomes are observed when amastigotes are switched back to promastigote culture conditions. Decreased protein levels are observed prior to any changes in A2 mRNA levels, indicating that translation control is the main and fastest mechanism of regulation of A2 protein levels (Cloutier et al. 2012). However, this SIDER1 element cannot account for the full temperature regulation of A2 protein expression, and other factors are also likely involved (Boucher et al. 2002).

5.1.3 Role of A2 in Visceral Disease

Down-regulation of A2 mRNA using antisense RNA (Zhang and Matlashewski 1997) or partial knockout of A2 genes (Zhang and Matlashewski 2001) decreased liver parasite burden during infection of *L. donovani*. Conversely, introducing A2 genes into *L. major* enhanced the ability of *L. major*-infected cells to migrate out of the dermis and increased parasite survival in mouse visceral organs (Zhang et al. 2003). Likewise, A2 is absent in *L. tarentolae* (a lizard *Leishmania* species) (Azizi et al. 2009) and expression of *L. donovani* A2 in *L. tarentolae* enhanced the ability of *L. tarentolae* to survive in mouse visceral organs (Mizbani et al. 2011). In addition, A2-expressing *L. tarentolae* parasites displayed enhanced infectivity in BALB/c cultured macrophages. Although transfected A2 sequences are functional in *L. tarentolae*, lack of A2 has been one of the factors that partly contribute to the loss of infectivity and virulence of this parasite. Overall, these results indicate that A2 plays a key role in parasite survival in the visceral organs in animal models. Since A2 is expressed during human visceral infection (Ghedin et al. 1997), it may play a similar role in the human host. Moreover, A2 is expressed in *L. amazonensis*, a New World species that has been also associated with visceral leishmaniasis in both humans and dogs (Almeida et al. 1996; Miro et al. 2008; Dias et al. 2011).

As seen in Fig. 3.2, acquision of A2 repetitive sequences overlaps with the ability of *Leishmania* species to visceralize. In this figure, sequences of the dihydrofolate reductase-thymidilate synthase (DHFR-TS), a conserved housekeeping gene that is functionally equivalent among all *Leishmania* species, were aligned and used to portrait *Leishmania* phylogeny. The most parsimonious tree was obtained by using DHFR-TS gene sequences of *Crithidia fasciculata*, a monogenetic trypanosomatid, as outgroup (Fernandes and Beverley, unpublished results). This tree topology reveals that A2 sequences were acquired by the *L. donovani*/*L. infantum*/*L. amazonensis* common ancestor after its divergence from the ancestor shared with *L. major*. A similar tree topology has been generated by using the glycoprotein (gp) 63 gene

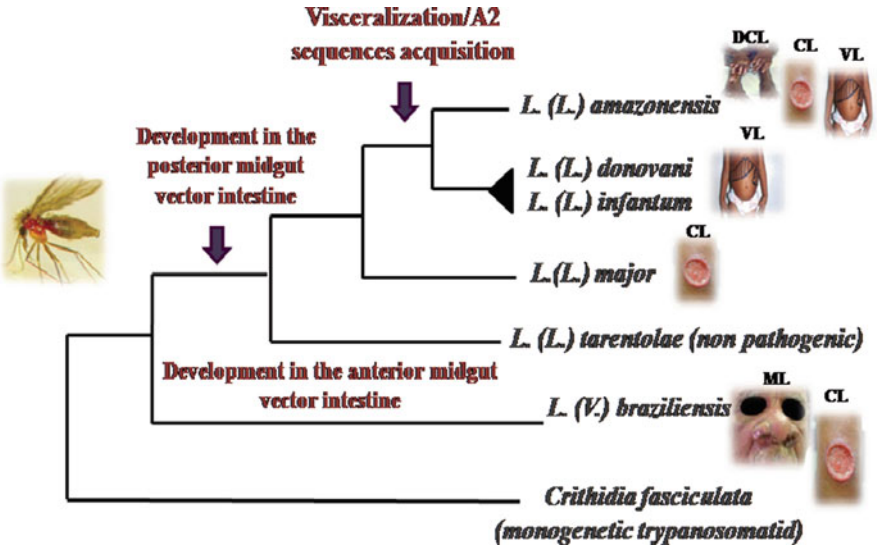

Fig. 3.2 Phylogenetic tree of *Leishmania* species according to the sequences of *DHFR-TS* gene. Gene sequences of the DHFR-TS of representative *Leishmania* species were aligned and used to portrait *Leishmania* phylogeny and to show that visceralization capacity coincides with gain of complete A2 gene sequences, therefore generating species that are able to visceralize and others that are not. The most parsimonious tree was obtained by using DHFR-TS sequences of *Crithidia fasciculata*, a monogenetic trypanosomatid, as outgroup (Fernandes and Beverley, unpublished results). Images of sand fly and different clinical manifestations of visceral and tegumentar leishmaniasis associated to each species were obtained from Brasil 2006b, 2007

(Mauricio et al. 2007). Since parts of the A2 locus are found in *L. major*, including N and C terminal sequences and one repeat unit (Zhang et al. 2003), gain of the A2 locus seems to have occurred following divergence of the *L. donovani*/*L. infantum*/*L. amazonensis*/*L. major* common ancestor from *L. tarentolae*. Expansion of the A2 locus, acquisition of the repeat units and the ability to visceralize would then have occurred following divergence of the *L. donovani*/*L. infantum*/*L. amazonensis* common ancestor from *L. major*. This however remains to be established since using the heat shock protein (hsp) 70 gene, *L. major* is evolutionarily closer to *L. donovani* than is *L. amazonensis* (Fraga et al. 2010). Regardless of the position of *L. amazonensis* in these trees, i.e., inside *L. donovani*/*L. infantum* clade or not, visceralization capacity appears to coincide with the gain or loss of complete A2 gene sequences, generating species that are able to visceralize and others that are not.

Another way of thinking the role of A2 sequences is that they may inhibit cutaneous disease: expressing A2 in *L. major* was associated with decreased footpad parasite burden and swelling (Zhang et al. 2003) and A2 was down-regulated in human PKDL cases (Sharma et al. 2010). A2 may therefore contribute to the inability of *L. donovani* to cause cutaneous leishmaniasis.

A2 has some sequence homology with the repetitive S-antigen of *Plasmodium falciparum* (Charest and Matlashewski 1994), but the function of this antigen is

unknown (Anderson and Day 2000). Although A2 has an N-terminal secretory leader sequence, A2 proteins are not secreted and colocalize very similarly with BiP (binding immunoglobulin protein), a endoplasmic reticulum chaperone, suggesting an endoplasmic reticulum localization for A2 (McCall and Matlashewski 2010). A2 promotes parasite survival following heat shock (McCall and Matlashewski 2010) and oxidative stress (McCall and Matlashewski 2012) and may therefore allow visceral *Leishmania* species to survive within the more stressful environment of the visceral organs.

These results established that species-specific genes may be involved in tissue tropism and pathology of *Leishmania* infection and set a model for testing the role of additional *L. donovani*-specific genes potentially involved in visceral infections.

5.2 Other Visceralizing Proteins

This functional approach has been applied to test 21 different *L. donovani*-specific genes so far (Zhang et al. 2008, 2013; Zhang and Matlashewski 2010) and has led to the identification of at least three additional candidate genes: a cytolosic protein of unknown function (LinJ.28.0340), a nucleotide sugar transporter (LinJ.15.0900) and the cytosolic GAPDH (glyceraldehyde 3-phosphate dehydrogenase, LinJ.36.2480).

LinJ.28.0340 encodes a hypothetical protein that is localized in the cytoplasm of *L. donovani*. Similar to the effect of expressing A2 in *L. major*, over expression of LinJ.28.0340 also resulted in recovery of increased numbers of *L. major* parasites from liver and spleen of BALB/c mice, indicating its potential effect in virulence and tropism to visceral organs (Zhang et al. 2008; Zhang and Matlashewski 2010). Moreover, knocking out the LinJ.28.0340 gene from *L. donovani* significantly impaired the ability of *L. donovani* to survive as axenic amastigotes in culture and to survive in visceral organs in BALB/c mice, confirming their role in virulence and visceralization of infection (Zhang and Matlashewski 2010).

LinJ.15.0900 is a nucleotide sugar transporter localized in the Golgi apparatus. Transfection of LinJ15.0900 into *L. major* increased significantly liver and spleen parasite burden following intravenous infection. However, unlike LinJ.28.0340, knocking out LinJ.15.0900 in *L. donovani* had no effect on *L. donovani* virulence (Zhang et al. 2011).

The third protein is the GAPDH, LinJ.36.2480. One GAPDH is localized in the glycosomes in *Leishmania*. In *L. donovani* and *L. mexicana* there also exists a second GAPDH enzyme present in the cytosol that is absent in *L. braziliensis* and has become a pseudogene in *L. major*. To further investigate the role of the cytosolic GAPDH (cGAPDH), a *L. donovani* cGAPDH null mutant was generated and conversely the functional *L. donovani* cGAPDH was introduced into *L. major*. The *L. donovani* cGAPDH null mutant was able to proliferate at the same rate as the wild type parasite in glucose deficient medium. However, in the presence of glucose, the *L. donovani* cGAPDH null mutant consumed less glucose and proliferated slower than the wild type parasite and displayed reduced infectivity in visceral organs of

experimentally infected mice. This demonstrates that cGAPDH is functional in *L. donovani* and required for survival in visceral organs. In contrast, restoration of cGAPDH activity in *L. major* had an adverse effect on *L. major* proliferation in glucose-containing medium providing a possible explanation for why it has evolved into a pseudogene in *L. major*. These findings suggest that differences in glucose metabolism between *L. donovani* and *L. major* may represent an important factor in the ability of *L. donovani* to cause visceral disease (Zhang et al. 2013).

However, none of these genes could restore *L. major* visceral virulence to levels comparable to *L. donovani*. In addition, transfecting most *L. donovani* species-specific genes individually into *L. major* did not improve *L. major* survival within the visceral organs (Zhang and Matlashewski 2010). Other factors are then involved. One possibility is that full visceral virulence requires the combination of these species-specific genes. In addition, significant differences in chromosome ploidy are observed between *Leishmania* strains and species (Zhang and Matlashewski 2010; Rogers et al. 2011), possibly altering protein levels. Differences in protein levels between cutaneous and visceral strains may also determine parasite tropism.

5.3 Application of Species-Specific Genes for Vaccine Design: A2 as a Vaccine Antigen

Studying species-specific genes has improved our knowledge of parasite biology and the determinants of tissue tropism. These genes can also be used for a number of clinical applications, and A2 in particular has been extensively studied as a vaccine candidate. Vaccination with A2 antigen either as recombinant protein associated to different adjuvants (Ghosh et al. 2001b; Coelho et al. 2003), DNA (Ghosh et al. 2001a; Zanin et al. 2007), attenuated non-replicative viruses (Resende et al. 2008), non-pathogenic bacteria (Yam et al. 2011) or non-virulent parasites (*L. tarentolae*) (Mizbani et al. 2009), has provided evidence of its protective effect in mice. Therefore, A2 antigen has emerged as one of the most promising candidates, among the few amastigote antigens tested so far as vaccine against VL.

In general, the protective immunity induced by A2 vaccines was associated to parasite-specific Immunoglobulin (Ig) G2a antibodies, as well as high levels of IFN-γ and low levels of IL-10 produced by T cells in recall response to parasite antigens, resulting in reduced lesion size and numbers of parasites in protected animals (Ghosh et al. 2001a, b; Coelho et al. 2003; Zanin et al. 2007; Resende et al. 2008; Mizbani et al. 2009; Yam et al. 2011). Given the impressive reductions in parasite burdens at the site of infection and in distant body sites, anti-A2 immune responses may act by promoting clearance of parasites instead of only preventing their dissemination.

In silico prediction analyses, mapped two $CD4^+$ T cell epitopes (SASAE-PHKAAVDVGPLS/PHKAAVDVGPLSVGPQS) in the N terminal portion of 34 amino acids that correspond to the non-repetitive segment of A2 (Resende et al. 2008), as shown in Fig. 3.3. Figure 3.3 also shows other features of A2. The VGPQSVGPL

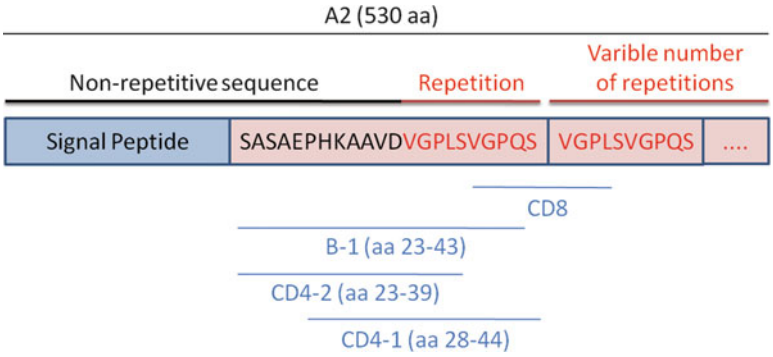

Fig. 3.3 Virtual B and T cell epitope mapping of *L. donovani* A2 protein

motif has high affinity for different MHC (Major Histocompatibility Complex) I class haplotypes, and TAP (Transporter associated with Antigen Processing), constituting an epitope for $CD8^+$ cytotoxic T cells. The two $CD4^+$ T cell epitopes induced IFN-γ production in a recall response of splenocytes from infected or A2 vaccinated BALB/c mice, whereas the $CD8^+$ T cell epitopes induced IFN-γ production and cytotoxic activity in mice that were vaccinated with adenovirus-expressing A2. The mapped main B cell epitope of A2 was a secondary structure of a peptide containing two repetitive units of A2 (Resende et al. 2008).

The A2 antigen also contains B and T cell epitopes recognized by human cells, which is an important requirement for induction of protection against leishmaniasis (Martins et al. 2006). In addition, anti-A2 antibodies are present in *L. infantum* infected dogs and are higher in asymptomatic animals as compared with the symptomatic ones (Porrozzi et al. 2007; Costa et al. 2012).

Supported by the evidence gathered from pre-clinical trials in mice, a recombinant protein formulation consisting of saponin as adjuvant and the recombinant protein A2 (Leish-Tec®) has been tested in a phase II trial in dogs (Fernandes et al. 2008). Vaccinated dogs presented high levels of anti-A2 IgG and IgG2, but not IgG1 antibodies, and high IFN-γ and low IL-10 levels in response to A2. After challenge, five out of seven control animals presented severe symptoms of VL as early as 3 months, such as bloody diarrhea and intense weight loss. In contrast, five out of seven vaccinated animals remained asymptomatic throughout the trial. The two symptomatic animals presented low grade symptoms, which appeared 1 year after the infection. After vaccination, the dogs remained negative in serological tests using promastigote antigens, which are usually applied in routine serological tests for CVL, indicating that vaccination with A2 allows serological distinction between immunized and infected dogs, by means of serological tests with non A2 related antigens, such as promastigote or other recombinant antigens.

A double-blinded randomized phase III trial was also performed to test the efficacy of Leish-Tec® in an endemic area of VL, located in the city of Porteirinha, Minas Gerais, Brazil. In this study, 1,650 healthy dogs were included, following statistical analysis. Dogs were followed up for 1 year, when a significant protective

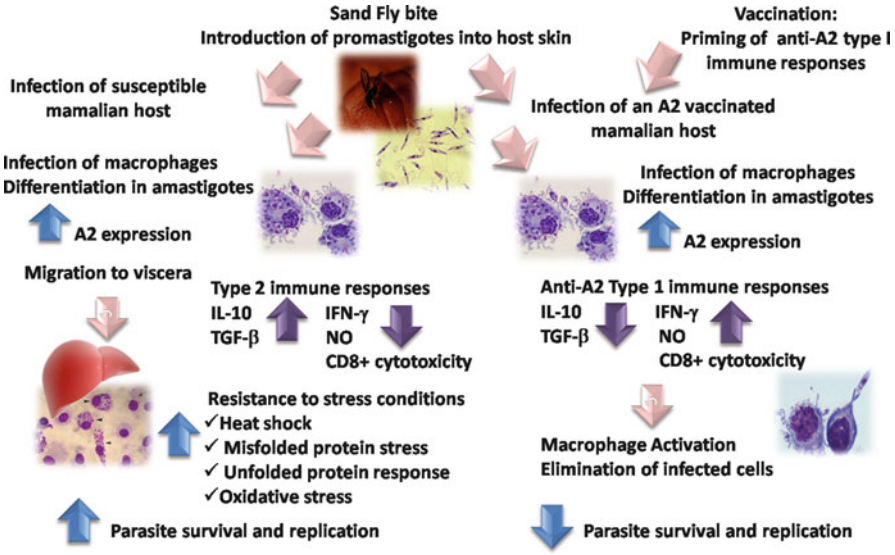

Fig. 3.4 Summary of the A2 main biological and immunogenic properties in a susceptible *Leishmania* infected or in a vaccinated host, respectively. *Blue arrows*: indicate A2 associated conditions. *Pink arrows*: host associated conditions. *Purple arrows*: associated immune responses. *Upside arrows* indicate increased processes and *down side arrows*, decreased processes

efficacy (71 %) was observed in Leish-Tec® vaccinated animals, based on the recovery of parasites through culture of bone marrow aspirates. Amongst the animals that presented anti-A2 antibodies in response to Leish-Tec®, 82 % of protection was achieved [Machado-Coelho et al., unpublished results]. Furthermore, Leish-Tec® was also shown to be safe for a heterogeneous dog population and was licensed for commercialization in Brazil, being the first recombinant protein based vaccine to be licensed for dogs in the world (Fernandes et al. 2012).

Since Rhesus monkeys are one of the closest infection models to human VL, demonstration of protective responses in this model is therefore an important step towards developing vaccines against human VL. A pre-clinical trial in this infection model was conducted, aiming to test, through homologous or heterologous prime-boost protocols, the protective responses induced by adenovirus expressing A2, plasmids expressing A2 and the recombinant A2. In comparison to the control groups, the vaccinated monkeys displayed a significant capacity to control parasite replication. The vaccination with recombinant protein and adenovirus expressing A2 provided the best results, leading to complete granuloma resolution and elimination of parasites in hepatic biopsies, as confirmed by real time PCR (Polimerase Chain Reaction) analysis [Grimaldi et al. manuscript in preparation].

Figure 3.4 summarizes of A2 biological and immunogenic properties in susceptible and vaccinated animals.

5.3.1 Other Applications of A2

A2 was originally identified as an amastigote-specific protein (Charest et al. 1996). Monoclonal antibodies produced against the A2 protein (Charest and Matlashewski 1994) has, therefore, been used by laboratories around the world as markers of differentiation from promastigotes to amastigotes (Saar et al. 1998; Gupta et al. 2001; Somanna et al. 2002; Goyard et al. 2003; Debrabant et al. 2004; Barak et al. 2005). The A2 5′ and 3′ UTR sequences have also been incorporated into a *Leishmania* expression vector for high level expression in transgenic amastigotes (Ghedin et al. 1998). In addition, the A2 3′UTR has also been used to direct the expression of suicide genes such as a truncated 3′nucleotidase/nuclease. Similarly, the herpes thymidine kinase was targeted to the A2 chromosomal locus. These were expressed specifically in amastigotes and led to decreased amastigote viability under drug pressure with acyclovir (Ghedin et al. 1998). Such negative selection may be useful for the generation of live attenuated *Leishmania* vaccines.

Finally, A2 has also been investigated for diagnostic purposes in humans and dogs, by detecting anti-A2 antibodies by Western blot, ELISA (Enzyme-Linked Immunosorbent Assay) and immunoprecipitation (Porrozzi et al. 2007; Zhang and Matlashewski 2010; Costa et al. 2012; Zhang et al. 2013). These could be used to diagnose *L. donovani* and *L. mexicana* infections in humans and were up to 92 % sensitive by immunoprecipitation. Anti-A2 antibodies were also detected post-treatment. *T. cruzi*, *L. tropica* and *L. braziliensis*-infected patients all gave negative results (Zhang and Matlashewski 2010). An ELISA to detect anti-A2 antibodies in dogs could detect both symptomatic and asymptomatic infections with a sensitivity of 94 %. Although sensitivity was high in this test, specificity was lower: positive results were also obtained in significant number of mucosal and cutaneous leishmaniasis cases as well as in 10 % of samples from leprosy patients (Zhang et al. 2013).

6 Conclusion

The investigation of species-specific proteins in *Leishmania* is an effective approach to study the biology and pathogenesis of this important human pathogen. Several such proteins have so far been implicated in the development of visceral disease, in which A2 is one of the most extensively studied. Further study is warranted to identify additional determinants of visceral disease. In addition to providing important insight into pathogenesis, A2 represents a promising vaccine antigen and emerging results have demonstrated that it is effective in vaccination against visceral leishmaniasis in dogs in Brazil. This may further suggest that it could also provide protection against human leishmaniasis. It will be interesting to determine whether other virulence factors may also represent vaccine candidates.

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Chapter 4
Arginase in *Leishmania*

Maria Fernanda Laranjeira da Silva and Lucile Maria Floeter-Winter

Abstract The presence of different sets of several enzymes that participate in the Krebs-Henseleit cycle has been used to identify several genera of trypanosomatids. One of these enzymes is arginase (L-arginine amidinohydrolase, E.C. 3.5.3.1), a metalloenzyme that catalyzes the hydrolysis of L-arginine to L-ornithine and urea. Arginase activity has been detected in *Leishmania*, *Crithidia* and *Leptomonas* but not in *Trypanosoma*, *Herpetomonas* or *Phytomonas*. The ureotelic behavior of some trypanosomatids is not due to urea excretion but to the production of ornithine to supply the polyamine pathway, which is essential for replication. *Leishmania* is found inside macrophages in the mammalian host and to live in these cells, the parasite must escape from several microbicidal mechanisms, such as nitric oxide (NO) production mediated by inducible nitric oxide synthase (iNOS). Since arginase and iNOS use the L-arginine as substrate, the amount of this amino acid available for both pathways is critical for parasite replication. In both promastigotes and amastigotes, arginase is located in the glycosome indicating that arginine trafficking in the cell is used to provide the optimal concentration of substrate for arginase. Arginine uptake by the parasite is also important in supplying the arginase substrate. *Leishmania* responds to arginine starvation by increasing the amino acid uptake. In addition to the external supply, the internal L-arginine pool also governs the uptake of this amino acid, and the size of this internal pool is modulated by arginase activity. Thus, arginine uptake and arginase activity are important in establishing and maintaining *Leishmania* infection.

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Abbreviations

AD	Arginine deiminase
AGM	Agmatinase
ARG	Arginase
ASL	Argininosuccinate lyase
ASS	Argininosuccinate synthetase
CAT	Cationic amino acid transporter
CH	Citrulline hydrolase
CPS1	Carbamoyl phosphate synthetase
EGFP	Enhanced green fluorescent protein
eNOS	Endothelial nitric oxide synthase
IFNγ	Interferon gamma
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LOHA	N^{ω}-hydroxy-L-arginine
LPG	Lipophosphoglycan
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
OCT	Ornithine carbamoyltransferase
ORF	Open reading frame
PEX	Peroxin
PST1	Peroxisomal targeting signal type 1
PV	Parasitophorous vacuole
SSU rRNA	Small subunit ribosomal RNA
UTR	Untranslated region

1 The Arginase Pathway

L-Arginine is one of the most versatile amino acids. It is used as a precursor not only for protein synthesis but also for the synthesis of NO, urea, ornithine, citrulline, creatinine, agmatine, glutamate, proline and polyamines (Wu and Morris 1998). This amino acid was first isolated from lupulus seeds in 1886 (Schulze and Steiger 1886) and was later identified as a constituent of proteins in animals (Herdin 1895). In 1897, Schulze and Winterstein established the structure of L-arginine by alkaline hydrolysis, which generates ornithine and urea. In 1910, Sorensen described the synthesis of arginine from benzoylornithine. In 1904, Kossel and Dakin identified the enzyme that hydrolyzes arginine to yield ornithine and urea in the liver, arginase. However, the elucidation of the prominent roles of arginine in important metabolic and physiological pathways did not occur until 1932, when the urea cycle was discovered by Krebs and Heiseleit (1932).

Arginase is a trimeric metalloenzyme that requires two atoms of manganese per subunit for full activity (Reczkowski and Ash 1992; Di Costanzo et al. 2005;

da Silva et al. 2008). This enzyme has regulatory roles, as it modulates the arginine availability in the cells that express this enzyme and it regulates polyamine synthesis due to the production of ornithine, a precursor of polyamines, which are essential for cell replication (Wu and Morris 1998).

Mammals have two arginase genes that encode two distinct isoforms, type I and II, that are similar with respect in enzymatic properties; however, these enzymes have distinct subcellular locations, tissue distribution, expression patterns and immunological reactivities (Grody et al. 1987; Jenkinson et al. 1996). Arginase I is cytosolic and is highly expressed in the liver, where it participates in the urea cycle, whereas arginase II is localized in the mitochondria and is expressed in the brain, kidneys, mammary glands, intestines and macrophages (Wu and Morris 1998). Some cell types express both isoforms, such as aorta endothelial cells in rats and murine macrophages (Wu and Morris 1998).

Protozoan organisms can eliminate nitrogen directly as ammonia; however, organisms of the Trypanosomatidae family have an interesting organization regarding urea cycle and arginine-citrulline-ornithine interconversion enzymes (Fig. 4.1a), whose activities are genus-specific (Camargo et al. 1992; Camargo 1999). Arginase is one of the urea cycle enzymes that is expressed in some trypanosomatids, including *Leishmania* spp., *Leptomonas* spp. and *Crithidia* spp.; remarkably, arginase is not expressed by *Trypanosoma* spp. (Camargo et al. 1978) (Fig. 4.1b). Interestingly, the arginase-expressing genera were found to form a monophyletic branch in the phylogenetic analysis of several trypanosomatids using SSU (small subunit) rRNA sequences (Briones et al. 1992) (Fig. 4.1c). This observation leads to the question of when during evolution did the arginase gene appear in these organisms.

Nevertheless, the genome projects of *Trypanosoma cruzi* and *Trypanosoma brucei* pointed the presence of arginase sequences in these organisms (XM_800602 and XM_841942) (Berriman et al. 2005; El-Sayed et al. 2005). However, given the similarity of arginase to agmatinase, we conducted an evolutionary analysis of arginase and agmatinase, and the sequences listed as arginase in the genomes of *T. cruzi* and *T. brucei* were found to be more similar to agmatinase than to arginase, even when using agmatinase sequences from distant organisms such as *Homo sapiens* and *Xenopus* sp. (Fig. 4.2). Like arginase, agmatinase is a metalloenzyme that hydrolyses agmatine to urea and putrescine in the presence of Mn^{2+} ions. This enzyme has been characterized in several organisms, from bacteria to vertebrates, such as humans (Hirshfield et al. 1970; Satishchandran and Boyle 1986; Sastre et al. 1998; Uribe et al. 2007). The alignment of the amino acid sequences of distinct arginases and agmatinases highlights conserved and semi-conserved residues, including the residues important for Mn^{2+} coordination (Fig. 4.3). Therefore, it is feasible that the trypanosomatids that do not express arginase produce instead putrescine as the result of agmatinase activity. The phylogenetic analysis (Fig. 4.2) does not allow knowing if the two sequences are products of gene duplication or even when that event occurred. The organisms that express arginase also harbor an agmatinase-like sequence, and agmatinase activity could function as a salvation

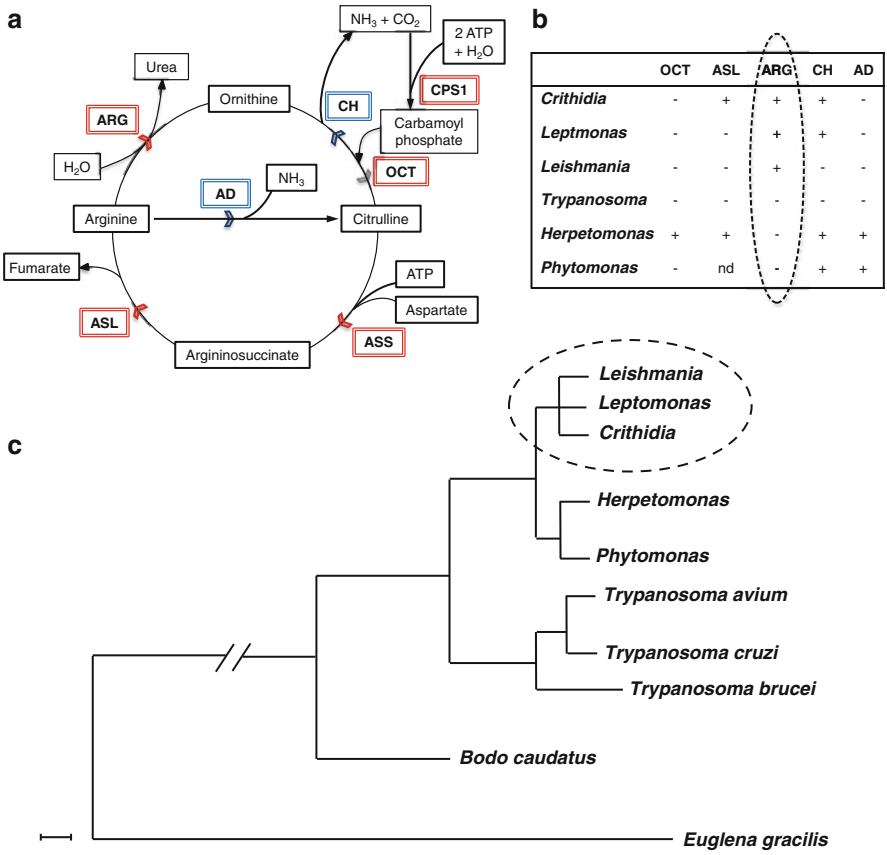

Fig. 4.1 (**a**) Urea cycle and arginine-citrulline-ornithine interconversion pathways. *CPS1* carbamoyl phosphate synthetase, *OCT* ornithine carbamoyltransferase, *ASS* argininosuccinate synthetase, *ASL* argininosuccinate lyase, *ARG* arginase, *CH* citrulline hydrolase, *AD* arginine deiminase. *Red boxes* indicate enzymes of the urea cycle. *Blue boxes* indicate enzymes of arginine-citrulline-ornithine interconversion. (**b**) Enzymes of the ornithine-arginine metabolism in trypanosomatids (Modified from Camargo et al. (1978), Batistoti et al. (2001)). + and −, indicate detectable and undetectable activity of the indicated enzyme, respectively. *nd* not determined. The *dashed circle* highlights ARG activity. (**c**) Phylogenetic tree based on SSU rRNA data of trypanosomatids obtained by Fitch and Margoliash method. The *bar* represents 0.01 substitution/sequence position (Modified from Briones et al. 1992)

route in the putrescine synthesis when arginase is knocked out (da Silva et al. 2012b). Most likely, in evolutionary terms, the physiological role of arginase may have provided some selective advantage that led to the maintenance of this gene in some genera.

The *Leishmania amazonensis* arginase gene, its transcript and the translated protein were characterized and used to predict the three-dimensional structure of the

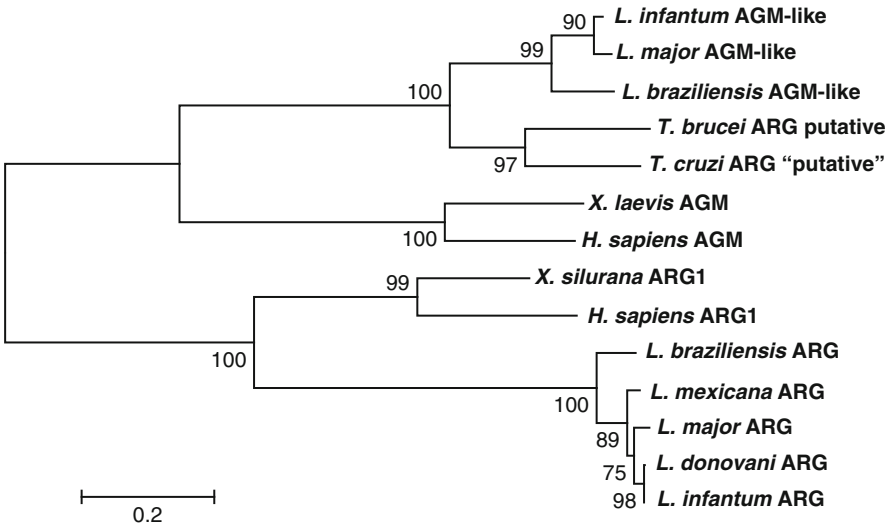

Fig. 4.2 Evolutionary tree of arginase/agmatinase of trypanosomatids. The evolutionary history of arginase (ARG) and agmatinase (AGM) coding region was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). The tree with the highest log likelihood (−9262.5640) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale (the bar), with branch lengths measured in the number of substitutions per site. The nucleotide sequences analysed are: *L. infantum* AGM XM_001465648.1, *L. major* AGM XM_001683277.1, *L. braziliensis* AGM XM_001565031.1, *T. brucei* ARG XM_841942.1, *T. cruzi* ARG XM_800602.1, *X. laevis* AGM NM_001095741.1, *H. sapiens* AGM NM_024758.4, *X. tropicalis* ARG NM_001006713.1, *H. sapiens* ARG NM_001244438.1, *L. braziliensis* ARG XM_001568200.1, *L. mexicana* ARG XM_003879095.1, *L. major* ARG XM_003722493.1, *L. donovani* ARG XM_003864686.1, *L. infantum* ARG XM_001468931. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011)

Leishmania enzyme by computational analysis of the deduced amino acid sequence. The inferred structure allowed the comparison of functional sites with those described for human (liver and macrophage) arginase, revealing a high degree of similarity, especially at the catalytic center (da Silva et al. 2002, 2008). Two important differences were detected: there are two non-conserved amino acids between human and *Leishmania* arginases that form different channel-like structures, and a difference in the charge in a cleft. It was suggested that these differences in the neighborhood of the active site could be exploited in the design of *Leishmania-specific* arginase inhibitors (da Silva et al. 2002, 2008). Although some studies identified inhibitors that are more effective against the parasite arginase, this task has proved to be tricky because these molecules also inhibit human arginase to a significant degree (Iniesta et al. 2001; da Silva et al. 2012a). However, irrespective to specificity, the subcellular location of the enzyme must be taken into account to identify effective drugs.

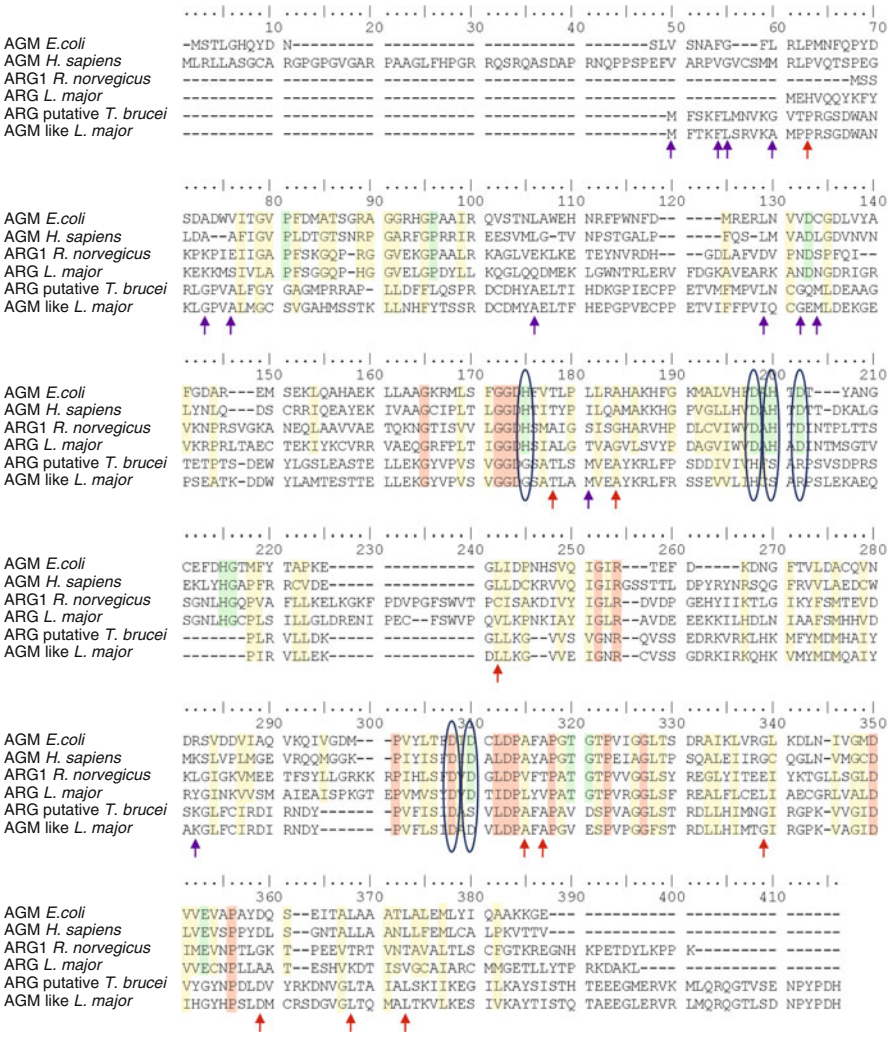

Fig. 4.3 Mapping of conserved and semi-conserved residues on the multiple alignment of arginase (ARG) and agmatinase (AGM) proteins: *E. coli* (P60651) and *H. sapiens* AGM (Q9BSE5), *R. norvegicus* ARG1 (P07824), *L. major* ARG (XP003722541.1), *T. brucei* putative ARG (XP847035.1) and *L. major* AGM-like proteins (XP001683329.1). Conserved and semi-conserved residues are highlighted in *red* and *yellow*, respectively. Residues conserved among non-putative sequences are highlighted in *green*. *Blue circles* highlight the position of the residues that coordinate Mn^{2+} ions in non-putative sequences (Manikandan et al. 2008). *Red* and *purple arrows* indicate conserved and semi-conserved residues, respectively, between AGMs and *T. brucei* putative ARG

2 Arginase's Role in Host Defense During *Leishmania* Infection

At first, the role of arginase was thought to be exclusively metabolic, related to arginine-ornithine-citrulline interconversion in the trypanosomatid genera expressing this enzyme (Camargo 1979). In contrast, arginine is also the precursor for the synthesis of nitrite, nitrate and NO in mammals (Hibbs et al. 1987).

In mammals, three nitric oxide synthase (NOS) isoenzymes have been described: neuronal NOS (nNOS, type I), inducible NOS (iNOS, type II) and endothelial NOS (eNOS, type III). Most animals express nNOS and eNOS constitutively in diverse tissues, and these enzymes are Ca^{2+}/calmodulin-dependent. iNOS is not constitutively expressed in most cells but is highly induced in macrophages by endotoxins and inflammatory cytokines (Wu and Morris 1998), and the main effect of NO produced by this pathway is antiproliferative (Boucher et al. 1999). Based on these data, the hypothesis arose that arginase could be essential for the regulation of macrophage iNOS activity by modulating the availability of L-arginine. Several studies support this hypothesis. First, it was shown that arginase II induction resulted in a decrease in NO production, which jeopardized the defense functions of macrophages (Wang et al. 1995). Another group observed diminished infectivity of *L. major* after treatment with an arginase inhibitor, N^{ω}-hydroxyl-L-arginine, that is an intermediate of NO synthesis (Iniesta et al. 2001).

In the vertebrate host, *Leishmania* parasites invade macrophages, cells that belong to the family of professional phagocytes in animals. Inside macrophages, *Leishmania* differentiate into the amastigote form that inhabits digestive vacuoles, which fuse to macrophage lysosomes, generating phagolysosomes. Therefore, these parasites are able to evade the humoral immune response, and the immunological response against them is cell-mediated (Rey 1992). One of the defense strategies of macrophages is the oxidative burst induced by phagocytosis. This process consists of NADPH oxidase activation, which leads to the transfer of protons to oxygen molecules, generating several highly reactive molecules, such as superoxides, hydrogen peroxides and hydroxyl radicals (Cunningham 2002). Another macrophage defense mechanism is vesicular acidification due to the activation of proton ATPases. This acidification causes protein denaturation, making parasites susceptible to acid hydrolases (Cunningham 2002). The induction of iNOS is another important macrophage defense mechanism and results in L-arginine oxidation and the production of citrulline and NO. As previously stated, NO is one of the most important highly reactive microbicidal molecules produced to combat invading pathogens (Qadoumi et al. 2002). To survive inside macrophages, *Leishmania* parasites have to evade all of these macrophage defense mechanisms.

Among *Leishmania*'s evasion strategies is the expression of lipophosphoglycans (LPGs) on the metacyclic promastigote surface, conferring resistance to lysis

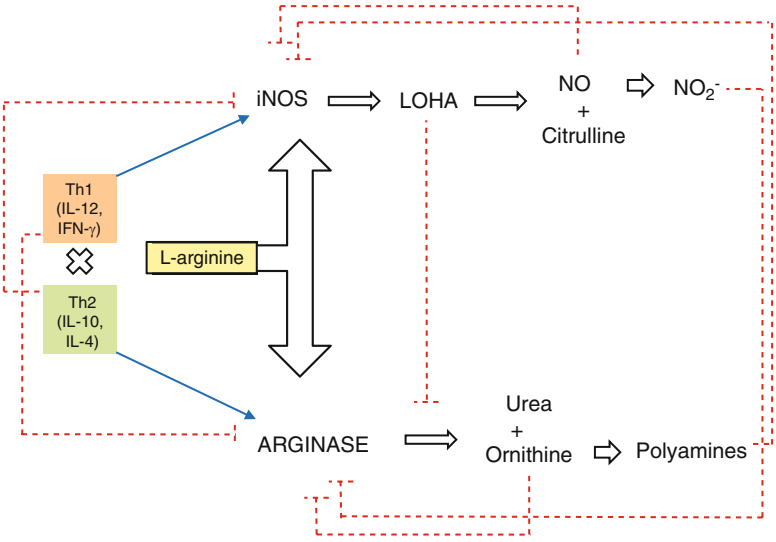

Fig. 4.4 Th1/Th2 immune responses cross-regulation and induction of arginase and iNOS. Classical activation of macrophages with Th1 cytokines (IL-12 and IFN**γ**) induces L-arginine metabolism by iNOS. Conversely, activation of macrophages with Th2 cytokines (IL-10 and IL-4) induces L-arginine metabolism by arginase. Arginase and iNOS products regulate reciprocally the activity of the each enzyme. Stimulatory routes are represented by continuous *blue arrows* and inhibitory routes by *dashed red arrows*. LOHA, N^{ω}-hydroxy-L-arginine

mediated by the complement system (Sacks et al. 1995). Once inside macrophages, the parasite LPGs participate in the inhibition of phagosome – endosome fusion, enabling the escape from lysosomal enzymes (Descoteaux and Turco 1999). In addition, these parasites also have mechanisms to diminish iNOS enzyme activity and hence NO production (Bogdan and Rollinghoff 1999). The regulation of NO production is crucial in this scenario, and the fate of the infection depends on the balance of Th1 and Th2 immune responses. The Th1 response implies a cellular immune response that leads to macrophage activation and antibody opsonization. In contrast, the Th2 response directs the organism's defense to a humoral response that is ineffective against *Leishmania* infection (Abbas and Lichtman 2007). The Th1/Th2 balance also regulates arginine metabolism, directing the cells to favor hydrolysis by arginase or oxidation by iNOS. IFN-γ is produced during a Th1 response and induces iNOS expression, whereas IL-4 and IL-10 are produced during a Th2 response and induce arginase expression (Corraliza et al. 1995). The induction of one of these enzymes leads to the inhibition of the other one, indicating the existence of two competitive metabolic states (Hrabak et al. 1996; Munder et al. 1998) (Fig. 4.4).

Vertebrates' susceptibility to *Leishmania* infection is mediated by the Th2 response via arginase activity and polyamine synthesis, which favor parasite replication in macrophages and reduce the amount of arginine available for NO synthesis (Iniesta et al. 2005; Kropf et al. 2005). *Leishmania* infection resolution is mediated

by the Th1 response, which induces iNOS, thus promoting parasite killing by macrophages (Alexander and Bryson 2005; Wanasen and Soong 2008). *Leishmania* parasites also express their own arginase (da Silva et al. 2002, 2008; Roberts et al. 2004), adding another piece in the competition for arginine. In addition to the hypothesis regarding the importance of the host arginase, the role of the parasite arginase must also be considered. This role would be to modulate L-arginine availability in infected cells by shifting arginine consumption to polyamine production through the activity of the parasite arginase, thus favoring parasite survival and replication (da Silva et al. 2012b).

3 Arginase's Role in *Leishmania* Physiology

The genomic characterization of arginase enabled the construction of arginase knockout and add-back parasites that yielded significant information about the functional role of this enzyme and about how the subcellular location of this enzyme influences its function (da Silva et al. 2012b). The characterization of *L. mexicana*, *L. major* and *L. amazonensis* arginase knockout parasites demonstrated that the arginase pathway is essential for *in vitro* proliferation because the absence of enzyme activity made these knockout parasites auxotrophic for polyamines. Confirming the importance of *Leishmania* arginase for all parasite life stages, these mutants also exhibited attenuated *in vitro* and *in vivo* infectivity (Roberts et al. 2004; Reguera et al. 2009; da Silva et al. 2012b). The attenuated profile observed during infection with *L. mexicana* knockout parasites was attributed to an increase in host NO production due to greater L-arginine availability (Gaur et al. 2007). In contrast, the attenuated profile of *L. major* knockout infection was not due to NO overproduction, and the profile of induced cytokines was not different from that induced by wild-type parasites. These results suggest that the effect of arginase on *L. major* infection is not associated with the host immune response (Muleme et al. 2009). These results also stress the important differences among *Leishmania* species.

The analysis of *Leishmania* arginase genes revealed the presence of one of the most frequent glycosome import signals, the PST1 carboxyl signal, which consists of tree amino acids – SKL (Opperdoes and Szikora 2006; da Silva et al. 2008). Glycosomes are peroxisome-like organelles found only in kinetoplastids, and the presence of glycosomes represents one of the main differences between the parasite and the host. A portion of carbohydrate metabolism is compartmentalized in this organelle (Opperdoes 1987; Sommer et al. 1996; Opperdoes and Szikora 2006). The compartmentalization of the first seven enzymes of the glycolysis pathway (hexokinase, phosphoglucose isomerase, phosphofructokinase, aldolase, triosephosphate isomerase, glyceraldehyde-phosphate hydrogenase and phosphoglycerate kinase) and of two other enzymes that metabolize glycerol (glycerol kinase and glycerol 3-phosphate dehydrogenase) contributes to a significant increase in the level of ATP production by glycolysis, in addition to protecting the parasites from the accumulation of toxic glycolysis intermediates (Haanstra et al. 2008). Thus, through this

compartmentalization, the parasites manage to overcome the limited energy yielded by the conversion of glucose into pyruvate during the vertebrate phase of the life cycle (Sommer et al. 1996).

The glycosomal proteins are synthesized by free ribosomes in the cytoplasm and are then imported into glycosomes (Hart et al. 1987) without post-transcriptional processing (Moyersoen et al. 2003). The protein importation into the glycosome requires the same carboxy-terminal peptidic signals described for peroxisomes, confirming that both organelles have a common ancestor and that the importation mechanism is conserved (Sommer et al. 1996). Moreover, the glycosome import mechanism also depends on several peroxins (PEX), as also observed for peroxisomes, and these proteins were shown to be essential for trypanosomatids (Guerra-Giraldez et al. 2002; Moyersoen et al. 2003; Krazy and Michels 2006).

In addition to the presence of the SKL signal at the C-terminus, the use of arginase fused to EGFP (enhanced green fluorescent protein) suggested that arginase is compartmentalized in the glycosomes of *L. mexicana* promastigotes (Roberts et al. 2004). Another strategy using *L. amazonensis* promastigotes that express a glycosomal-targeted EGFP confirmed the location based on the co-localization of the fluorescent protein with arginase immunolabeling (da Silva et al. 2008). Importantly, during macrophage infection, arginase remains compartmentalized in the glycosomes of amastigotes (da Silva et al. 2012a).

Previous data have suggested that the glycosomal milieu is not essential for the role of arginase in polyamine biosynthesis (Roberts et al. 2004). However, a further study revealed that the incorrect localization of arginase impairs parasite proliferation and attenuates infection (da Silva et al. 2012a). The results of this study showed that the proper subcellular compartmentalization of arginase in the glycosome of *L. amazonensis* is important for enzyme activity and proper physiological functioning during parasite infection.

4 Arginine Trafficking Through *Leishmania*-Infected Macrophages

To favor either arginase or iNOS activity, it is necessary to increase the influx of L-arginine into the host cell (Yeramian et al. 2006). In mammals, the transport of L-arginine occurs through a family of cationic amino acid transporters known as CATs (cationic amino acid transporters), the properties of which resemble that of the y + amino acid transport system. There are six CAT family members, CAT1, CAT2A, CAT2B, CAT3, CAT4 and CAT14. The functions of CAT4 and CAT14 are not well known, and the remaining members carry L-arginine, L-lysine and L-ornithine. CAT2A and CAT2B are the cationic carriers relevant to macrophages and are produced by the alternative splicing of the same gene (Closs et al. 2006). CAT2B is responsible for the influx of arginine in response to cytokines produced during Th1 or Th2 immune responses (Visigalli et al. 2004). CAT2B blockage leads to undetectable levels of iNOS and arginase activity, suggesting that the

intracellular arginine concentration is important for this amino acid metabolism by both enzymes (Yeramian et al. 2006). This study also reinforced the hypothesis that the respiratory burst in macrophages can be regulated by the cross-competition between arginase and iNOS for substrate (Hrabak et al. 1996).

In macrophages infected with *Leishmania*, when a Th2 response occurs, there is an increase in CAT2B expression and a repression of iNOS expression (Wanasen et al. 2007; Tuon et al. 2008; Calegari-Silva et al. 2009). The increase in arginine influx without iNOS activation leads to an increase in parasite replication, most likely due to a consequent increase in the uptake of the amino acid by the parasite and its use by arginase to produce ornithine for replication. This hypothesis relies on the existence of an arginine transporter in the parasite and is reinforced by the ability of *Leishmania* to uptake arginine from external medium (Kandpal et al. 1995). The AAP3 arginine symporter, which is made up of 480 amino acids and 11 transmembrane domains, has also been described for *L. donovani* (Shaked-Mishan et al. 2006) and for *L. amazonensis* (Castilho-Martins et al. 2011).

The characterization of the AAP3 coding region revealed the duplication of the gene, with the two copies organized *in tandem* in the genome of both parasites. In *L. amazonensis*, the open reading frames (ORFs) of the two copies exhibit a similarity of 98 %, but the 5 ′and 3′ UTRs of each copy exhibit differences that were exploited to design probes to quantify the transcripts from each copy. One of the transcripts is present at a low copy-number along the growth curve of promastigotes. The other transcript is more abundant and exhibits regulated expression throughout the growth curve, increasing by two orders of magnitude in the stationary phase. Given that a decrease in nutrient supply in culture medium occurs in this phase, the expression of this transcript, as well as the transport activity, were measured in parasites submitted to arginine deprivation. Under these conditions, both arginine uptake and the amount of the regulated transcript increased significantly (Castilho-Martins et al. 2011).

The deprivation experiment was repeated using *L. amazonensis* null and add-back arginase mutants, for which the endogenous concentrations of arginine have also been evaluated. Both the amount of the regulated transcript and the amount of arginine taken up were inversely correlated with the intracellular amount of arginine. The mutants lacking arginase activity had an intracellular arginine concentration that was approximately fourfold greater than that in the wild-type parasite or the add-back mutant (da Silva et al. 2012b). Likewise, the null mutants exhibited a decreased amount of transcript and a lower arginine uptake (Castilho-Martins et al. 2011). Thus, the transport of arginine is regulated by the amount of the amino acid present in the external medium and by the intracellular concentration.

5 Concluding Remarks

Arginase activity is essential for *Leishmania* proliferation and infectivity. Besides, its proper glycosome compartmentalization is important for activity and role in parasite physiology. Parasite arginase participates in a complex system, along with

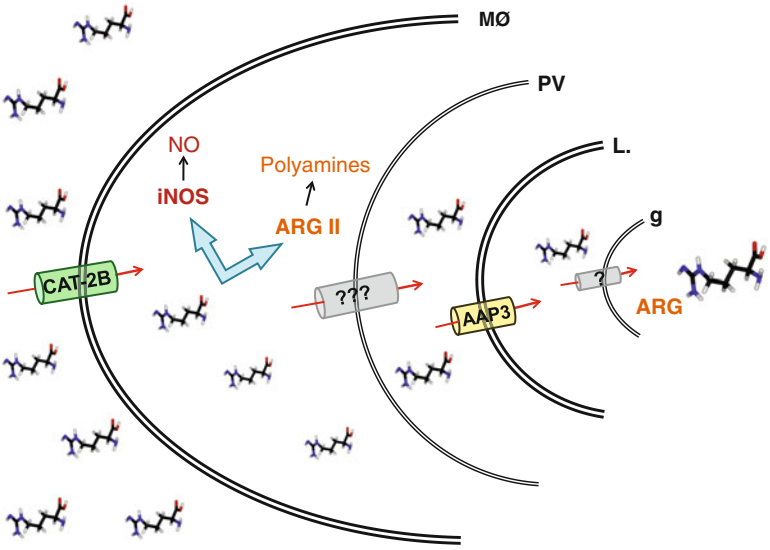

Fig. 4.5 L-arginine traffic through macrophages infected with *Leishmania*. Arginine is taken up by macrophage (Mø) through CAT-2B membrane transporter. In the cytoplasm of the Mø the amino acid is metabolized by Mø's arginase (ARG II) or iNOS, or is taken up by parasitophorous vacuolum (PV) through an unknown mechanism. In order to achieve *Leishmania*'s arginase (ARG), L-arginine must be taken up by parasite through the AAP3 transporter and then must reach the glycosome (g) by unknown mechanisms

host arginase and iNOS besides parasite own NO synthesis pathway, and then L-arginine fate is a crucial step defining host or parasite success.

Therefore, *Leishmania* arginase can be considered as a possible target for chemotherapy, but the compartmentalization of this enzyme in the glycosome and the mechanisms of arginine trafficking (Fig. 4.5) indicate that a better strategy for the development of new treatments for leishmaniasis may reside in the inhibition of the arginine trafficking or even arginase import into the glycosome.

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Chapter 5
The Heat Shock Proteins of *Trypanosoma cruzi*

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Abstract *Trypanosoma cruzi* is the causal agent of Chagas' disease, a debilitating disorder affecting millions of people in several countries. A flagellated protozoan parasite, *T. cruzi* has a complex life cycle that involves infecting an insect and a mammalian host. During its life cycle, the parasite undergoes several kinds of stress, prominent among which is heat stress. To deal with this environmental challenge, molecular chaperones and proteases, also known as heat shock proteins (HSPs), are induced as part of the stress response. Several families of HSPs are synthesized by *T. cruzi*, including members of the major HSP classes such as HSP70, HSP90, HSP100, HSP40, chaperonins and small HSPs, and these proteins show conserved and unique features. *In this review* we describe these proteins and the corresponding gene expression patterns and discuss their relevance to the biology of the parasite.

Abbreviations

Clp	Caseinolytic protease
CHR	Cellular homeostasis response
CSR	Cellular stress response
HSP	Heat shock protein

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LIT	Liver infusion tryptose
NBD	Nucleotide-binding domains
ROS	Reactive oxidative species
sHSP	Small heat shock protein
UTR	Untranslated region

1 The Cellular Stress Response

Variations in environmental conditions must be dealt with by the organism to maintain the structural and functional integrity of cellular components. At the cellular level, two kinds of responses can be discerned: the cellular stress response (CSR) and the cellular homeostasis response (CHR) (reviewed by Kültz 2005). CSR is transient and deals with macromolecular integrity and redox potential, while CHR is environmental stressor-specific and leads to adaptation until the conditions change. CSR is not specific to the type of stress but to the type of damaged macromolecule, and it leads to stress tolerance. Damage to proteins, DNA, and membrane lipids is monitored by the cell, and the response involves: (a) cell cycle control; (b) protein chaperoning, repair or degradation; (c) DNA and chromatin stabilization and repair; and (d) modifications of cellular metabolism. The basic CSR is very evolutionarily conserved, but several species-specific aspects and components are present that reflect the particular circumstances of the organism. Typical CSR inducers are heat and oxidative stress. Here we review the current knowledge on *T. cruzi*'s response to heat stress with emphasis on experimentally characterized proteins and genes.

2 Environmental Stresses Associated with *T. cruzi*'s Life Cycle

During its life cycle, *T. cruzi* has to cope with several environmental changes, including shifts in temperature, low availability of nutrients, changes in osmolarity, and the presence of highly reactive oxidative species (ROS). The stress response in all these situations is similar to that of other protozoan parasites, and is varied and sophisticated (see reviews by Vonlaufen et al. 2008; Ürményi et al. 2012). For example, *T. cruzi* increases the synthesis of heat shock proteins (HSPs) or DNA repair enzymes when some macromolecules are damaged, and synthesizes non-enzyme redox molecules and increases the activity of superoxide dismutases and peroxidases when ROS are present. Although harmful in general, environmental stresses may be crucial for *T. cruzi*'s development, since nutritional stress can induce metacyclogenesis (Contreras et al. 1985). In any case, the parasite's stress response is essential for viability in various situations, such as the heat and osmotic

stress responses during transition from the insect to the mammalian host, and the oxidative stress response when invading mammalian cells. The parasite's stress response is therefore important for disease transmission and pathogenesis. It is also relevant to treatment, since the drugs of choice for Chagas' disease, nifurtimox and benznidazole, are thought to interfere with the parasite's oxidative stress response (Wilkinson and Kelly 2009).

3 Genome Organization and Gene Expression in *T. cruzi*

T. cruzi's stress response involves modulation of gene expression, which shows a number of peculiar features. The genome of the parasite contains more than 12,000 protein-coding genes and is composed of a large percentage of repetitive sequences (El-Sayed et al. 2005). The diploid genome is estimated to be comprised of 41 pairs of chromosomes (Weatherly et al. 2009), and genome size varies from 44 MB to 55 MB, depending on the strain (Franzén et al. 2011). Protein-coding genes are arranged head-to-tail with usually small intergenic regions in large, directional gene clusters that may span a considerable portion of the chromosome and are separated by convergent or divergent strand-switch regions. Divergent strand-switch regions have specific epigenetic marks, such as acetylated H3K9/H3K14 and H4K10 and methylated H3K4, in a pattern that suggests the presence of bidirectional promoters (Respuela et al. 2008). *T. cruzi*'s gene clusters are transcribed as multicistronic RNAs that are subsequently processed into mature mRNAs by *trans*-splicing and polyadenylation. In *trans*-splicing, a capped, short spliced leader RNA is added to the 5′ untranslated region (UTR) of all coding sequences via two transesterification reactions (reviewed by Martínez-Calvillo et al. 2010). The pervasive generation of individual mRNAs by post-transcriptional processing from polycistronic transcripts implies that gene regulation is exerted by post-transcriptional mechanisms such as modulation of mRNA stability and translation (Kramer 2012). Cell form-specific gene expression has also been investigated (Atwood et al. 2005; Cuervo et al. 2010). Proteome analysis of epimastigotes (present in the insect vector), amastigotes, metacyclic trypamostigotes and bloodstream trypomastigotes (present in the mammal) showed that 30 % of detected proteins are present in all developmental stages, and that there is a shift from carbohydrate- to lipid-based energy metabolism in the transition from trypomastigotes to amastigotes. In addition, metacyclogenesis is accompanied by a reduction in ribosomal proteins, consistent with trypomastigotes being unable to divide, and by an increase in antioxidant enzymes, adequate for the intracellular environment of phagocytic cells. However, despite the wealth of biological information described above, sequence similarity-based annotation of the genome shows that at least half of the protein-coding genes identified are of unknown function, highlighting low little we understand this organism at the molecular level.

4 Heat Shock Proteins

Proteins need to be conformationally flexible to execute their cellular functions, and are optimized to be marginally stable at growth temperatures (reviewed by Richter et al. 2010). Organisms are therefore sensitive to small temperature changes, which cause protein unfolding and, due to the very high intracellular protein concentration, aggregation and in general loss of protein homeostasis. Presumably unfolded proteins trigger the stress response, and so any denaturing agent besides heat, such as oxidative species, heavy metals, ethanol or organic substances, also induces the stress response. Heat stress causes defects of the cytoskeleton, fragmentation of the endoplasmic reticulum and the Golgi apparatus, decreased number of lysosomes and mitochondria, changes in membrane permeability, and decreased translation. The stress response protects the cell from these adverse effects, and a previous heat shock results in the cell being much less affected by a subsequent heat shock, a phenomenon called thermotolerance. Heat stress-induced proteins are grouped in seven classes: (a) molecular chaperones, which help other proteins return to their native conformation; (b) components of the proteolytic system, to degrade irreversibly denatured and/or aggregated proteins; (c) DNA- and RNA-modifying enzymes, to remove heat-induced modifications of nucleic acids; (d) metabolic enzymes, to reorganize and stabilize the energy supply of the cell; (e) regulatory proteins that initiate further stress response pathways or inhibit expression cascades; (f) cytoskeleton-sustaining proteins; and (g) transport proteins, detoxifying enzymes and membrane-modulating proteins that are needed to restore membrane stability and function (Richter et al. 2010 and references therein). Most HSPs are molecular chaperones, which comprise the best studied and model group for the cellular response to heat stress. This class of proteins is divided in several conserved families according to molecular weight and broad function, among which the five major are: HSP104, HSP90, HSP70, chaperonins, and small HSPs (reviewed by Young et al. 2004; Kriehuber et al. 2010). In addition, HSPs have been shown to be antigenic and immunogenic in various situations, including cancer and infectious diseases. The immunogenicity of HSPs is a consequence of the peptide- and protein-binding properties of these proteins, which facilitates interaction with the intracellular antigen-presenting machinery of the major histocompatibility complex type I. This interaction, in turn, affects innate and adaptive immunity and may influence survival of intracellular pathogens (reviewed by Srivastava 2002).

HSPs and the heat stress response of protozoan parasites have been the subject of several recent reviews (Folgueira and Requena 2007; Vonlaufen et al. 2008; Shonhai et al. 2011). Digenetic parasites frequently are submitted to temperature changes during their life cycles, and HSPs are, as expected, part of their heat stress response. Although intracellular organisms and parasites frequently undergo genome reduction, the chaperone gene complement is sometimes expanded in protozoan parasites, highlighting the importance of these proteins in the biology of these organisms. HSP70 and HSP40 gene families are examples of these expansions (Folgueira and Requena 2007; Shonhai et al. 2011). HSPs have also been shown to

be important for parasite intracellular survival, differentiation and virulence. In fact, anti-HSP antibodies are present in Chagasic patients and in patients with other parasitic and bacterial infectious diseases.

4.1 Heat Stress in T. cruzi

T. cruzi's response when exposed to elevated temperatures in culture has been extensively studied (Carvalho et al. 1987; Alcina et al. 1988; Requena et al. 1992; de Carvalho et al. 1994; Olson et al. 1994; Rondinelli 1994). Reduced motility and morphological changes to a round shape can be seen at 40 °C or above (Requena et al. 1992; de Carvalho et al. 1994; Pérez-Morales et al. 2012). Some variability is observed depending on the strain, but in general increased levels of HSP100, HSP90, HSP70 and HSP60 can be detected when epimastigotes are incubated from 37 °C to 43 °C. Different from higher eukaryotes, however, these proteins are already present in non-heat shocked parasites (Dragon et al. 1987; Requena et al. 1992; de Marval et al. 1993). The typical general inhibition of protein synthesis during heat shock can be seen at 40 °C but not at 37 °C, and it can be prevented by incubating the parasite in serum (Carvalho et al. 1987). The mechanism by which serum protects protein synthesis remains to be explained. *T. cruzi*'s heat stress response also confers thermotolerance, since preincubation at a high but permissible temperature allows the parasite to withstand a subsequent otherwise lethal temperature (J.F. Carvalho et al. unpublished). In addition, a dose-dependent induction of HSP90 was seen after ethanol treatment, showing that a non-heat stress can also induce HSPs in this organism (Giambiagi-de-Marval et al. 1993b). Finally, incubation of epimastigotes at 40 °C for 2 h leads to an inhibition of *trans*-splicing and to a reversible and partial accumulation of mRNAs in the nucleolus (Názer et al. 2012). The HSP70 mRNA, however, remains distributed throughout the cytoplasm, suggesting that components of the stress response may bypass such nucleolar retention (Názer et al. 2012). Recently, a proteomic approach was used to investigate proteins induced by elevated temperatures in epimastigotes (Pérez-Morales et al. 2012). Using 2D gel electrophoresis followed by mass spectrometry, 24 proteins were identified whose levels changed after incubation at 37 °C or 42 °C, 19 of them increasing and 5 decreasing. The proteins were assigned to the following functional categories (with the protein with the largest level change in parenthesis): metabolism (transitional endoplasmic reticulum ATPase), cell defense (HSP70), interaction with the cellular environment (mucin-associated surface protein), protein fate (ubiquitin hydrolase), cellular transport (Rab6 GTPase-activating protein), protein synthesis (cysteinyl-tRNA synthetase), cell cycle (retrotransposon hot-spot protein) and unknown function (Pérez-Morales et al. 2012). As seen in other eukaryotes, these changes adapt the organism's metabolism and subcellular structures to the new condition.

Since *T. cruzi* naturally undergoes a heat shock when it transits from an insect to a mammalian host during its life cycle, it is assumed that the parasite synthesizes HSPs in preparation for infection. In fact, metacyclic trypomastigotes, the infective

form, show increased levels of HSP70, HSP60 and HSP90 when compared to epimastigotes (Paba et al. 2004), although it is still unclear whether HSP induction is part of the differentiation process or is just an adaptation to the new environment (Folgueira and Requena 2007). However, elevated temperatures inhibit metacyclogenesis in infected insects but do not affect parasite survival (Garcia et al. 2011). These results suggest that any preparedness to higher temperatures is restricted to the metacyclic trypomastigote. The possibility that elevated temperature by itself could trigger a differentiation event was also investigated. Consistent with the results in infected insects, heat treatment does not induce differentiation to the infective form in *T. cruzi* (Contreras et al. 1985; Rondinelli et al. 1988). However, incubation temperature can promote differentiation in an *in vitro* cycle of the parasite in axenic culture (Rondinelli et al. 1988). In this cycle, the authors have shown that they are able to induce differentiation of *in vitro*-generated amastigote-like cells to epimastigotes or to trypomastigote-like cells depending on the temperature of incubation (29 °C or 37 °C) and culture medium type (liver infusion tryptose (LIT) or serum). These results suggest that temperature can influence a differentiation program in this parasite, although it is unclear whether the heat stimulus has such an effect in its natural life cycle. Recently, the effects of severe heat treatment on RNA-binding proteins have been investigated. In epimastigotes incubated at 40 °C, TcSR62 and TcPTB2, an SR (serine/arginine rich)- and a polypyrimidine sequence-binding protein, respectively, partially accumulate in the nucleolus in an active transport-dependent manner (Názer et al. 2011). This accumulation is reversible upon return to normal temperatures, and suggests the participation of the nucleolus in the heat stress response of the parasite (Názer et al. 2011).

The main HSP families of *T. cruzi* are reviewed in the following sections.

4.1.1 HSP100 Family

The HSP100 family of chaperone proteins is a member of the AAA+ superfamily of ATPases. The family is comprised of hexameric protein complexes that act on proteins in different states of folding and/or aggregation, leading to protein unfolding, disassembly and disaggregation or degradation. The proteins are present in bacteria (called Clps, for caseinolytic proteases) and in eukaryotes both in a mitochondrial and a cytosolic form (called HSP100s). Two classes are described based on domain organization and biochemical activity. Class 1 proteins contain two nucleotide-binding domains (NBDs) and promote either disaggregation or proteolysis. Class 2 proteins have only one NBD and promote proteolysis. Bacterial ClpB and its human ortholog, HSP104, are class 1 HSP100s that are characterized by the presence of a Walker-type NBD. These proteins help organisms survive severe stress by being capable of disentangling even insoluble protein aggregates. It is believed that the HSP100 complex pulls the misfolded protein through the central pore of the hexameric ring, reinitiating folding. ClpB and HSP104 are induced by heat shock and act in concert with HSP70. They are also required for thermotolerance (reviewed by Doyle and Wickner 2009; Richter et al. 2010).

Orthologs of both HSP104 and of bacterial ClpB have been identified in *T. cruzi*'s genome sequence, the latter presumably having a function in the mitochondrial protein metabolism (Folgueira and Requena 2007). With the gene sequence available as a starting point, a member of the HSP104 family has been recently characterized in *T. cruzi* (Campos et al. 2012). A single copy gene is present in the genome, and the primary sequence of 869 amino acid residues contains the characteristic N-terminal domain and the Walker A- and B-type NBD domains. A 3D structure of the hexameric protein was obtained by comparative modeling, and the predicted structure is markedly different from the human ortholog, which may be relevant for potential vaccine or drug targets. The HSP104 gene expression pattern was also investigated, and the protein is induced at 37 °C and 40 °C, accumulating at least up to 24 h. mRNA levels also increase 3.5-fold and almost twofold after 3 h at 37 °C and 40 °C, respectively, suggesting an increase in mRNA processing and/or stability. Additional work is required to determine the subcellular compartmentalization of the protein, its relevance to thermotolerance in the parasite and the corresponding mechanisms of gene expression.

4.1.2 HSP90 Family

HSP90 is present at very high levels in the cytosol of both bacteria and eukaryotic cells, and their levels are increased further during heat stress. Five different isoforms are described in eukaryotes: an inducible cytosolic HSP90, a constitutive cytosolic HSP90, an endoplasmic reticulum glucose-regulated protein 94 (Grp94), a mitochondrial tumor necrosis factor receptor-associated protein 1 (TRAP1) and a membrane-associated HSP90N. Unlike other HSPs, HSP90 has several peculiar characteristics: (a) it is more selective regarding protein substrates; (b) it does not bind unfolded proteins, only native-like polypeptides; and (c) it is the most sophisticated chaperone machine of eukaryotes, interacting with several cochaperones in a defined order. Of the more than 300 known HSP90 substrates, most are transcription factors and kinases involved in cell cycle control and signal transduction. HSP90 functions in an ATP-dependent manner associated with the cochaperones HSC70, HSP40, HOP and p23. HOP, or STI-1, is also induced upon heat shock and is believed to keep HSP90 in a conformational state that facilitates its interaction with the protein substrates. The mechanistic details of HSP90 function, however, are less well understood (reviewed by Young et al. 2004; Richter et al. 2010).

The HSP90 genes of *T. cruzi* are organized as six copies arranged in tandem in the genome (Dragon et al. 1987; Folgueira and Requena 2007). The genes give rise to a 704-amino acid residue protein of 85 kDa, which is present in both epimastigotes and trypomastigotes and is usually called HSP83 (Dragon et al. 1987). The protein is already present in non-heat-shocked cells, and is induced in elevated temperatures (Carvalho et al. 1987; Dragon et al. 1987). Unlike mammalian HSP90, which binds ATP but has no ATPase activity, the parasite's HSP83 is a highly active ATPase (Nadeau et al. 1992). In addition, its ATPase activity is stimulated up to fivefold by 6-mer and 24-mer peptides, and the protein is probably

autophosphorylated (Nadeau et al. 1992). HSP83 can also functionally complement an *hsp90* yeast mutant (Palmer et al. 1995). Treatment of *T. cruzi* with the HSP90 inhibitor geldanamycin produces a dose-dependent increase in HSP100, HSP83 and HSP70 levels and proliferation arrest at G1, indicating the importance of HSP83 in the parasite's cell cycle control (Graefe et al. 2002). Since it has been described that inhibition of HSP83 induces differentiation in *L. donovani* (Wiesgigl and Clos 2001), the effects of geldanamycin on *T. cruzi*'s differentiation were also tested. Bloodstream trypomastigotes showed morphological changes (round cell shapes) in the presence of the drug, but no differentiation to epimastigotes occurred (Graefe et al. 2002). The ortholog of HSP90-associated factor HOP, TcSTI-1, was also characterized in the parasite and shown to interact with HSP83 (Schmidt et al. 2011). *TcSTI-1* is present as a single copy gene, and the protein is composed of 556 amino acid residues with a predicted molecular weight of 63.1 kDa. *TcSTI-1* does not appear to be readily induced by different stressors, as protein and mRNA levels remain unchanged under heat, nutritional or pH stress conditions in exponentially growing epimastigotes. However, TcSTI-1 is induced by nutritional stress in late-phase growth epimastigotes, indicating that growth phase affects the stress response in epimastigotes. In addition, the presence of additional members of the HSP90 family was investigated in the parasite's genome. Three orthologs of the endoplasmic reticulum (Grp94) and two of the mitochondrial (TRAP-1/HSP75) members of the HSP90 family have been found in the genome sequence (Folgueira and Requena 2007; Shonhai et al. 2011), although these genes have not yet been characterized experimentally. It remains to be seen whether HSP83 is involved in signal transduction in the parasite.

4.1.3 HSP70 Family

The HSP70 family is the most conserved and studied of HSPs, and one of the most conserved families of proteins in general. There are induced and constitutive paralogs (the latter called HSC70, or HSP70 cognate protein), and family members localized in the cytoplasm, mitochondria and endoplasmic reticulum (where it is called Grp78). Two major families have been recognized in the eukaryotic HSP70 superfamily: HSPA, with 13 typical HSP70 proteins, and HSPH, with four HSP110/Grp170 protein members. HSP70 proteins exert several different functions: (a) binding to nascent polypeptide chains to prevent premature folding; (b) disassembling of protein aggregates and assisting refolding of misfolded proteins; (c) directing proteolytic degradation of denatured or unstable proteins; (d) assisting membrane translocation of organelle and export proteins; and (e) modulating the activity of regulatory proteins. HSP70s typically contain a 45-kDa N-terminal ATPase domain and a C-terminal substrate-binding domain, which contains the conserved EEVD motif that is necessary for association with co-chaperones such as HSP40 and HOP/STI-1. HSP70 functions by binding and releasing, in an ATP-dependent manner, an extended polypeptide substrate, in association with HSP40 (reviewed by Hartl and Hayer-Hartl 2002; Young et al. 2004). It has also been described that HSP70 may

act as a signal transducer by being released extracellularly and interacting with cells of the immune system with immunoregulatory effects, known as the chaperokine activity of the protein (Asea 2003).

In *T. cruzi*, HSP70 proteins are composed of approximately 650 amino acid residues, and possess the typical ATPase domain. The HSP70 genes of different subfamilies were initially characterized as present in ten tandemly repeated copies in the genome (Requena et al. 1988, 1989; Engman et al. 1989a, b, 1992). Analysis of the draft sequence of the parasite's genome (El-Sayed et al. 2005) showed that 11 HSP70 genes are present, with the HSP70. a, HSP70. b, HSP70. c, mitochondrial HSP70, HSP110, Grp170 and Grp78 subfamilies being represented together with some unusual members (Folgueira and Requena 2007; Louw et al. 2010; Shonhai et al. 2011). There appears to be some stage-specific HSP70 members, although its biological significance is unclear. The cytosolic HSP70.4 is highly enriched in amastigotes but undetectable in trypomastigotes, while HSP70. a was found exclusively in trypomastigotes (Atwood et al. 2005). Differences in activity and behavior of HSP70 members were also described. Both cytosolic and mitochondrial HSP70s are potent ATPases, being 100 times more active than human HSP70, and the ATPase activity is stimulated by peptides (Olson et al. 1994) and by HSP40 (Edkins et al. 2004). In addition, the mitochondrial HSP70 possesses autophosphorylation activity (Olson et al. 1994). HSP70 can be readily detected in epimastigotes, trypomastigotes and amastigotes, although in lower levels in metacyclic trypomastigotes, and more intense labeling can be seen at 37 °C in epimastigotes and amastigotes (Giambiagi-deMarval et al. 1996). Cytosolic HSP70 migrates to the nucleus upon heat shock or when cells reach the stationary phase; mitochondrial HSP70, on the other hand, remains associated with the kinetoplast (Martin et al. 1993; Olson et al. 1994; Klein et al. 1995). The Grp78 member has also been characterized, and shown to possess a hydrophobic ER leader and a terminal MDDL sequence (which is responsible for retention of the protein at the endoplasmic reticulum lumen) and to be located in the endoplasmic reticulum. *Grp78* mRNA is also strongly induced by tunicamycin, a glycosylation inhibitor, but not by heat, a typical behavior of this subfamily (Tibbetts et al. 1994). Finally, Grp78 was shown to bind and assist folding of the lysosomal protease cruzipain and delivery of this glycoprotein to calreticulin (Labriola et al. 2011).

Consistent with published reports that describe the immunogenicity of HSPs (Srivastava 2002), the presence of anti-*T. cruzi*'s HSP70 antibodies was shown in animals experimentally infected with the parasite and in patients with Chagas' disease. Antibodies against the cytosolic, mitochondrial and endoplasmic reticulum HSP70 members have been detected (Engman et al. 1990; Levy Yeyati et al. 1992; Tibbetts et al. 1994). Although these results indicate the immunogenicity of the protein, it does not appear to play a role in the pathogenesis of the disease (Engman et al. 1990; Levy Yeyati et al. 1992). Attempts have been made, however, to use the immune system's response to the parasite's HSP70s for diagnostic and vaccine-related purposes. Grp78 has a potential as a diagnostic antigen in Chagas' disease, and absence of reactivity to cytosolic, mitochondrial and endoplasmic reticulum HSP70s may be indicative of effective treatment (Krautz et al. 1998). Although

anti-HSP antibodies are unable to distinguish Chagasic patients from those infected by other trypanosomatids, antibodies against a fragment of *T. cruzi*'s HSP70 can distinguish between healthy and infected people and between acute and chronic Chagasic patients (Flechas et al. 2009). In addition, the parasite's HSP70 induces functional maturation of murine dendritic cells, and the authors suggest that *T. cruzi*'s HSP70 may be useful as a vehicle for dendritic cells-based immunoprophylaxis and therapy against infections (Planelles et al. 2002).

The heat shock response in *T. cruzi* has been used as a model for studies of gene regulatory mechanisms and components, and HSP70 gene expression has been studied in greater detail. At least eight HSP70 protein isoforms have been experimentally detected, of which five are inducible by incubation for 2 h at 37 °C (Giambiagi-deMarval et al. 1996). No new isoforms were detected at elevated temperatures, suggesting that the heat-inducible protein isoforms are already present in non-heat-shocked cells. In fact, both HSP70 protein and mRNA are present at normal temperatures. HSP70 synthesis is increased in response to heat shock, which is accompanied by an increase in mRNA levels (Engman et al. 1989a; de Carvalho et al. 1990; Requena et al. 1992). Severe heat shock at 40 °C, however, leads to somewhat reduced HSP70 mRNA levels and accumulation the polycistronic precursor RNA, indicating an inhibition of RNA processing (Engman et al. 1995). Since the mRNA increase in polysomes is greater than it is in total RNA at elevated temperatures, preferential translation of HSP70 mRNA during heat shock has been suggested (de Carvalho et al. 1990). The mechanism responsible for the increase in mRNA levels during heat shock was investigated (Rodrigues et al. 2010). HSP70 mRNA half-life increases from 1 h at 29 °C to 2 h at 37 °C, and this increase in stability is dependent on protein synthesis. Consistent with these results, a 15-fold greater degradation of the 3′-UTR of the mRNA occurs at 29 °C than at 37 °C in an *in vitro* assay. In addition, the results are consistent with a U-rich sequence being the target of endonucleolytic cleavage. Interestingly, U-rich sequences can be found in the 3′-UTR of all heat-inducible mRNAs identified so far, which was first pointed out by (Sullivan et al. 1994). Finally, heat shock-responsive elements were found in the HSP70 mRNA's UTRs, and both 5′- and 3′-UTRs act cooperatively to stabilize the mRNA during heat shock (Rodrigues et al. 2010). Additional work is required to further characterize these RNA elements.

4.1.4 HSP40 Family

HSP40 proteins, also called J proteins, are the largest class of cofactors of HSP70 and are responsible for much of the functional diversity of the HSP70 family by conferring substrate specificity. Several HSP40s may function with a single HSP70, and may target HSP70 to specific proteins. Like HSP70, the protein is also distributed in all cellular compartments. HSP40 is characterized by an N-terminal, 70-amino acid residue sequence known as the J-domain, which interacts with HSP70 and stimulates its ATPase activity. In the HSP70's ATP-dependent protein-binding and release cycle, HSP40 binds to the unfolded peptide region and delivers it to HSP70 (reviewed by Hartl and Hayer-Hartl 2002; Young et al. 2004).

In *T. cruzi*, four HSP40 genes were initially characterized, named *TcJ1* to *TcJ4* (Tibbetts et al. 1998). TcJ2 and TcJ4 proteins contain the C-terminal CaaX prenyl modification motif, suggesting that they are associated with the plasma membrane. Only the *TcJ2* gene expression responds to heat shock, with increased mRNA levels after incubation at 37 °C for 3 h (Tibbetts et al. 1998). TcJ2 protein was shown to stimulate the ATPase activity of HSP70 *in vitro*, and the corresponding gene is capable of complementing the *ydj1* yeast mutant, suggesting that TcJ2 is the ortholog of the yeast HSP40 protein YDJ1 (Edkins et al. 2004). A mitochondrial HSP40, TcDJ1, has also been characterized, and shown to possess a mitochondrial leader peptide and the characteristic J-domain (Carreira et al. 1998). The corresponding gene was shown to be developmentally regulated, since both mRNA and protein levels are higher in epimastigotes than in metacyclic trypomastigotes. The authors suggest that the protein may participate in the biosynthesis of the mitochondria (Carreira et al. 1998). A member of the HSP40 family named TcJ6 was described and shown to be the ortholog of yeast Sis1cochaperone, required for translation initiation (Salmon et al. 2001). The authors show that the TcJ6 protein is cytosolic, concentrated around the nucleus and probably associated with the endoplasmic reticulum, and present at constant levels in epimastigotes and metacyclic trypomastigotes. In addition, the protein was found to be associated with ribosomal subunits, 80S monosomes and smaller polysomes, and the corresponding gene is capable of complementing a yeast mutant deficient in the ortholog gene *Sis1* (Salmon et al. 2001). Finally, similarity searches in the parasite's genome sequence led to the identification of 67 HSP40 genes. This gene complement represents a greatly expanded family, but the significance of the expansion is unclear (Folgueira and Requena 2007). Presumably, a larger number of HSP40 proteins implies a larger number of HSP70 protein substrates. It would be interesting to determine why a single-celled organism requires an apparently more complex HSP70/HSP40 system.

4.1.5 Chaperonin Family

The chaperonins form a large, two-ring barrel-shaped structure that encloses unfolded or misfolded proteins. Chaperonins are commonly classified in two groups. In group I chaperonins, two heptameric HSP60 rings and one heptameric HSP10 ring comprise the structure, which provide an isolated environment where nonnative proteins can achieve their final conformation without risk of aggregation. Group I chaperonins are found in bacteria, mitochondria and chloroplasts, and the protein levels increase substantially during heat shock. Group II chaperonins have a similar structure, but have a more limited substrate spectrum and are not induced by elevated temperatures. These proteins are found in archea and the eukaryotic cytosol, called thermosome and TRiC, respectively. Folding of the protein inside the chaperonin chamber is assisted in an ATP-dependent binding and release cycle, where exposed hydrophobic regions bind to the internal surface of the barrel, trapping the partially folded protein. Once the protein reaches its native conformation, hydrophobic regions are no longer exposed and the protein is released

to the cytoplasm (reviewed by Hartl and Hayer-Hartl 2002; Young et al. 2004; Richter et al. 2010).

In *T. cruzi*, both components of group I chaperonins have been characterized. *HSP60* genes are organized in nine copies in tandem (Giambiagi-de Marval et al. 1993a; Sullivan et al. 1994), and the protein is located throughout the mitochondrial matrix (de Marval et al. 1993; Sullivan et al. 1994). This distribution is different from that of the mitochondrial HSP70, which localizes to the kinetoplast (Sullivan et al. 1994). Up to five HSP60 isoforms have been described (Sullivan et al. 1994; Giambiagi-deMarval et al. 1996), only one of which is heat-induced (Giambiagi-deMarval et al. 1996). HSP60 gene expression in epimastigotes has also been investigated, and some conflicting results have been reported. de Marval et al. have described an increase in protein levels and unchanged mRNA levels at 37 °C, which suggests translational control (Giambiagi-de Marval et al. 1993a). On the other hand, Sullivan et al. reported essentially constant protein levels and a concomitant sixfold increase in mRNA levels at the same temperature (Sullivan et al. 1994). The source of this discrepancy is unclear, but may be related to different culture medium compositions.

The other component of group 1 chaperonin, HSP10, is encoded in *T. cruzi*'s parasite's genome by three tandemly repeated genes intercalated by genes of unknown function (Fernandes et al. 2005). Interestingly, this arrangement is conserved in trypanosomatids, being present in *Leishmania* spp. and *Trypanosoma brucei* (Folgueira and Requena 2007). The HSP10 protein of 100 amino acids contains a mitochondrial targeting sequence and shows predicted structural features that are conserved in the HSP10 family, although a 5-residue deletion conserved in trypanosomatids is present (Fernandes et al. 2005). A putative 3D structure of the protein was obtained by comparative modeling, and in the model the 5-residue deletion results in a larger central orifice of HSP10 heptameric ring. The functional significance, if any, of this change remains to be determined. The pattern of HSP10 gene expression in the parasite was also investigated. The levels of the main HSP10 mRNA of 1.0 kb remain unaltered during heat shock, similarly to those of HSP60, and a smaller mRNA resulting from alternative polyadenylation is observed at 37 °C and 40 °C (Fernandes et al. 2005). Since both HSP60 and HSP10 are part of the same molecular structure, and presumably are present in equimolar amounts, it would be interesting to investigate the regulatory mechanisms to determine whether both genes are coordinately regulated.

4.1.6 Small HSP Family

Small HSPs (sHSPs) are found in all groups of organisms and comprise the least conserved family of molecular chaperones. sHSPs are heterogeneous in size and sequence and are characterized by low monomeric molecular mass and the presence of a 90-amino acid conserved region called the alpha-crystallin domain. sHSPs form large oligomers of often 24 subunits that bind unfolded proteins in an ATP-independent manner and prevent aggregation. It is believed that sHSPs associate

with partially folded proteins during heat stress and store these proteins until another chaperone assists their refolding. sHSPs are one of the most strongly induced among the HSP families (reviewed by Richter et al. 2010).

A single *T. cruzi* sHSP has recently been characterized and named sHSP16 (Pérez-Morales et al. 2009). The sHSP16 protein is 142-amino acid residues long with a predicted 16 kDa molecular weight and contains the characteristic alpha-crystallin domain. The corresponding single-copy gene shows some sequence polymorphism among four strains investigated. In addition, *sHSP16* gene expression studies showed that mRNA levels are moderately increased after heat treatment. To test for chaperone activity, recombinant sHSP16 was incubated with malate dehydrogenase and shown to prevent aggregation of the enzyme *in vitro* (Pérez-Morales et al. 2009). Further work is needed to determine the biological role of this protein in the different forms of the parasite.

5 Conclusions

The complex life cycle of *T. cruzi* shows cell forms with different morphology and functional characteristics that interact with an insect and a mammalian host, undergoing several environmental variations in the process. Transition from the insect to the mammalian host involves a shift in temperature, and as expected the parasite is capable of inducing members of all the major classes of HSPs. The heat shock response in general and the HSPs in particular have both conserved and unique features in this organism, and the immunogenicity of these proteins may be relevant for pathogenesis. An improved understanding of *T. cruzi*'s heat shock response would be of great value in the context of the parasite's basic biology. Investigation of the regulation of the stress response traditionally led to a better understanding of gene regulatory mechanisms, and progress has been made in this respect in *T. cruzi*. However, a great deal of work remains to be done. The role of regulatory RNAs in the modulation of the stress response in eukaryotic organisms has recently been emphasized (Leung and Sharp 2010). Although microRNAs have not been found in *T. cruzi*, which lacks the necessary protein machinery for RNA interference, a ssRNA-mediated pathway has been proposed to be present in this organism (Batista and Marques 2011). Given that sequence-specific RNA-binding proteins are surprisingly difficult to identify in trypanosomatids, it is possible that RNA molecules may participate in gene regulation in *T. cruzi*. Future studies may shed light on these and other intriguing issues.

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Chapter 6
The gp82 Surface Molecule of *Trypanosoma cruzi* Metacyclic Forms

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Abstract Gp82 is a surface glycoprotein expressed in *Trypanosoma cruzi* metacyclic trypomastigotes, the parasite forms from the insect vector that initiate infection in the mammalian host. Studies with metacyclic forms generated *in vitro*, as counterparts of insect-borne parasites, have shown that gp82 plays an essential role in host cell invasion and in the establishment of infection by the oral route. Among the gp82 properties relevant for infection are the gastric mucin-binding capacity and the ability to induce the target cell signaling cascades that result in actin cytoskeleton disruption and lysosome exocytosis, events that facilitate parasite internalization. The gp82 sequences from genetically divergent *T. cruzi* strains are highly conserved, displaying >90 % identity. Both the host cell-binding sites, as well as the gastric mucin-binding sequence of gp82, are localized in the C-terminal domain of the molecule. In the gp82 structure model, the main cell-binding site consists of an α-helix, which connects the N-terminal β-propeller domain to the C-terminal β-sandwich domain, where the second cell binding site is nested. The two cell binding sites are fully exposed on gp82 surface. Downstream and close to the α-helix is the gp82 gastric mucin-binding site, which is partially exposed. All available data support the notion that gp82 is structurally suited for metacyclic trypomastigote invasion of host cells and for initiating infection by the oral route.

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Abbreviations

ACD	Acute Chagas' disease
DAG	Diacylglycerol
EIEC	Enteroinvasive *Escherichia coli*
GST	Glutathione S transferase
IP3	Inositol 1,4,5-triphosphate
MT	Metacyclic trypomastigotes
mTOR	Mammalian target of rapamycin
PI3K	Phosphatidylinositol 3-kinase
PTK	Protein tyrosine kinase
TCT	Tissue culture trypomastigotes
PKC	Protein kinase C
PLC	Phospholipase C

1 Introduction

Trypanosoma cruzi metacyclic trypomastigotes (MT) are the developmental forms responsible for the initial parasite-vertebrate host interaction. They are found in the terminal portions of the triatomine insect digestive tract as well as in the anal gland of opossum (*Didelphis marsupialis*), which is an important reservoir of *T. cruzi* (Deane et al. 1984). Since the first report on an outbreak of acute Chagas' disease (ACD) attributed to ingestion of food contaminated with opossum secretion (Silva et al. 1968), ACD outbreaks due to consumption of food or drink contaminated with MT have been reported in recent years with increasing frequency in Brazil, Venezuela and Colombia (Dias et al. 2008; Steindel et al. 2008; Beltrão et al. 2009; Cavalcanti et al. 2009; Nóbrega et al. 2009; Bastos et al. 2010; Alarcón de Noya et al. 2010; Ríos et al. 2011). Oral infection is possibly an ancient mode of *T. cruzi* transmission, prevalent among the large variety of mammalian species that can host this protozoan parasite.

Studies on experimental oral *T. cruzi* infection in mice using MT derived from the triatomine insect have shown that the parasites invade the gastric mucosal epithelium as a unique portal of entry to systemic infection, without evidence of invasion anywhere within the oropharynx or gastrointestinal tract (Hoft et al. 1996). Attempts to elucidate the mechanisms of gastric mucosal cell invasion by MT have revealed that gp82, a MT-specific surface molecule, plays a critical role in the establishment of *T. cruzi* infection by the oral route. Here we summarize the data on the structure and function of gp82 relevant for MT migration though the gastric mucin layer and for host cell invasion.

2 High Conservation of Gp82 Sequence Among *T. cruzi* Strains from Divergent Genetic Groups

The MT surface molecule gp82, which is specifically identified by the monoclonal antibody (mAb) 3F6 (Teixeira and Yoshida 1986), is a member of a multigene family belonging to the gp85/*trans*-sialidase superfamily (Araya et al. 1994). Although the gene is transcribed in MT and in epimastigotes, gp82 mRNA is barely detectable in epimastigotes because these parasite forms lack the stabilizing mechanisms that function in MT (Gentil et al. 2009). More recently, it was demonstrated that the 3′ untranslated region (3′UTR) of the gp82 transcript is involved in regulating gp82 expression in MT (Bayer-Santos et al. 2012).

Gp82 is highly conserved among genetically divergent *T. cruzi* strains, such as G, CL and BAT, which share >90 % sequence identity (Maeda et al. 2011). These strains, originated from different sources in distant geographical regions, have been used to study the multiple aspects of parasitism. G strain derived from an opossum in the Brazilian Amazon (Yoshida 1983) is associated with the sylvatic cycle of transmission, as opposed to CL strain isolated in the southern state of Rio Grande do Sul from *Triatoma infestans* captured in a dwelling where people were infected (Brener and Chiari 1963), whereas BAT strain isolated in São Paulo is a member of a *T. cruzi* lineage that infects bats (Marcili et al. 2009). Gp82 contains in its C-terminal domain the cell adhesion sites P4 and P8 (Manque et al. 2000), as well as the gastric mucin-binding sequence P7 (Staquicini et al. 2010), which are 100 % identical in G and CL strains (Fig. 6.1). The amino acid substitutions found in BAT strain sequences P4, P7 and P8, are mostly conservative and apparently do not affect the gp82 function (Maeda et al. 2011). On the other hand, consistent with the sequence/function relationship, a gp82 family member C03 that displays considerable differences in P4 and P8 sequences (Fig. 6.1) exhibits reduced cell adhesion capacity (Atayde et al. 2007). C03 protein is not recognized by mAb 3F6 and its cellular localization varies depending on the parasite strain. It is present on the surface of CL strain but not of G strain metacyclic forms, and flagellar components are predominantly detected by anti-C03 antibodies in permeabilized G strain parasites, whereas in CL strain the flagellum is not the preferential target for these antibodies (Atayde et al. 2007).

3 The Structural Basis of Cell Binding Property of Gp82

Native gp82, as well as the recombinant protein containing the full length gp82 sequence, can bind to host cells in a dose-dependent and saturable manner (Ramirez et al. 1993; Ruiz et al. 1998). The 3D structure of gp82, determined by homology


```
                                                                                P3
                                                                                    P4
R31(CL)   : QRGYASGEKRVNPLYLWVTDNNRSFYFGPIAMGNAANSMFVSSLLYSDGSLHLLQRRANDRGSVISLARLTEELKTIKS : 343
J18(G)    : ...............................P.............................................. : 266
F11(BAT)  : ---------------------------------.....N.....G.....S.....S..R. :  41
CO3(CL)   : .....LR..NA.................V.VEDNM.WE.A.A.....R.....Q.DD...R....S.....R... : 473

                                    P7
          P4                                P8
R31(CL)   : VLSTWSRLDASFSASSTPTAGLVGLLSNSASGDAWIDDYRSVNAKVMNAVKVHDGFKFTGFGSGAIWFVNNRESNGPHT : 422
J18(G)    : ..................................................................... : 345
F11(BAT)  : .....TR..T.....I.....T..... : 120
CO3(CL)   : ......QR.VFL.KL.I.....AA..DA..DGR.Y.E.LCL..T.T..R..K...QL.EPD..VM....TQGN.VR.V : 552


R31(CL)   : FVNYNFTLVATVIVHRVPRNSTTLLGAVLAEFISTLFIGLSYGTDGTWETVFNGETTTSGSTWMPGKEYQVALMLQDGN : 501
J18(G)    : ..-IT...CD-..... : 422
F11(BAT)  : ....D.......L.....L.....N.....E....H...I..... : 199
CO3(CL)   : .LGHD.....S.TIEEA.TADAP....A.GDTN.P.TM.VL.TA.KE.V.M.K.KR..KSG..E.K..H.....-.. : 630


R31(CL)   : KGSVYVDGMSVGSLATLPTFEVRGAEIADFYFVGGEDEEDKRSSSVTVKNVFLYNRPLGADELRMVR--KIDGSMHGGV : 578
J18(G)    : ..........--..... : 499
F11(BAT)  : ........V.....---------- : 257
CO3(CL)   : .A...I..KLL.EEV.QL.G.TP-L.YVH.C.GAC.MHN----.P.....NST.MTAI.DR.S..... : 704
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Fig. 6.1 Sequences of gp82 C-terminal domain of different *T. cruzi* strains. Aligned are the amino acid sequences deduced from cDNA clones R31 (GenBank AF128843), J18 (GenBank L14824) and F11 (GenBank JN116557) derived from CL, G and BAT strains, respectively, which exhibit a high degree of conservation. The sequence identified as P3 corresponds to the epitope for mAb 3F6, the sequences P4 and P8 represent the host cell binding sites, and P7 constitutes the main gastric mucin-binding site. Also shown is the sequence deduced from cDNA clone C03 (GenBank EF445668) derived from CL strain, which displays much lower sequence identity. Points represent residues that are conserved, non conserved amino acids are indicated, and dashes represent residues that are missing

modeling using as template the high resolution crystal structure of inhibitor-bound *Trypanosoma rangeli* sialidase (Amaya et al. 2003), which is closely related to *T. cruzi trans*-sialidase (Buschiazzo et al. 2002), revealed that the main cell adhesion site P4 is part of an α-helix that connects a β-propeller domain at the N-terminal region to a β-sandwich domain at the C-terminal region, where the cell binding site P8 is located (Cortez et al. 2012a, Fig. 6.2a). According to that model, both P4 and P8 sites are almost fully exposed (Fig. 6.2b), compatible with the presence of amino acids containing charged polar side chains with high solvent accessibility (Cortez et al. 2012a), and therefore available for interaction with target cells. Deletion of the N-terminal domain of gp82 does not affect the cell adhesion property, which is fully preserved in the recombinant protein corresponding to the C-terminal domain containing P4 and P8 sites (Santori et al. 1996a, Fig. 6.2c), whereas deletion of the sequence containing P4 and P8 sites results in complete loss of cell binding capacity (Manque et al. 2000, Fig. 6.2c). It appears that the gp82 construct containing the C-terminal domain preserves the conformation required for cell adhesion. On the other hand, in the construct lacking the central domain (Fig. 6.2c), there may be a change in protein folding leading to considerable conformational alteration.

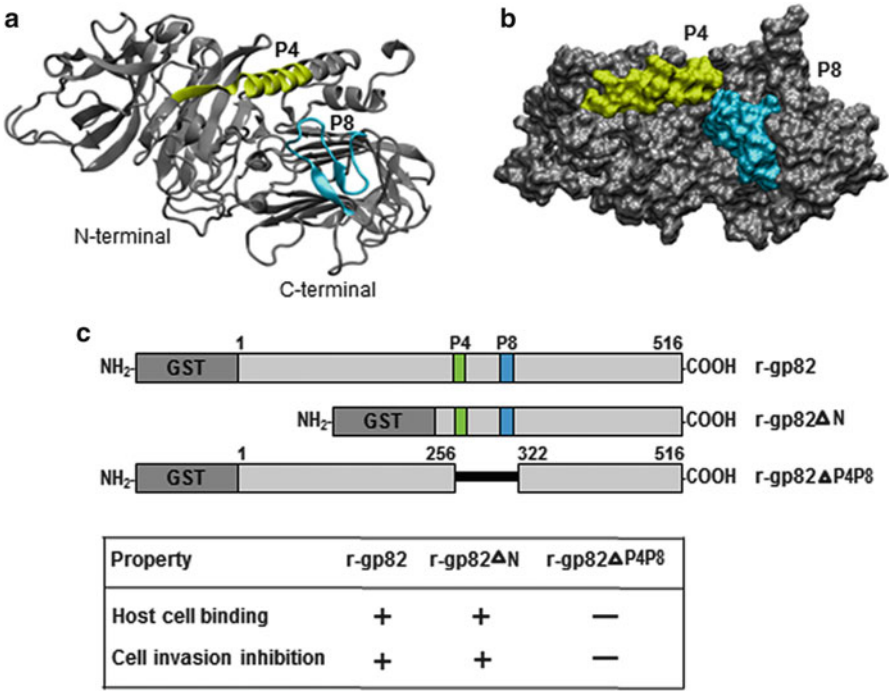

Fig. 6.2 The structural model of *T. cruzi* gp82 molecule and the cell adhesion sites. (**a**) Cartoon representation highlighting the cell binding sites P4 (*green*) and P8 (*blue*). (**b**) Surface representation of sites P4 and P8. (**c**) Schematic representation of gp82 recombinant proteins fused to GST, containing the full-length gp82 sequence or the C-terminal domain only, which display similar cell adhesion capacity, and the construct lacking P4 and P8 sites, which is unable to bind to host cells and to inhibit MT invasion

4 Gastric Mucin-Binding Property of Gp82 and MT Migration

Mucins are the main macromolecular component of the mucus that protects the gastrointestinal mucosa. Binding to mucin constitutes a pre-requisite for colonization by diverse enteropathogenic microorganisms. *Shigella*, which invades and multiplies within colonic epithelial cells, binds specifically to human colonic mucin but not to small intestine mucin (Rajkumar et al. 1998), whereas *Helicobacter pylori*, which colonizes gastric mucosa, binds to human gastric mucin (Tzouvelekis et al 1991). In oral *T. cruzi* infection, MT binding to gastric mucin is the first step for migration toward the underlying target epithelial cells. MT, as well as the recombinant protein based on gp82, bind to gastric mucin but not to submaxillary mucin (Staquicini et al. 2010). This binding property is associated with the parasite ability to migrate through the gastric mucus barrier. *In vitro*, MT traverse a gastric mucin-coated transwell filter

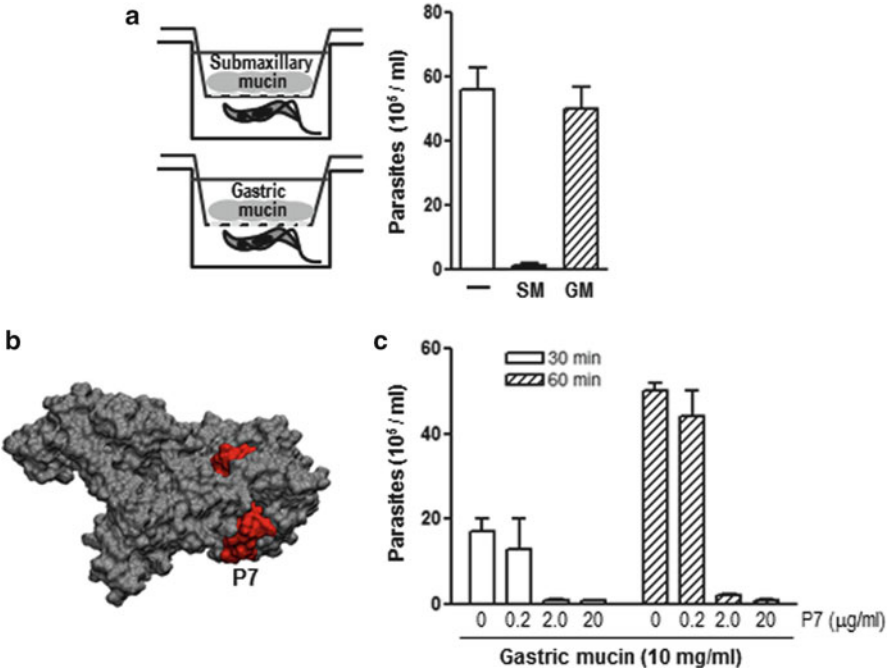

Fig. 6.3 Gp82-dependent *T. cruzi* MT migration through gastric mucin layer. (**a**) In MT migration assays, transwell filters coated with gastric mucin (GM) or submaxillary mucin (SM) were placed onto 24-well plates containing parasites. After 60 min incubation, samples from the filter chamber were collected and the number of parasites was counted. Values are the means ± SD of three independent assays. (**b**) Surface representation of gp82 structural model showing the gastric mucin-binding site P7 highlighted in *red*. (**c**) Assays were performed as in (**a**) using transwell filters coated with gastric mucin (10 mg/ml) alone, or mixed with the synthetic peptide P7 at the indicated concentrations. Values are the means ± SD of three independent experiments

as efficiently as the empty filter, but are blocked by a submaxillary mucin-coated filter (Fig. 6.3a), confirming previous findings (Staquicini et al. 2010). Gp82 binds to gastric mucin mainly through the surface-exposed portion of sequence P7 (Fig. 6.3b), which is critical for MT migration. Transwell filter coated with gastric mucin mixed with the synthetic peptide based on P7 blocked MT migration, whereas gastric mucin mixed with control peptide P7*, with the same composition as P7 but with a scrambled sequence, allowed parasite traversal (Cortez et al. 2012a, Fig. 6.3c). The relevance of gp82 sequence P7 in oral *T. cruzi* infection was demonstrated in experiments in which mice that received peptide P7, before oral MT administration, exhibited in the histological sections of the stomach much fewer amastigote nests at day four post-infection, and later on much fewer parasites circulating in the bloodstream, as compared to mice given the scrambled peptide P7* (Staquicini et al. 2010).

Metacyclic forms of *T. cruzi* strains that do not express gp82 on the surface have reduced gastric mucin-binding capacity and, as compared to gp82-expressing MT, they migrate less efficiently through the gastric mucin-coated transwell filter and are poorly infective in mice by the oral route (Cortez et al. 2003, 2012b).

5 Ca^{2+} Signal-Inducing Activity of Gp82 and Target Cell Lysosome Exocytosis

Gp82 is engaged by MT of highly infective *T. cruzi* strains to promote host cell invasion (Ramirez et al. 1993; Ruiz et al. 1998). During MT invasion, gp82 triggers the target cell signaling cascades that result in cytosolic Ca^{2+} mobilization in both cells (Ruiz et al. 1998; Yoshida et al. 2000; Neira et al. 2002). Ca^{2+} signal, which is required for *T. cruzi* internalization (Docampo and Moreno 1996; Burleigh and Andrews 1998) is detectable in MT-susceptible mammalian cells such as HeLa and Vero cells, but not in K562 cells that are resistant to MT invasion (Ruiz et al. 1998). In MT, gp82 triggers the activation of signaling cascades involving phospholipase C (PLC), which generates diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3), the latter promotes Ca^{2+} release from IP_3-sensitive compartments such as endoplasmic reticulum (Yoshida et al. 2000; Neira et al. 2002, Fig. 6.4). In addition to protein kinase C (PKC), which may be activated by DAG, phosphatidylinositol 3-kinase (PI3K) and protein tyrosine kinase (PTK) also participate in MT invasion process (Maeda et al. 2012). PTK phosphorylates p175, a protein undetectable in noninvasive epimastigote forms (Favoreto et al. 1998). Distinct from the parasite response, host cell PTK is not implicated in gp82-dependent MT invasion, rather signaling pathways involving lipid kinase PI3K, mammalian target of rapamycin (mTOR), PKC and PLC may be activated and Ca^{2+} appears to be released from IP_3-sensitive compartments or in IP_3-independent manner (Ferreira et al. 2006; Martins et al. 2011; Maeda et al. 2012, Fig. 6.4).

Increase in cytosolic Ca^{2+} concentration triggered by gp82 leads to Ca^{2+}-dependent actin cytoskeleton disruption, followed by lysosome recruitment to the cell periphery and exocytosis (Cortez et al. 2006; Martins et al. 2011). Lysosome exocytosis contributes for parasitophorous vacuole biogenesis during *T. cruzi* invasion (Tardieux et al. 1992; Rodríguez et al. 1999). The lysosome exocytosis triggered by gp82-mediated MT entry into target cells is associated with the signaling pathways involving PI3K, mTOR or PKC, provided that the pretreatment of cells with drugs that affect the activity of these kinases inhibits lysosome exocytosis and MT internalization (Martins et al. 2011).

6 Inhibitory Effect of Gp82 on Cell Invasion by Enteroinvasive *Escherichia coli* (EIEC)

Cell invasion by enteropathogenic microorganisms such as *Shigella*, *Salmonella*, *Listeria* and *Yersinia*, is associated with actin cytoskeleton rearrangements (Stebbins and Galán 2001; Cossart et al. 2003; Cossart and Sansonetti 2004). As gp82-mediated MT internalization induces F-actin disassembly, Cortez et al. (2006) investigated whether gp82 exerted any effect on bacterial uptake, by using the EIEC, which has an invasion plasmid similar to that harbored by *Shigella* and the same

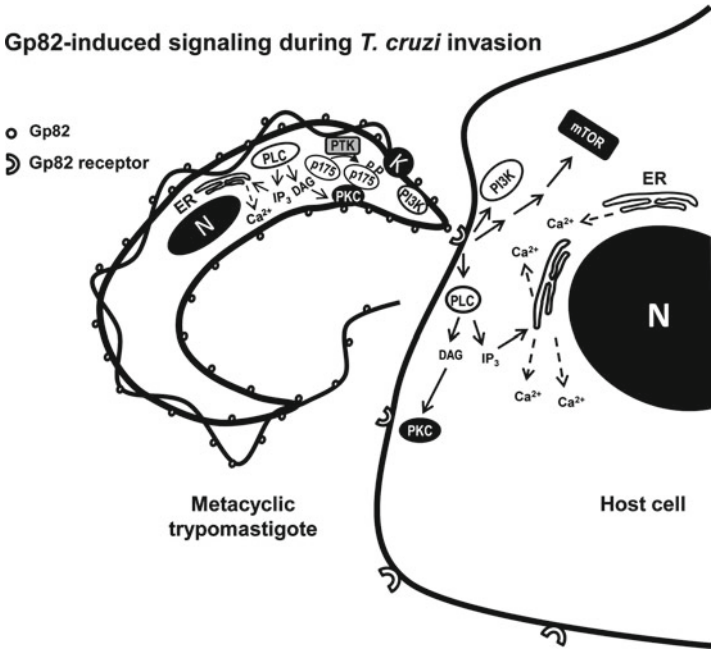

Fig. 6.4 Schematic representation of signaling molecules and pathways that may be activated during gp82-mediated *T. cruzi* MT entry into non-phagocytic mammalian cells. In metacyclic forms, the interaction of gp82 with its as yet undefined receptor triggers the activation of phospholipase C (PLC), generating diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). DAG stimulates protein kinase C (PKC) and IP_3 promotes Ca^{2+} release from IP_3-sensitive compartments. Lipid kinase phosphatidylinositol 3-kinase (PI3K) and protein tyrosine kinase (PTK) are also activated. PTK phosphorylates p175. In the host cell, PI3K, mammalian target of rapamycin (mTOR) and PLC are activated. DAG and IP_3 generated by PLC stimulate PKC and IP_3-mediated Ca^{2+} release from endoplasmic reticulum (ER), respectively

mode of pathogenesis (Sansonetti et al. 1983; Lan et al. 2001). Invasion of HeLa cells by EIEC, was drastically reduced by a recombinant protein containing full length gp82 sequence fused to glutathione S transferase (GST), whereas GST had no effect (Cortez et al. 2006). In contrast to the inhibitory effect of gp82, the mucin-like gp35/50 molecules, which are devoid of actin cytoskeleton disruption-inducing activity, increased EIEC internalization (Ferreira et al. 2006). Gp35/50 molecules expressed in MT have been implicated in host cell invasion by poorly invasive *T. cruzi* strains (Ramirez et al. 1993; Ruiz et al. 1998). MT binding to target cells mediated by gp35/50 triggers the activation of signaling pathways distinct from those induced by gp82 (Neira et al. 2002; Ferreira et al. 2006). Rather than promoting the lysosome exocytosis, gp35/50 binding to HeLa cells impaired lysosome mobilization (Maeda et al. 2011). The properties of gp35/50 molecules, quite distinct from gp82, have contributed to further reinforce the role played by gp82 in MT invasion of host cells.

7 Apoptotic Cell Death-Inducing Activity of Gp82 Toward Melanoma Cells

Several studies have revealed that actin cytoskeleton functions as a key regulator of apoptosis (Gourlay and Ayscough 2005), an evolutionary conserved physiological process of cell death triggered by intracellular or extracellular signals, in which a family of proteases known as caspases is involved (Chang and Yang 2000). Treatment with cytochalasin D, a drug that prevents actin filament elongation, was reported to induce apoptosis in adherent epithelial cells (White et al. 2001), and in human leukemia CMK-7 cells by accelerating caspase-3 activation (Yamazaki et al. 2000). Because the recombinant gp82 protein can induce actin cytoskeleton disorganization, its potential to promote apoptosis was tested in tumorigenic Tm5 cells derived from mouse melanocytes melan-a. The recombinant gp82 protein inhibited the growth of Tm5 cells, ultimately leading to death by apoptosis, but did not affect melan-a cells (Atayde et al. 2008). In addition to actin cytoskeleton disruption, alterations associated with apoptosis, such as exposure of phosphatidylserine in the outer leaflet of the plasma membrane, altered nuclear morphology, DNA fragmentation, increase in mitochondria depolarization and in caspase-3 activity were detected in Tm5 melanoma cells treated with gp82 protein but not in GST-treated cells (Atayde et al. 2008). Another relevant finding concerned NF-κB, the nuclear transcription factor that is upregulated in melanoma cells (Dhawan and Richmond 2002; McNulty et al. 2004; Amiri and Richmond 2005). Translocation of NF-κB to the nucleus was inhibited in Tm5 cells treated with gp82 protein (Atayde et al. 2008). The effect of gp82 in inhibiting melanoma cell growth was also observed *in vivo*. C57BL/6 mice that were injected with Tm5 cells and were treated *in situ* with the recombinant protein during 10 days, starting on day 1 or 8 post-injection, developed tumors of smaller size than mice treated with PBS or GST and survived longer (Atayde et al. 2008).

8 Immunogenic Property of Gp82

The first study to determine the immunogenicity of gp82, using the native antigen purified from metacyclic forms, showed that specific antibodies are produced and T cell response is elicited in BALB/c mice, without inducing protective immunity (Yoshida et al. 1993). To further investigate the immunological properties of gp82, Santori et al. (1996) immunized BALB/c mice with the recombinant gp82 protein, containing the carboxy-terminal domain corresponding to amino acids 224–516 fused to GST (Fig. 6.2c), which elicited a response capable of protecting 85 % of animals against acute infection, upon challenge with MT. As the native gp82 used in the first study contains N-linked oligosaccharides (Ramirez et al. 1993), it appears that the carbohydrate-free recombinant protein is more effective in inducing protective immune response. Antibodies generated by immunization with the recombinant gp82

protein lacked MT neutralizing effect, whereas $CD4^+$ T cells from the spleen displayed an intense proliferative activity upon stimulation with the native gp82, accompanied by increased production of IFN-γ, a cytokine associated with resistance to *T. cruzi* infection (Santori et al. 1996b). In addition, intranasal CpG-adjuvanted gp82 vaccination has been performed. CpG-gp82-immunized mice were significantly protected from a biologically relevant oral MT challenge, and spleen cells from immunized mice proliferated and secreted IFN-γ in a dose-dependent manner in response to *in vitro* stimulation with gp82 and parasite lysate (Eickhoff et al. 2010).

9 Distinctive Properties of MT Gp82 and TCT Surface Molecule Tc85-11

Tissue culture-derived trypomastigotes (TCT), which correspond to the parasite forms that circulate in the bloodstream and disseminate to diverse organs and tissues, express on the surface molecules of the gp85/*trans*-sialidase superfamily (Alves and Colli 2008). The structure of Tc85-11, a family member with laminin-binding property implicated in host cell invasion, has been determined (Giordano et al. 1999; Marroquin-Quelopana et al. 2004). As compared to MT gp82 sequence, Tc85-11 shares considerable identity but the cell adhesion sites are localized in N-terminal domain and are poorly conserved (Cortez et al. 2012a), suggesting that MT and TCT molecules interact with distinct cell surface receptors, what may trigger diverse mechanisms of parasite internalization. Whether Ca^{2+} mobilization is induced by Tc85-11 is not known. On the other hand, a TCT secreted factor of unknown identity can trigger IP_3 formation, intracellular Ca^{2+} mobilization and a Ca^{2+} dependent F-actin rearrangement (Rodriguez et al. 1995). One possibility is that Tc85-11-mediated attachment of TCT to target cells induces the secretion of Ca^{2+} signal-inducing factor. Unlike the gp82-dependent MT invasion, which is associated with mTOR activation (Martins et al. 2011), TCT entry into mammalian cells is enhanced when mTOR is inhibited (Romano et al. 2009). Another difference between gp82 and Tc85-11 concerned the ability to bind laminin. In gp82, the sequences equivalent to Tc85-11 laminin-binding sites were poorly conserved and, accordingly, the ability of gp82 in binding laminin was significantly lower as compared to Tc85-11 (Cortez et al. 2012a). This differential property is compatible with the interactions that bloodstream trypomastigotes have to establish with components of extracellular matrix and basal laminae, in order to overcome this barrier and reach the target cells.

10 Concluding Remarks

T. cruzi metacyclic stage-specific surface molecule gp82, with its gastric mucin-binding property and host cell invasion-promoting activity, plays a critical role in the establishment of infection by the oral route. Compatible with its relevance, gp82

is highly conserved among genetically divergent *T. cruzi* strains. The mechanisms of gp82-mediated host cell invasion by metacyclic forms have been partially disclosed. Understanding the whole process in more detail is far ahead and will demand enormous efforts. Gp82, in its recombinant form, has been extremely useful in elucidating diverse aspects of infection by MT. An interesting possibility, emerging from the inhibitory effect of recombinant gp82 protein on enteroinvasive bacteria internalization, is that it could be used to modulate cell invasion by other pathogenic microorganisms.

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Chapter 7
The Gp85 Surface Glycoproteins from *Trypanosoma cruzi*

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Abstract *Trypanosoma cruzi* strains show distinctive characteristics as genetic polymorphism and infectivity. Large repertoires of molecules, such as the Gp85 glycoproteins, members of the Gp85/*Trans*-sialidase superfamily, as well as multiple signaling pathways, are associated with invasion of mammalian cells by the parasite. Due to the large number of expressed members, encoded by more than 700 genes, the research focused on this superfamily conserved sequences is discussed. Binding sites to laminin have been identified at the N-terminus of the Gp85 molecules. Interestingly, the *T. cruzi* protein phosphorylation profile is changed upon parasite binding to laminin (or fibronectin), particularly the cytoskeletal proteins such as those from the paraflagellar rod and the tubulins, which are both markedly dephosphorylated. Detailed analysis of the signaling cascades triggered upon *T. cruzi* binding to extracellular matrix (ECM) proteins revealed the involvement of the MAPK/ERK pathway in this event. At the C-terminus, the conserved FLY sequence is a cytokeratin-binding domain and is involved in augmented host cell invasion *in vitro* and high levels of parasitemia *in vivo*. FLY, which is associated to tissue tropism and preferentially binds to the heart vasculature may somehow be correlated with the severe cardiac form, an important clinical manifestation of chronic Chagas' disease.

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Abbreviations

AEP	Aminoethylphosphonate
Asp Box	SxDxGxTW amino acid sequence
CK	Cytokeratin
DGF	Disperse gene family
DTU	Discrete typing units
ECM	Extracellular matrix
ERK1/2	Extracellular signal-regulated protein kinases 1 and 2
FAY motif	VTVxNVFAYNR amino acid sequence
FL-160	*T. cruzi* 160 kDa flagellum-associated protein
FLY motif	VTVxNVFLYNR amino acid sequence
FLY-phage	Bacteriophages expressing the FLY motif
FN	Fibronectin
FRIP motif	xRxP amino acid sequence
Gal	Galactose
Galf	Galactofuranose
GIPC	Glycosylinositolphosphoceramide
GIPL	Glycoinositolphospholipid
GlcN	Glucosamine
Gp	Glycoprotein
GPI	Glycophosphatydilinositol
H1A10	mAb that recognizes members of the Tc85 glycoprotein family
HBP	Heparin binding proteinase
IFN-γ	Interferon gamma
IL-10	Interleukin 10
LPPG	Lipopeptidophosphoglycan
Man	Mannose
MAP	Mitogen-activated protein
MASP	Mucin-associated surface protein
Mbp	Mega base-pair
NO	Nitric oxide
PD98059	Selective inhibitor of MAP kinase kinase
PFR	Paraflagellar rod protein
PI	Phosphatidylinositol
PIPLC	Phosphatidylinositol phospholipase C (PI-PLC)
PK	Protein kinase
PKAc	Protein kinase A catalytic subunit
PLD	Phospholipase D
RGD motif	Amino acid sequence within fibronectin that mediates cell attachment
RNAi	Interference RNA
SAPA	Shed acute phase antigen
Tc	*T. cruzi*
Tc80 POP	*T. cruzi* 80 kDa prolyloligopeptidase

Tc85-11	A clone member from the *T. cruzi* Gp85 glycoprotein family
TcMUC	*T. cruzi* mucin gene family
TCNA	*T. cruzi* neuraminidase
TcSMUG	*T. cruzi* small mucin-like gene family
TNF	Tumor necrosis factor
Treg	Regulatory T cells
TS	*Trans*-sialidase
TS	Trypomastigotes
U0126	Selective inhibitor of MAP kinase kinase
VSG	Variant surface glycoprotein
WGA	Wheat germ agglutinin

1 Introduction: General Considerations on the Variability of the Infection

Trypanosoma cruzi is the causative agent of Chagas' disease, affecting approximately ten million people in South and Central America. Due to considerable increase in migration, individuals suffering from Chagas' disease are now spread worldwide. It is estimated that there are approximately 300,000 individuals infected with *T. cruzi* in the United States, 80,000 in Europe and Western Pacific, 5,500 in Canada, 3,000 in Japan and 1,500 in Australia (Coura and Viñas 2010). *T. cruzi* is transmitted to humans by the feces of a contaminated triatomine insect, by blood transfusion, by the placenta or by the ingestion of contaminated insects occasionally present in fresh vegetables or fruits (oral infection). The human infection consists of an acute phase, with or without symptoms and a chronic phase, which presents distinct clinical manifestations (cardiac, digestive or cardiac-digestive forms) or could be asymptomatic. Severe cardiac or digestive forms cover 10–50 % of the cases, with a great regional variation (Coura and Viñas 2010). The causes of the diverse clinical manifestations are unclear, but most probably, the well known genetic variability of the pathogen plays an important role.

T. cruzi is a dixenic protozoon, having two hosts – an insect and a mammal – and distinct parasite forms are part of the parasite life cycle. Briefly, trypomastigotes, the classical infective and non-dividing forms of *T. cruzi*, are present in the triatominae (called metacyclic trypomastigotes) and in mammals (bloodstream trypomastigotes). Epimastigotes in the digestive tract of the insect and amastigotes inside mammalian cells are the dividing forms of the parasite. Differentiation steps from epimastigotes to metacyclic trypomastigotes in the insect and from trypomastigotes to amastigotes, and from the latter back to trypomastigotes in the cytoplasm of mammalian cells, is responsible for maintaining the parasite life cycle.

In addition to these classical and very well defined forms, intracellular epimastigotes in mammalians (Tonelli et al. 2004) and amastigote-like forms in the insect tract (Souza et al. 2010) are intermediate forms present in the life cycle, suggesting

that parasite differentiation evolves as a continuum rather than by discrete steps. Moreover, infection of cells by extracellular amastigotes is being characterized in recent years (Alves and Mortara 2009).

An extensive literature emphasizes important differences in the invasion process of cells by metacyclic trypomastigotes, bloodstream trypomastigotes and extracellular amastigotes. Considering the complex process in each case, different molecules triggering distinct signaling pathways were described to be involved in *T. cruzi* infection and were the subject of numerous reviews (Burleigh and Andrews 1995; Alves and Colli 2007; Alves and Mortara 2009; Villalta et al. 2009; Yoshida 2009; Epting et al. 2010; Souza et al. 2010; Butler and Tyler 2012). Moreover, strains of *T. cruzi* showed distinct biological characteristics like infectivity, genetic and protein polymorphism (Buscaglia and Di Noia 2003; Pena et al. 2009; Yoshida 2009; Ramirez et al. 2010; Telleria et al. 2010; Lima et al. 2012). The high heterogeneity amongst the strains leads to distinct initiatives for their classification. Genotyping strategies resulted in the recent classification of *T. cruzi* in six groups (DTUs, discrete typing units I–VI) (Zingales et al. 2009). As happen with other classifications attempted before, a great deal of effort is being made to associate the prevalence of a given DTU or strain in a geographic area with specific clinical manifestations of Chagas' disease (Zingales et al. 2012). However, such putative correlations are not clearly established, as yet (Mantilla et al. 2010; Zafra et al. 2011). The genetic background of the human host, the genome of each strain, with its extensive repertoire of multigene families (see below) or even genetic exchange among the parasites (Bogliolo et al. 1996; Gaunt et al. 2003; Sturm and Campbell 2010; Minning et al. 2011) may all contribute to the outcome of the disease, making the association strain-clinical manifestations a complex task. Another aspect to be considered for the establishment of *T. cruzi* infection is the survival of the parasite inside each type of mammalian cell, since it was established, in most of the cases, that *T. cruzi* is found inside almost all tissues and organs analyzed, in the acute phase. In contrast, in the chronic phase, detectable parasites are restricted to few organs (Coura and Viñas 2010). Why the parasite is practically eliminated from a given organ but not from others is a question poorly addressed in the literature, but certainly the immune system plays a central role in the process, as shown in rodent models (Franco et al. 2003; Sardinha et al. 2010).

In vitro, *T. cruzi* parasitizes almost any cell type and two main routes of invasion were reported for tissue cultured – derived cells. One route apparently undertaken by the majority of the parasites in the population occurs with the engulfment of the parasite by the plasma membrane, followed by later fusion with components of the lysosome path; the second route (Andrade and Andrews 2005), reported to be less frequent (20–25 % of the parasite population (Burleigh 2005)), occurs with lysosome recruitment to the local of invasion, thus contributing, together with the plasma membrane, to the initial formation of the parasitophorous vacuole. In both cases, an internal acidic pH is necessary for the parasite to leave the vacuole, as shown by the drastic reduction of infection of host cells treated with weak bases (Ley et al. 1990;

Andrade and Andrews 2005). It is unknown whether the specific surface composition directs the parasite to one of the routes or, conversely, the route undertaken could influence the fate of the parasite inside the host cell.

As pointed out above, one of the important characteristics described for *T. cruzi* is the variability between strains and the large amount of genes that constitute its repertoire of multigene families. One of the largest is constituted by the Gp85/*Trans-sialidase* (TS) superfamily, encoding surface glycoproteins. The chemical structure of the Gp85 glycoproteins and their role in parasite-host interaction are the main focus of this chapter.

2 Involvement of Gp85 Glycoproteins in *T. cruzi* Infection

2.1 Gp85 Glycoproteins: GPI and Carbohydrate Structure of Tc85

Molecules belonging to the Gp85/TS superfamily are the most abundant surface glycoproteins involved in the infectivity of *T. cruzi*. This superfamily is composed of glycoproteins that do not have *trans*-sialidase activity (Gp85 glycoproteins) and *trans*-sialidase, with enzymatic activity. Almost all members are glycophosphatydilinositol (GPI)–anchored to the plasma membrane and are encoded by a large number of genes. Molecular heterogeneities at the protein or carbohydrate levels were extensively described in the literature, when individual parasites in the population, different stages or strains are compared.

As is known, GPIs are GIPLs. The first GIPL (glycoinositolphospholipid) described was LPPG, discovered in 1974 (Alves and Colli 1974; Lederkremer et al. 1976), with a structure later classified as an inositolphosphoceramide, having sphinganine as an alcoholic base with a fatty acid (lignoceric acid or palmitic acid) forming an amide bond with the base amino group in carbon 2. Typically, carbon 1 is linked to inositol followed by a glucosamine (Lederkremer et al. 1978; Previato et al. 1990; Lederkremer et al. 1991). Free GIPLs exist abundantly in kinetoplastidae (Ferguson 1997).

Concomitantly, it was realized that the structures of protein anchors (Ferguson et al. 1988), called GPI (glycophosphatidylinositol), and GIPLs had the same chemical pattern. Figure 7.1 depicts the structure of *T. cruzi* type-1 GIPL, formerly LPPG, and for comparison the structure of *T. cruzi* mucin-GPI anchors (Lederkremer and Colli 1995).

Note that in *T. cruzi* type-1 GIPL the carbohydrate moiety is decorated with galactofuranoses, and a molecule of aminoethylphosphonate (AEP) is linked to the non-acetylated glucosamine. The lipid moiety is linked to this structure by inositolphosphate and in epimastigotes harvested in the stationary phase of growth, is composed by a ceramide having lignoceric acid or palmitic acid linked to the amino

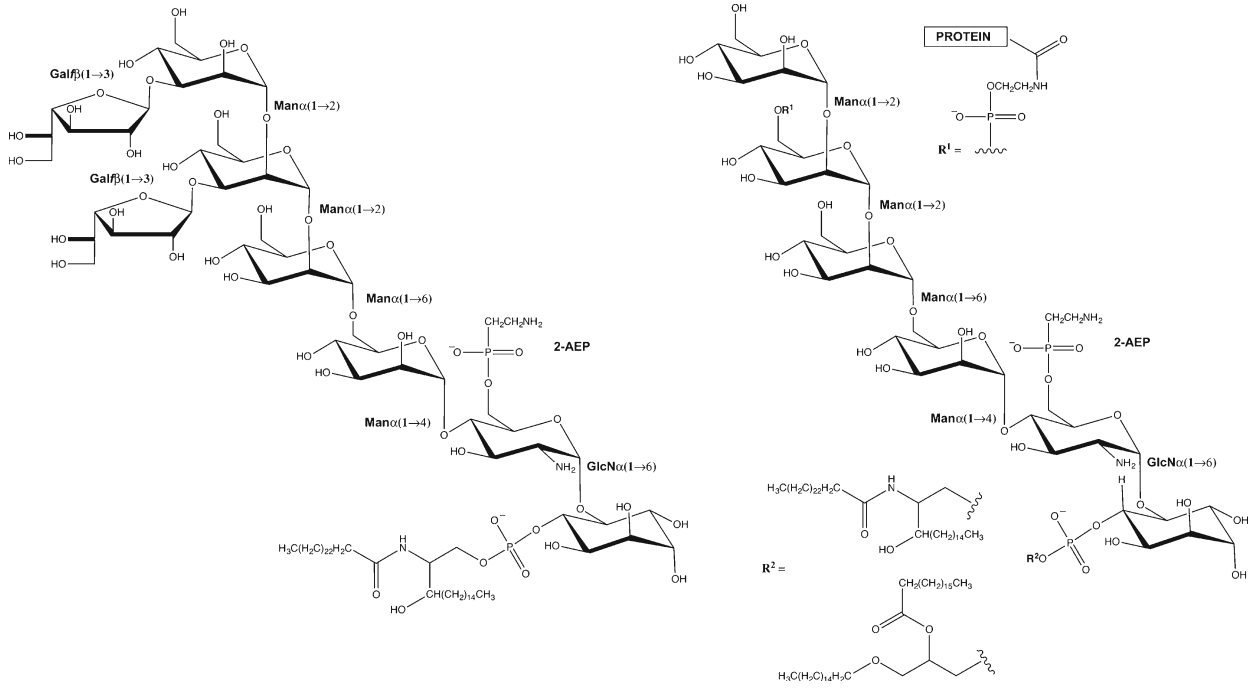

Fig. 7.1 Structures of GIPL-Type 1 and Mucin GPI-Anchor of *Trypanosoma cruzi*. (*Left*) GIPL-Type 1 (formerly LPPG) – a ceramide is linked to the glycan chain through a phosphate-inositol bridge linking the hydroxyl in sphinganine C1 to the hydroxyl in inositol C6; a saturated fatty acid is linked to sphinganine C2 through an amide bond; this fatty acid can be lignoceric acid ($C_{24:0}$) or palmitic acid ($C_{16:0}$); the structure is decorated with 2-aminoethylphosphonate (2-AEP) bound to C6 of glucosamine and galactofuranoses linked to mannoses ($Gal_f\beta(1\rightarrow3)Man$); (*Right*) Mucin GPI-Anchor – two lipid structures (R^2) may be linked (see Table 7.1) to the glycan core through a phosphate-inositol bridge: (*1*) a ceramide similar to GIPL-1; (*2*) a glycerol lipid in which a fatty acid (usually $C_{16:0}$) is linked to C1 by an ether bridge (alkyl), and another fatty acid ($C_{16:0}$, $C_{18:1}$ or $C_{18:2}$) to C2 by an ester bridge (acyl). Mucin anchors are also decorated with 2-AEP and are linked to the protein by one of the mannoses (C6-OH) through an ethanolamine phosphate (R^1) that establishes an amide bond with the amino acid at the protein carboxyl terminus

Table 7.1 Free and protein linked GIPLs in *Trypanosoma cruzi* (*apud* Lederkremer and Agusti 2009)

	IPC structures	PI structures		
Compound	Amide bond in C-2 of SP or DHS	Glycerol C-1	Glycerol C-2	References
T. cruzi GIPL-1 (LPPG)	($C_{16:0}$, $C_{24:0}$)	–	–	(Lederkremer et al. 1978; Lederkremer et al. 1990)
GIPLs	–	Alkyl ($C_{16:0}$)	Acyl ($C_{16:0}$)	(Lederkremer et al. 1993)
Tc85	–	Alkyl ($C_{16:0}$)	–	(Couto et al. 1993; Abuin et al. 1996b)
TS (metacyclics)	($C_{16:0}$, $C_{18:0}$)	–	–	(Agusti et al. 1998)
TS (trypomastigotes)	($C_{16:0}$)	Alkyl ($C_{16:0}$)	–	(Agusti et al. 1997)
1G7	–	Alkyl ($C_{16:0}$)	Acyl ($C_{16:0}$, $C_{18:0}$)	(Heise et al. 1995)
Mucins (epimastigotes)	–	Alkyl ($C_{16:0}$)	Acyl ($C_{16:0}$)	(Acosta-Serrano et al. 1995; Previato et al. 1995)
Mucins (metacyclics)	($C_{16:0}$, $C_{24:0}$)	–	–	(Acosta-Serrano et al. 1995)
Mucins (trypomastigotes)	–	Alkyl ($C_{16:0}$)	Acyl ($C_{16:0}$, $C_{18:1}$, $C_{18:2}$)	(Camargo et al. 1997)
NETNES (epimastigotes)	–	Alkyl ($C_{16:0}$)	Acyl ($C_{16:0}$)	(MacRae et al. 2005)

IPC inositolphosphoceramide, *PI* phosphatidylinositol, *SP* sphinganine, *DHS* dihydrosphinganine

group at sphinganine carbon 2. This GIPL is, thus, a glycosylinositolphosphoceramide (GIPC) (Lederkremer and Colli 1995).

GIPLs that serve as protein anchors (GPI), might be either GIPC or a glycerol-derived phospholipid with a phosphatidylinositol (PI) usually substituted in carbons 1 and 2 of the glyceryl moiety by fatty acids through ether (alkyl) or ester (acyl) bonds. GPI anchors also differ from free GIPLs in the carbohydrate moiety due, generally, to the absence of the galactofuranose and aminoethylphosphonate decorations, and acquisition of an ethanolamine at the nonreducing end of the glycan moiety to serve as a bridge with an amino acid of the anchored protein. In Table 7.1 the lipid structures of free GIPLs and GPI-anchors of *T. cruzi* main surface glycoproteins shown in Fig. 7.1 are summarized.

Phosphatidylinositol phospholipase C (PI-PLC) cleaves the phosphate bond releasing the lipid residue and leaving the phosphate group attached to the inositol-glycan residue. A phospholipase D (PLD) from rat blood plasma cleaves the phosphate bond releasing ceramide 1-phosphate (Lederkremer et al. 1996).

GPI anchors are linked enzymatically to the protein carboxyl terminus by a transpeptidase (Lederkremer and Agusti 2009) usually 9–12 amino acids upstream

from the carboxyl terminal stretches of hydrophobic amino acids, a characteristic of GPI-anchored proteins (Cross 1990; Colli 1993; Cross and Takle 1993). As an example, putative anchor insertion sites (ω sites) (Pierleoni et al. 2008) have been identified in Tc85-11, a cloned member of the Gp85/*Trans*-sialidase superfamily (Giordano et al. 1999):

(751) GDGGAN**G**↓DA**G**↓SAYGRELLPMLLLLGLWALATA$_{COOH}$ (786)

The existence of structural lipid remodeling in mucin anchors, without changes in the glycan portion, has been well established. In fact, as seen in Table 7.1 while the lipid moiety of mucin anchors from epimastigotes and trypomastigotes is an alkylacylglyceroinositol phospholipid, the mucin anchor of metacyclic trypomastigotes is inositolphosphoceramide (Acosta-Serrano et al. 1995). Both formation and remodeling of inositolphosphoceramide also occur during the trypomastigote-amastigote transition (Salto et al. 2003). Another modification that is worth commenting is the existence of an unsaturated fatty acid in the composition of trypomastigote mucin GPIs as an essential feature for the immune response by the host (Almeida and Gazzinelli 2001).

The members of the Gp85/*Trans*-sialidase superfamily are glycoproteins. However, to our notice, no studies on the glycan antennae composition have been made in *trans*-sialidases, the catalytic members of the family. Tc85, a subset of the non-catalytic Gp85 contains, at least, one oligosaccharide antenna, although 12 putative glycosylation sites have been identified in a cloned member of the family (Giordano et al. 1999). Studies were conducted on a *T. cruzi* trypomastigote extract chromatographed in a WGA-column, which is known to bind sialic acid. The eluted material was desialylated and the glycan structure was analyzed by a combination of enzyme and chemical degradation. The results indicate the existence of a N-linked complex type oligosaccharide chain linked to the protein by N-acetyl-D-glucosamine. The chain contained also fucose and αGal-(1→3)-Gal. More studies are needed to elucidate the complete chemical structure of the carbohydrate chains of this glycoprotein family. However, there is no reason to believe that they differ from each other in this aspect.

The Tc85 subset was defined by the monoclonal antibody H1A10 (Alves et al. 1986). This antibody was able to immunoprecipitate the Tc85 bound and Tc85 unbound to the WGA-column, indicating that part of the pool did not contain sialic acid. This suggests that the Gp85 superfamily members may also be substrates, in addition to mucins, for sialic acid addition by *trans*-sialidase.

It was demonstrated that an endogenous phospholipase C cleaves the link between GPI and mucins with the consequent shedding of mucins into the medium (Pollevick et al. 2000). However, Tc85, and probably all Gp85 glycoprotein family members, are mainly shed to the medium through membrane vesicles (20–80 nm) that bud constantly from the parasite surface (Torrecilhas et al. 2012). A half-life of 3.5–4 h for Tc85 was found by pulse-chase experiments with ^{35}S-methionine (Abuin et al. 1996a) suggesting that this may be the half-life time for the shed vesicles.

2.2 Gp85/TS Glycoproteins: Multigene Family

Approximately 50 % of the *T. cruzi* genome from the CL Brener strain (DTU VI), the first *T. cruzi* genome published, is composed by repetitive sequences, mainly large gene families of surface proteins, subtelomeric repeats and retrotransposons. The Gp85/TS superfamily is one of the largest gene families formed by ~700 genes and equal number of pseudogenes, a characteristic shared by other *T. cruzi* families, as for example, MASP (mucin-associated surface proteins) (~1,377genes/433 pseudogenes) and mucins (~863 genes/201 pseudogenes) (El-Sayed et al. 2005). It is important to note that the complexity of the gene families and repeated elements introduce a strong component of uncertainty for the correct assembly of these regions, that are frequently incomplete and misleading (Andersson 2011). Consequently, this adds an extra degree of difficulty for proteomic studies, resulting in ~50 % of "unknown proteins" in all trypanosomatid proteomes recently reported (Nakayasu et al. 2009; Nett et al. 2009; Marchini et al. 2011).

Genome sequencing of other *T. cruzi*, *T. brucei* and *Leishmania* strains, with distinct biological and pathological outcomes, are underway. The comparison of their whole genomes should contribute to complete the genome assembly and, importantly, to provide some clues for the understanding of genome-pathological/biological relationship, as well as to point out unique genes for *T. cruzi*, *T. brucei* or *Leishmania* (Alsford et al. 2011; Choi and El-Sayed 2012). To reach these goals, an ambitious sequencing program was launched, with the rationale for strain selection assigned in: (www.genome.gov/Pages/Research/DER/PathogensandVectors/Pathogens of Trypanosomatid.pdf). In this context, a recently published draft of the *T. cruzi* Silvio X10/1 strain genome reported that no major gene differences in the core gene content were found between the Silvio X10/1 strain (classified as DTU I; haploid genome size ~44 Mbp) and CL Brener, a hybrid strain resulted from DTU II/III hybridization (classified as DTU VI, haploid genome size ~55 Mbp) (Franzen et al. 2011). Although a significant reduction in the gene content of some multigene families, as MASP, mucin, DGF and Gp63 was reported for Silvio X10/1, only a slight decrease was observed when the Gp85/TS superfamily was analyzed (Franzen et al. 2011). The generation of new variants from the Gp85/TS superfamily was presumed to occur in the chromosomes subtelomeric regions, which are also enriched in retrotransposons. In *T. cruzi*, 9 % of the total Gp85/TS sequences were found in the subtelomeric regions of 39 chromosome ends. However, not all multigenic families are abundant in the subtelomeric regions, since mucins and MASP-coding genes are poorly found in these regions (Moraes Barros et al. 2012).

Similarly to *T. cruzi*, other trypanosomatids such as *Leishmania* or *T. brucei* deal with distinct environments in both mammalian and invertebrate hosts and also display distinct morphological stages during their life cycle. One important issue is how they modulate specific gene expression in response to these environmental changes, since regulation of transcription is absent. RNA polymerase II transcribes almost all protein coding genes. One of the few exceptions is the transcription of

T. brucei Variant Surface Glycoproteins (VSG) genes, which are transcribed by RNA polymerase I. VSG, a highly polymorphic family of surface proteins is associated with the evasion of the blood-living form of *T. brucei* from the immune system (Schwede et al. 2012).

The transcripts in trypanosomes are mainly polycistronic and may contain from tens to hundreds of genes, which are then processed by 5′-*trans*-splicing and 3′-polyadenylation. The regulation of the protein expression relies mostly on post-transcriptional mechanisms, in which the mRNA degradation is the main control. However, the mechanism that selects which specific mRNA should be degraded is unknown (Schwede et al. 2012). Interestingly, in *Giardia lamblia* the expression of one of its 190-variant specific surface proteins is regulated by a mechanism similar to RNA interference (Prucca et al. 2008). In trypanosomatids, the iRNA pathway has been suggested to promote genome stability by silencing mobile elements (Kolev et al. 2011). Although strong iRNA activity was detected in *L. braziliensis*, no iRNA pathway was detected in *L. major*, *L. infantum* or *T. cruzi*.

In this scenario, comparative proteomic analysis of the parasite stages under different physiological conditions, as well as comparison among strains, is fundamental to the understanding of pathogenicity and parasite biology. For instance, variability in the MASP family gene expression in *T. cruzi* was demonstrated by proteomic analysis (Atwood et al. 2006; Pablos and Osuna 2012; Santos et al. 2012). The expression levels in the two distinct DTUs analyzed (CL Brener – DTU VI lineage and PAN4 – DTU1 lineage) vary among stages and strains. The expression of the MASP family, using a conserved 5′-terminal region encoding the signal peptide of the MASP proteins, showed a relatively more homogeneous expression among the stages of PAN4 strain than among the CL Brener strain stages. Assigning the value 1 to the expression in epimastigotes, the following ratios have been found: PAN4 strain: trypomastigotes 3.94> metacyclic trypomastigotes 1.32> amastigotes 1.18> epimastigotes 1; CL Brener strain: amastigotes 6,495.48> trypomastigotes 1,796.98> metacyclic trypomastigotes 32.22> epimastigotes 1 (Pablos and Osuna 2012). Moreover, an increased expression of the MASP family was observed from 24 h onwards after infection, although this expression was heterogeneous among the population (Pablos and Osuna 2012). By sequencing seven cDNA libraries, a more heterogeneous expression of MASP genes was reported, including the differential expression of few genes among trypomastigotes derived from epithelial or myoblast cells and higher expression of MASP genes in bloodstream trypomastigotes in comparison to tissue-cultured derived trypomastigotes. More interestingly, a temporal change in the repertoire of MASP expressed in the population was reported in bloodstream trypomastigotes isolated from sequential passages in mice, exemplified by a higher expression of MASP16 after passage 2 and for MASP2 and MASP27 after passage 10. Accordingly, antibody levels against specific sequences of the MASP proteins also vary after sequential passages in mice (Santos et al. 2012).

Extensive polymorphism has also been described for the CL Brener strain mucins (Frasch 2000): *TcMUC* family, the largest one, which seems to be restricted to the parasite forms present in the mammalian host, and *TcSMUG*, a less diverse gene group. *TcSMUG* is formed by two subgroups: *TcSMUG S*, which codes for the major expressed Gp35/50 mucins, and *TcSMUG L*, coding for GPI-anchored mucins

(Nakayasu et al. 2009), expressed on the surface of epimastigotes. Interestingly, *TcSMUG L* products are refractory to sialylation by the parasite *trans*-sialidases and their expression vary among the isolates (Urban et al. 2011).

The polymorphism of the *T. cruzi* Gp85 glycoproteins was first described at the protein level in the subset of Tc85 glycoproteins, as discussed above. An anti-Tc85 monoclonal antibody inhibited between 50 % and 90 % the invasion of mammalian cells in a strain or stage-dependent way (metacyclic or tissue-culture-derived trypomastigotes). Moreover, the monoclonal antibody recognized in the parasite population approximately 50 % of the tissue-cultured trypomastigotes and 90 % of the metacyclic trypomastigotes (Alves et al. 1986; Abuin et al. 1989). The heterogeneity in the expression of these proteins among stages and among individual parasites in the population, as well as the observed polymorphism, raised suggestions that these events should facilitate a wide variety of interactions between parasite and host. Additionally, a large panel of monoclonal and polyclonal antibodies demonstrated the simultaneous expression of different subsets of 85 kDa glycoproteins (called SA85) by each parasite in the population (Kahn et al. 1999). In addition, each individual protein may interact with distinct host receptors, as shown e.g. for one family recombinant protein (Tc85-11), which binds at least to laminin and to components of the host cytoskeleton (see item 3.2).

An extensive number of reports including the genome sequencing, confirmed the polymorphism of the protein family and contributed with new data to the understanding of its role in parasite-host cell interaction (do Carmo et al. 2002; Alves and Colli 2007; Alves and Mortara 2009; Epting et al. 2010; Souza et al. 2010; Butler and Tyler 2012).

In conclusion, the protein expression encoded by large multigene families, herein illustrated for MASP, mucins and gp85 glycoproteins opens up a plethora of possibilities for the parasite to deal with the host, including cell invasion or host immune response.

2.3 Gp85/TS Superfamily: Genomic and Functional Signature Motifs

2.3.1 Grouping the Gp85/TS Gene Superfamily: General Structure

The Gp85/*Trans*-sialidase gene family was first identified in the 1980s and organized in four subgroups accordingly to the presence of conserved domains and functional properties of the proteins (Fig. 7.2, groups I-IV). Group I was composed by proteins described in the literature, as TCNA (Pereira 1983; Pereira and Hoff 1986) and SAPA (Pollevick et al. 1991) and only this group contains members with *trans-sialidase* activity, for which the residue Tyr^{374} at the catalytic site is essential. The replacement of Tyr^{374} by histidine results in inactive TS, devoid of catalytic activity, but with binding capacity to host cells in a lectin-like way (Cremona et al. 1995; Rubin-de-Celis et al. 2006; Carvalho et al. 2010) in spite of a residual hydrolytic activity being found while studying the crystal structure of a supposedly inactive TS (Oppezzo et al. 2011).

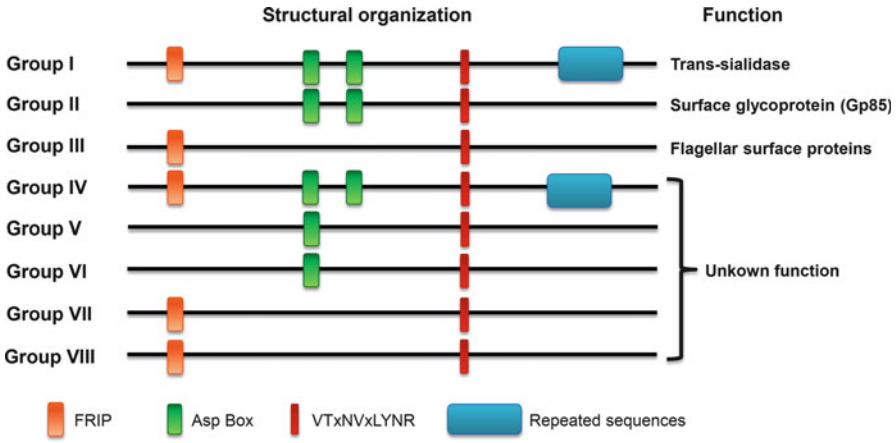

Fig. 7.2 Genomic distribution of Gp85/TS motifs. Schematic organization of the domains FRIP, Asp Box, FLY motif and repeated sequences to each Gp85/*Trans*-sialidase subgroups, adapted from Freitas et al. 2011

TCNA introduced the key signatures of the *trans*-sialidase proteins: *Asp Box motifs* (SxDxGxTW), *SAPA* (shed acute phase antigen) *motif*; *VTV* or *FLY motif* (VTVxNVxLYNR) and *FRIP motif* (xRxP) (Pereira et al. 1991; Colli 1993; Cross and Takle 1993; Frasch 1994; Schenkman et al. 1994). Group II was composed by glycoproteins called Gp85 located at the parasite surface. Members of this group, as Tc85-11, gp90 and gp82 for example, are related to adhesion and invasion of the host cell (Alves and Colli 2008). Group III contains surface proteins associated to the parasite flagellum. FL-160 is an important member of this group, able to inhibit the complement pathway in the host cell. Finally, group IV is also composed by proteins included in the superfamily due to the presence of the conserved domain VTxNVxLYNR at the C-terminal, as Tc13 protein (Cross and Takle 1993; Schenkman and Eichinger 1994; de Souza et al. 2010).

After the identification of new genes and based on 508 complete gene sequences analyzed from the Gp85/TS superfamily, the previous classification was expanded to eight subgroups (Fig. 7.2, groups I-VIII) (Freitas et al. 2011). Groups I–IV are identical to the original classification and the new genes were included in the additional groups V, VI, VII and VIII. Of the predicted proteins 96 % contain the motif VTxNVxLYNR, but only 328 out of the 508 predicted proteins have the canonical VTVxNVxLYNR sequence. A conserved or degenerated *Asp box* motif was found in 383 proteins belonging to groups I, II, IV, V and VI, from which the majority has one (220) or two (154) *ASP motifs*, while few members (9) have three. Asp box motifs are missing in groups III and in the majority of the members from groups VII and VIII. In relation to the *FRIP motif*, due to the small size and highly degenerated sequences, only the occurrence of the *FRIP motif* (xRxP) upstream the *Asp box* closest to the N-terminal region, was considered. Taken this into account, the majority of proteins from groups I, III, IV, VII and VIII have the *FRIP motif* (Freitas et al. 2011).

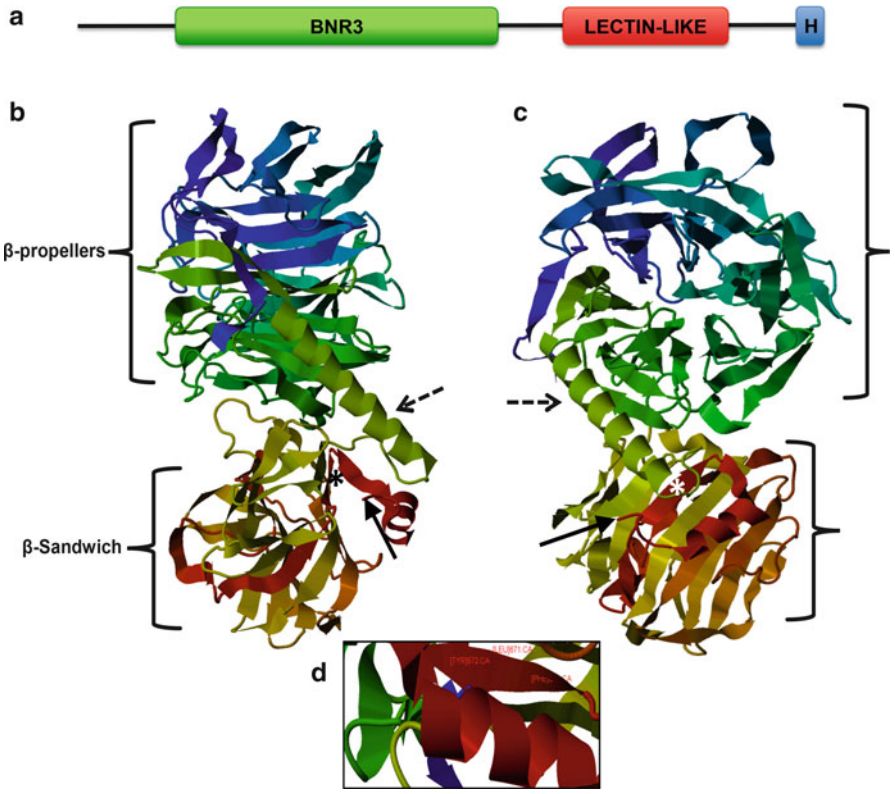

Fig. 7.3 General structure of the Gp85/TS members. (**a**) Localization of the domains: neuraminidase (BNR 3), Lectin-like and *H* Hydrophobic C-terminal of Tc85-11 protein. (**b**) and (**c**) Tc85-11 tridimensional structure model from amino acid 52-689 modeled after *T. rangeli* neuraminidase structure. The sequence arrangements β-Propeller and β-sandwich are indicated by *brackets*. *Dotted arrows* indicate the α-helix motif. *Bold arrows* show the C-terminal. *Black and white asterisks* represent the FLY motif localization, also represented in (**d**). The model was obtained by *PSI* The Protein Modal Portal (http://www.proteinmodelportal.org/)

The reported crystal structure of the *T. rangeli* sialidase (Buschiazzo et al. 2000) launched the basis for the subsequent studies on the *trans*-sialidase structures (Buschiazzo et al. 2002; Montagna et al. 2002; Oppezzo et al. 2011) or for modeling the structure of other members of the group (Marroquin-Quelopana et al. 2004; Cortez et al. 2012).

The overall structure of *T. cruzi trans*-sialidase follows the general pattern of *T. rangeli* sialidase, with two distinct structural domains connected by a long α-helical segment (Fig. 7.3): the catalytic site-containing N-terminal domain, named neuraminidase domain (BNR3), and the C-terminal lectin-like domain, not involved in the transglycosylation activity (Buschiazzo et al. 2002). The N-terminal domain is characterized by six β-propeller motifs with a very organized arrangement (Fig. 7.3) and the C-terminal domain contains a lectin-like topology, organized as a

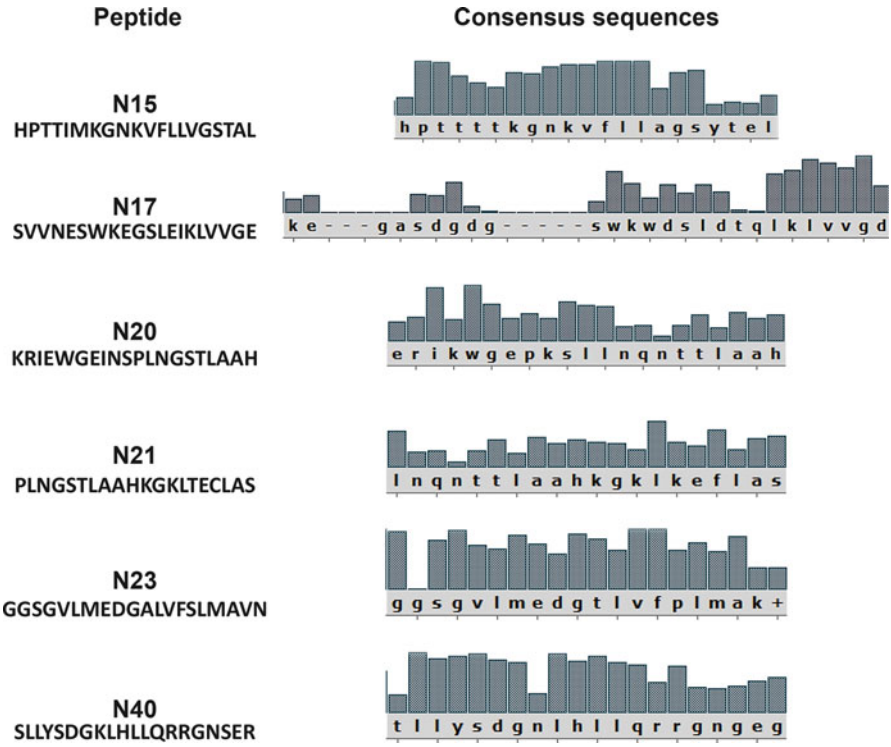

Fig. 7.4 Amino acid conservation of Group II Gp85/TS laminin binding peptides. All Group II Gp85/TS proteins (Boscardin et al. 2010) were aligned by Clustalw using UGene software. On left, the sequence of the laminin binding peptides described by Marroquin-Quelopana et al 2004. On the *right*, the consensus sequence of the peptides after alignment of all Group II glycoproteins

β-sandwich structure with β-sheet motifs (Fig. 7.3). The FLY motif is localized upstream to the C-terminal. The protein ends with a second α-helix which, in the members of Group I, is followed by the SAPA repeats, composed by 12 repeated amino acids (Frasch 1994) and associated with the stability of TS in the blood (Alvarez et al. 2004). SAPA, a highly antigenic region (Frasch 1994; Alvarez et al. 2004), is absent from the proteins of Group II. In TS, three putative glycosylation sites are localized at the N- terminus and two at the C-terminus domain (Buschiazzo et al. 2000). A TS-based model is shown in Fig. 7.4 for Tc85-11 protein structure, from Group II, involved in *T. cruzi* invasion.

The need of TS enzymatic activity in the invasion of host cells by *T. cruzi* is controversial. In addition to the carbohydrate-binding site present in the catalytic pocket of the inactive TS implicated in the infectivity of the parasite (Mendonça-Previato et al. 2005; Souza et al. 2010; Rubin and Schenkman 2012), binding to cell receptors due to the lectin-like domain was also reported for TS, as the nerve growth factor receptor (Trk) (Chuenkova and Pereira Perrin 2005; de Melo-Jorge and

PereiraPerrin 2007; Dias et al. 2008; Weinkauf et al. 2011). Notwithstanding, due to the high number of Gp85/TS proteins displaying extensive polymorphism, it is not unlikely that different members of the superfamily can perform different functions on a complex biological phenomenon, such as parasite adhesion to host cells. This led some investigators to focus on the possible role in biological function of peptide segments of the amino acid sequence, in order to delve on the role of the superfamily members (Magdesian et al. 2001; Marroquin-Quelopana et al. 2004; Cortez et al. 2012) (see Sects. 2.3.2 and 2.3.3).

2.3.2 Gp85 Glycoproteins Family: Tc85 Adhesion to ECM and Ensuing Post-translational Modifications in *T. cruzi* Proteins

Tc85 Adhesion to ECM Elements

Different reports showed the binding of *T. cruzi* to extracellular matrix components, as laminin-1 (Giordano et al. 1994a, b), collagen (Velge et al. 1988), fibronectin (Ouaissi et al. 1984), heparansulphate (Calvet et al. 2003; Oliveira et al. 2008; Bambino-Medeiros et al. 2011), galectin-3 (Moody et al. 2000) or thrombospondin (Johnson et al. 2012).

Fibrous proteins, as collagen and elastin, and structural proteins, like fibronectin and laminin, are the main components of the ECM, in addition to proteoglycans (Kadler 1995; Kielty et al. 2002). Fibronectin is an example of structural protein able to control many physical functions by interaction with different elements, such as growth factors and adhesion molecules. Fibronectin contains a heparin-binding motif (Ruoslahti 1988) and the major functional domain named FN III that includes the RGD (Arg-Gly-Asp) motif, responsible for recognition of and binding to integrin (Ruoslahti 1996). Many other ECM molecules contain the RGD motif, as laminin, vitronectin, thrombospondin and tenascin. Laminin, a major constituent of the basement membranes, is responsible for the tensile strength of the tissues. It is composed by three subunits (α, β and γ) that interact with other laminin molecules, as well as with other ECM elements (Colognato and Yurchenco 2000).

As pointed out, the importance of ECM-parasite interaction during the infection is well established in the literature. In this context, members of prolyloligopeptidase family (as oligopeptidase C and Tc 80 POP) may degrade ECM components and activate signaling pathways in the parasite and/or host cells important for parasite invasion (Grellier et al. 2001; Cazzulo 2002; Souza et al. 2010). Also, a heparin binding proteinase (HPB) localized at the parasite flagellar membrane can also trigger signaling pathways involved in the penetration of the parasite (Oliveira-Jr et al. 2013). Furthermore, the infection of cultured cardiomyocytes by *T. cruzi* leads to a reduction of fibronectin and a reorganization of laminin, suggesting the modulation of ECM during parasite invasion (Calvet et al. 2004). Additionally, molecules that interact with ECM, as transforming growth factor β have also been implicated in parasite infection (Araujo-Jorge et al. 2008).

The direct binding of *T. cruzi* 85 kDa surface proteins to fibronectin through the RGD motif and its relevance for parasite invasion has been shown decades ago (Ouaissi et al. 1986). *In vitro* inhibition of *T. cruzi* invasion in the presence of fibronectin or peptide-containing RGD led to the suggestion that RGD works as a bridge between the parasite and the host cell (Calvet et al. 2004).

The involvement of the protein portion of laminin in *T. cruzi* infection was initially shown by the partial inhibition of cell invasion by anti-laminin antibodies and by the isolation of a laminin-binding 85 kDa glycoprotein (Tc85) from *T. cruzi* trypomastigotes (Giordano et al. 1994a, b). Later on, one member of the Tc85 subset was cloned and the expressed recombinant protein (Tc85-11) bound to laminin with high affinity, but not to fibronectin or gelatin (Giordano et al. 1999). In accordance with the role of laminin in *T. cruzi* invasion, silencing of laminin γ1 decreases parasite binding to the host cell and intracellular amastigotes multiplication (Nde et al. 2006). Recently, a laminin-like molecule found in the salivary gland of the host insect has been described as the receptor for *Phytomonas*, an important plant parasite component of the Trypanosomatidae family (Dias et al. 2012).

The amino acid sequences responsible for Tc85-11 binding to laminin were determined by inhibition assays using synthetic peptides covering Tc85-11 primary structure and the laminin-binding site was located at the β-propeller region on the N-terminus model of Tc85-11 (Marroquin-Quelopana et al. 2004). The seven N-terminal peptides with high affinity to laminin are well conserved amongst all members of Gp85 (group II) (Fig. 7.4), but not on the other Gp85/TSprotein groups (not shown), strongly suggesting a major biological role of proteins from group II in the adhesion step of *T cruzi* trypomastigotes to ECM components.

Post-translational Modification of *T. cruzi* Proteins Due to the Adhesion of Trypomastigotes to Laminin and Fibronectin

The signaling triggered by ECM elements in mammalian cells is well studied (Kim et al. 2011) in contrast to scarce information available on the events triggered in *T. cruzi* trypomastigotes upon adhesion of the parasite to the ECM. One first report showed changes in the phosphorylation level of several trypomastigote proteins, which may be key elements during parasite adhesion to laminin and fibronectin (Mattos et al. 2012). Phosphorylation and dephosphorylation events may have an important role in the parasite as can be inferred from phosphatome and kinome studies that described the existence of 86 phosphatase genes (Brenchley et al. 2007) and 190 kinase genes (Parsons et al. 2005). Also, *trans*-sialidase interaction with the catalytic subunit of PKA (PKAc) was demonstrated recently, as well as the phosphorylation of TS by PKAc, probably involving the cAMP pathway. Additionally, a suggestion has been made that the *trans*-sialidase phosphorylation by PKA is involved in the regulation of protein intracellular traffic and turnover (Bao et al. 2010). Albeit less characterized, phosphorylation of a 175 kDa protein from metacyclic trypomastigotes (Favareto et al. 1998), as well as intensive dephosphorylation of proteins from trypomastigotes (Zhong et al. 1998) have also been reported.

Our group has recently described the involvement of protein dephosphorylation in the parasite upon adhesion to fibronectin and laminin (Mattos et al. 2012). It has been shown that dephosphorylation of the major parasite cytoskeletal proteins α-tubulin and paraflagellar rod protein (PFR), as well as of ERK 1/2, may be key events in the parasite during adhesion to ECM and may be responsible for the success of parasite infection.

The signal cascades that are activated in the parasite during the host cell recognition and invasion are far away from being known and should be further explored, since they are important steps responsible for the success of the parasite invasion.

2.3.3 Role of the Conserved FLY Motif

The specificity of Tc85 towards host cells is somehow determined by the conserved motif VTVxNVFLYNR (FLY motif), which binds to targets in a receptor-ligand manner. The first indication that the FLY motif was involved in the adhesion to and entry of trypomastigotes into host cells came from *in vitro* studies on Tc85-11, a member of the Tc85 family (Giordano et al. 1994a, b; Giordano et al. 1999; Magdesian et al. 2001). Most of the characterization of Tc85-11 function has been carried out in the context of trypomastigote-host cell interaction, reviewed below.

Identification of FLY as a Cell-Binding Domain

Cell-binding assays with the use of synthetic peptides (~15 amino acids long) that spanned the Tc85-11 protein showed that the FLY motif-containing peptide was a mammalian cell-binding domain (Magdesian et al. 2001). Indeed, micromolar concentrations of the synthetic FLY peptide inhibited the binding of the recombinant Tc85-11 protein to tissue culture cells. Furthermore, alanine scanning analysis to measure the effect on cell binding of the individual substitution of each amino acid with alanine within the FLY motif, demonstrated that substitution of the leucine residue for alanine (VTNVFAYNRPL, named FAY motif) eliminated completely the capacity of the peptide to inhibit binding of Tc85-11 to host cells. The discovery of the FLY motif came together with the identification of cytokeratin-18 (CK18) as its host cell receptor as revealed by mass spectrometry analysis of the receptor previously purified by affinity chromatography. Although the localization of CK18, a member of the keratin gene family present in the intermediate filaments of different cells (Fuchs and Weber 1994), on the plasma membrane is controversial in the literature, recombinant CK18 and anti-CK18 antibody completely inhibited FLY adhesion to epithelial cells. In addition, cell invasion assays in the presence of anti-CK18 antibody inhibited the invasion of epithelial cells by trypomastigotes in more than 60 % (Magdesian et al. 2001). However, cells transiently transfected with CK18-iRNA containing negligible levels of CK18 transcripts and CK18 protein were efficiently infected by *T. cruzi* trypomastigotes. Notwithstanding, the growth of amastigotes was severely arrested in CK18-RNAi treated cells (Claser et al.

2008). The relevance of these findings to the understanding of *T. cruzi* infectivity deserves further investigations. Since the CK8/CK18 pair provides cell protection against apoptosis (Caulin et al. 2000; Gilbert et al. 2001) it is reasonable to determine the viability of CK18-RNAi cells after 48 h of transfection, time in which amastigote numbers into the host cells were determined. Also, the possibility that *T. cruzi* trypomastigotes exploit other surface receptors to internalize in CK18-RNAi cells cannot be ruled out. A study demonstrating that incubation of cells with the synthetic FLY motif or the recombinant Tc85-11 protein stimulated epithelial cell invasion in a dose-dependent manner, suggested that these might act as the primary signals originated by *T. cruzi* to promote an environment that facilitates trypomastigote entry into epithelial cells (Magdesian et al. 2001).

Mechanism of FLY Potentiation of *T. cruzi* Infection

Intermediate filaments (IF) consist of a highly dynamic family of cytoplasmic (cytokeratins, vimentin, desmin, neurofilaments) and nuclear disease-associated cytoskeletal proteins (nuclear lamins). Depending upon the cell type, IFs are composed by different members of the cytoskeletal IF protein family: epithelial cells contain mainly cytokeratins; muscle cells, desmin; mesenchymal cells, vimentin; and neurons, neurofilaments (Hutton et al. 1998). Cytokeratins are encoded by a large multigene family (more than 40 functional genes were described in human and other mammalian genomes) whose individual members can be divided into two major types, the type I or acidic keratins (CK9–CK23) and type II or neutral-basic keratins (CK1–CK8) (Moll et al. 1982). In all epithelial cells, cytokeratin filaments are built from a type I and type II heterodimer expressed in a tissue-specific, and differentiation-specific manner. For example, simple (single-layered) epithelia express CK7, CK8, CK18, CK19, CK20, and CK23 as different pair partners, with CK8/CK18 being a pair common to all epithelial cells. Like all IF proteins, CK8 and CK18 consist of a central α-helical (rod) domain (where heterodimerization occurs) flanked by N- and C-terminal globular "head" and "tail" domains that serve to regulatory and functional purposes. Indeed, several lines of evidence indicate that post-translational modifications of CK8/CK18, particularly phosphorylation of specific serine residues within the "head" and "tail" domains, recognized by mitogen-activated protein (MAP) kinases and also by protein kinase C (PKC), affect IF dynamics, solubility, and organization (Fuchs and Cleveland 1998; Omary et al. 1998; Ridge et al. 2005; Sivaramakrishnan et al. 2009).

Considering these observations, it is reasonable to speculate that binding of the FLY domain to CK18 would alter its phosphorylation status somehow reorganizing the cytoskeleton to facilitate infection by the parasite. Latex beads covered with the synthetic FLY motif promoted a decreased CK18 phosphorylation and CK18 redistribution within epithelial cells, with its accumulation in the dorsal region near the FLY binding site (Magdesian et al. 2007). The mechanism governing the CK18 dephosphorylation and spatiotemporal relocation upon FLY binding was only partially elucidated. No change in Ca^{2+} homeostasis or in the intracellular levels of

cAMP was observed in cells incubated with the synthetic FLY peptide for different periods of time. However, a significant and reproducible increase in phosphorylated ERK1/2 was found in the presence of FLY. Inhibitors of the ERK1/2 pathway (U0126 and PD98059), inhibited FLY-beads adhesion to epithelial cells and U0126 blocked by 57 % the host cell infection by *T. cruzi* trypomastigotes. Although the precise mechanism involved in the FLY-mediated potentiation of infection is not completely understood at present, it might involve the activation of the ERK1/2 in host cells thus contributing to the establishment of infection.

Role of the FLY Motif in *T. cruzi* Tissue Tropism

The mouse model is widely used to study aspects of *T. cruzi* infection. By experimentally infecting mice, many groups showed marked differences in the tissue distribution of the distinct *T. cruzi* strains, also shown for mixed infections in patients (Mantilla et al. 2010), indicating that the parasite genetic background may contribute to the outcome of infection, but efforts to elucidate the molecular and the cellular bases associated to *T. cruzi* tissue tropism were unsuccessful.

Currently, it is well established that the vasculature in different parts of the body are morphologically and functionally different, including the expression of unique molecules that play important roles in health and disease (Ruoslahti and Rajotte 2000). The involvement of the FLY motif in tissue-specific homing was studied using the phage display approach. Phage display is a powerful tool to explore the vasculature trait *in vivo* (Rajotte et al. 1998; Pasqualini 1999). In short, the idea behind the technique is to engineer bacteriophages genetically in order to express and display foreign peptides or protein fragments fused with the capsidic proteins on the surface of the phage particle (Smith 1985). The resulting fusion bacteriophages are injected intravenously into mice, allowing them to circulate and bind to endothelial cells on the vascular bed. Target organs are then removed and homogenized and bound phages are rescued by infection of bacteria (Pasqualini and Ruoslahti 1996). Filamentous phages expressing the FLY motif at the tip of the pIII minor protein coat (FLY-phage) bound to immobilized CK18 protein and to epithelial cells in a dose-dependent manner, as well as to immortalized endothelial cells derived from the heart and the bladder, two organs known to lodge *T. cruzi* after infection (Tonelli et al. 2010). The importance of the FLY-phage selective binding to endothelial cells *in vitro* was underscored in homing assays *in vivo*. Specific enrichment of the FLY-phage particles was observed in the heart vasculature followed by the bladder and to a lesser extent to the colon after intravenous administration of FLY-beads. Importantly, FLY-phage was not enriched in other tissues like the brain, lung or bone marrow. Other intermediate filament proteins, as vimentin, a main component of endothelial cells, CK8, CK18 and CK20 were also tested in FLY-phage binding assays, with the latter adhering to all proteins analyzed. This, together with the observation that intermediate filament proteins were detected on the surface of live endothelial cells derived from bladder, heart and colon, but not with lung-derived endothelial cells (Tonelli et al. 2010), strongly suggests that the

preferential binding of FLY-phage to these cells may be due, at least in part, to the presence of extracellular exposed intermediate filament proteins.

These results imply an interaction between the FLY motif and vimentin exposed on the surface of endothelial cells that may play an important role in adhesion of trypomastigotes to the vascular bed of selected organs or tissues. Notwithstanding, although FLY may be part of the picture, it is conceivable that other molecules of the parasite might be involved in the tissue tropism observed in humans and animal models.

The Host Immune Response to FLY

Animal studies implicated a wide variety of immune responses associated to members of the Gp85/*Trans*-sialidase superfamily upon *T. cruzi* infection. These studies showed that epitopes of the Gp85/*Trans*-sialidase family of proteins are immunodominant, and elicit antibody, as well as $CD4^+$ and $CD8^+$ specific T-cells responses (Tarleton 2007; Boscardin et al. 2010). In the particular case of the enzymatically active *trans*-sialidase it is well established that transfer of sialic acid from host sialylated proteins to *T. cruzi* glycoproteins provides resistance to complement-mediated lysis and assists in host cell invasion (Tomlinson et al. 1994). In the latter, the virulence-enhancing effect was described to be associated to the enzyme sialic-binding site although the participation of other sites within the protein cannot be ruled out. Supporting this idea, the intraperitoneal administration of the FLY synthetic peptide into BALB/c mice 1 week before infection with *T. cruzi* resulted in higher levels of parasitemia, more parasite nests in specific organs like the heart, bladder and small intestine, anticipated mortality and strong inflammatory foci, mainly in the atria (Tonelli et al. 2011). A consequence of a pathogenic infection is the activation of an inflammatory process that is an essential part of the host protective response. By contrast, a persistent tissue inflammation may have deleterious effects on the infected individual. Indeed, in the case of chronic infections with *T. cruzi* tissue inflammation is accompanied by tissue fibrosis and determines the clinical outcome for patients with Chagas' disease.

In the heart of infected animals $CD4^+$ and $CD8^+$ T cells generate IFN-γ that, together with TNF, activates cardiomyocytes to synthesize nitric oxide (NO) that is important in the control of intracellular parasite multiplication (Machado et al. 2000). Both $CD4^+$ and $CD8^+$ T cells play important roles in the control of *T. cruzi* infection in humans and animal experimental models (Tarleton et al. 1994, 1996; Fuenmayor et al. 2005) and $CD8^+$ T cells were considered crucial to control *T. cruzi* proliferation, especially at the acute phase of Chagas' disease. An increase in $CD4^+$ T cells, but not of $CD8^+$ T cells, in the heart of FLY-treated and infected mice was observed, as well as a10 % increment of a subpopulation of the T cells, expressing the CD4, CD25 and Foxp3 markers (Treg cells) (Fontenot et al. 2003; Hori et al. 2003). These regulatory T cells are involved in shutting down immune responses to prevent the expansion of self-reactive lymphocytes or autoimmunity (Bluestone and Abbas 2003), but in pathogenic infections the role played by Treg

was somehow controversial in the literature. In addition to the increment of Treg cells FLY, in resident peritoneal cells, induced the production of IL-10, a cytokine that strongly inhibits the immune response to many eukaryotic intracellular pathogens (Cyktor and Turner 2011). All together, these data suggested the importance of Treg cells in relation to the specific effect of FLY in the exacerbation of the infection by *T. cruzi*, but the mechanism deserves further studies to be well understood.

3 Concluding Remarks

The published data regarding infection by *T. cruzi* emphasize the remarkable variety found *in vitro* or *in vivo*. Different members of several multigene protein families are expressed among the population, herein exemplified by gp85/TS, MASP or mucins present on the cell surface of *T. cruzi*. Each parasite can express more than one member and their expression may change during the time as shown e.g. for gp85 or MASP families, respectively. But how *T. cruzi* switches the expression of the proteins is an open question that has to be answered. Such variability is potentiated if different strains are considered, making the understanding of host-parasite interaction a formidable task. On one hand, the presence of conserved sequences among the members of each family may indicate functions to be preserved, which can be for example, essential for the protein structure or a specific biological function, as adhesion to a specific host molecule. On the other hand, the variable regions can be essential, for example, for the evasion of the parasite from the mammalian immune system or for the interaction with distinct molecules. The knowledge of the conserved regions functions is a proposal of some research groups aiming at a better understanding of each family function. Synthetic peptides or phage display methodology are good tools to be employed, as exemplified by the Tc85 amino acid epitopes involved in laminin adhesion or to the possible role of the FLY motif in tissue tropism in the mouse model.

Due to understandable reasons, most of the studies on the host-*T. cruzi* interaction have been focused in the mammalian response. This is now rapidly changing with new tools that allow large-scale approaches for the simultaneous analysis of modifications occurring in both parasite and host.

Finally, it is essential to probe into the protein posttranscriptional modifications under different conditions and environments to which the parasite is submitted. Protein phosphorylation and dephosphorylation are common events in signaling pathways, herein discussed for *T. cruzi* trypomastigotes upon adhesion to laminin or fibronectin. It is expected that other postrancriptional modifications that result from the interaction of *T. cruzi* with the host will be described in the near future.

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Chapter 8
Trypanosoma cruzi Trans-Sialidase: Structural Features and Biological Implications

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Abstract *Trypanosoma cruzi trans*-sialidase (TcTS) has intrigued researchers all over the world since it was shown that *T. cruzi* incorporates sialic acid through a mechanism independent of sialyltransferases. The enzyme has being involved in a vast myriad of functions in the biology of the parasite and in the pathology of Chagas' disease. At the structural level experiments trapping the intermediate with fluorosugars followed by peptide mapping, X-ray crystallography, molecular modeling and magnetic nuclear resonance have opened up a three-dimensional understanding of the way this enzyme works. Herein we review the multiple biological roles of TcTS and the structural studies that are slowly revealing the secrets underlining an efficient sugar transfer activity rather than simple hydrolysis by TcTS.

Abbreviations

4-MUNeu5Ac	4-methylumbelliferyl-N-acetyl neuraminic acid
Gal*f*	Galactofuranose
Gal*p*	Galactopyranose
GlcNAc	*N*-acetylglucosamine
Neu5Ac	*N*-acetylneuraminic
Neu5Gc	*N*-glycolylneuraminic acid
NGF	Nerve growth factor receptor
pNPNeu5Ac	p-nitrophenyl-N-acetyl-neuraminic acid
SAPA	Shed acute phase antigen

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Sias	Sialic acids
Siglecs	Sia-binding Ig-like lectin
TcTS	*Trypanosoma cruzi trans*-sialidase
TSs	*Trans*-sialidase family
UTR	Untranslated regions

1 Introduction

The initiation of communication between *Trypanosoma cruzi*, the etiologic agent of Chagas' disease, and mammalian cells requires contact between parasite molecules and host ligands. The parasite membrane is covered with a dense coat formed mainly of molecules of the *trans*-sialidase (TSs) family and of sialic acids (Sias) containing glycoproteins; this coat assures an interface with the host environment (Freitas et al. 2011; De Pablos and Osuna 2012). Sias are acidic monosaccharides found at the outermost ends of the sugar chains of glycoconjugates involved in a myriad of functions ranging from cell recognition to cell life and death (Varki 2006). *T. cruzi* is not able to synthesize Sias by the well-known route in which cytidine monophosphate-sialic acid is an intermediate. Instead, *T. cruzi* is part of a restrict group of parasites evolutionarily adapted to incorporate sialic acid from exogenous sialoglycoconjugates by means of a glycoside hydrolase known as *trans*-sialidase (TcTS) (Previato et al. 1985). Further evidences suggest that TcTS activity play several functions in the course of *T. cruzi* infection ensuring a life-long parasitism in humans. The multifunctional role of TcTS is due to its ability to dialogue with different cells from the mammalian host (Fig. 8.1) (Mendonça-Previato et al. 2010; dC-Rubin and Schenkman 2011; Chuenkova and Pereiraperrin 2011; Schauer and Kamerling 2011; Freire-de-Lima et al. 2012). Alongside, structural works are deciphering the way this enzyme works. TcTS was the first example of a retaining glycosidase utilizing an aryl glycoside intermediate (Watts et al. 2003; Amaya et al. 2004). Such finding had shed light in the catalytic mechanisms of other sialidases of medical importance (Kim et al. 2013; Vavricka et al. 2013).

Herein, we discuss the importance of this unique enzyme in curse of infection of mammalian host by *T. cruzi*, highlighting the studies that are deciphering the mechanism of TcTS catalysis.

2 Structure and Catalytic Mechanism of TcTS

TcTS is a retaining glycoside hydrolase (Todeschini et al. 2000) member of the family number 33 (GH-33) (http://www.cazy.org/GH33.html) that preferentially transfers sialic acid units to β-galactopyranosyl (β-Gal*p*)-containing molecules and exclusively synthesizes α2-3-linkages (Fig. 8.2a). In the absence of a galactoside, TcTS catalyzes sialoside hydrolysis (Fig. 8.2b) with retention of configuration (Todeschini et al. 2000).

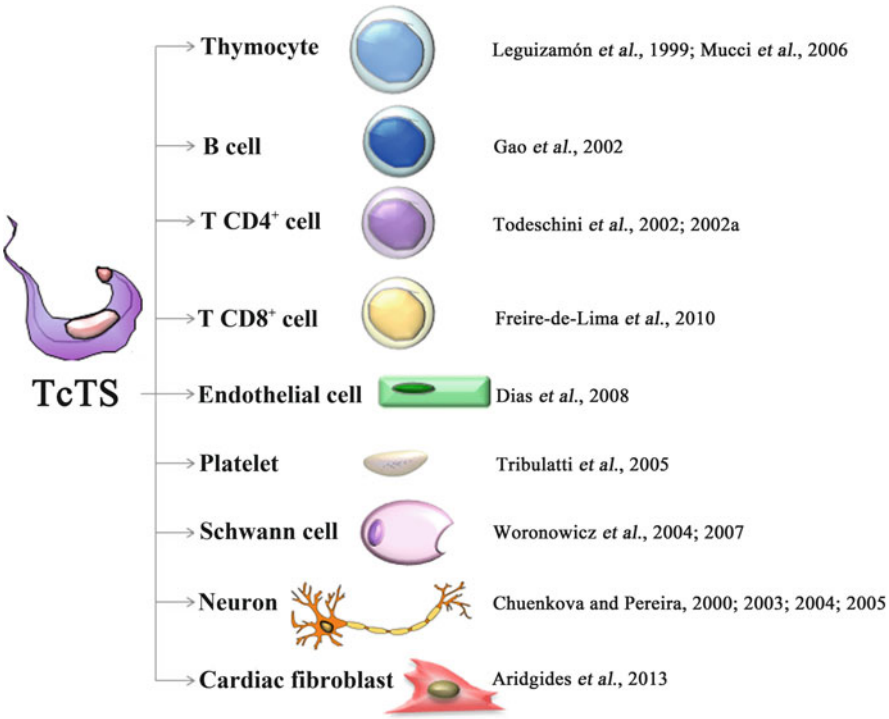

Fig. 8.1 Possible interactions of TcTS with different cells. The multifunctional role of TcTS during *T. cruzi* infection is due to its ability to dialogue with different cells from the mammalian host

The TcTS structure consists of three domains (Fig. 8.3a): (i) an N-terminal domain containing a binding site (green in Fig. 8.3a) that folds into a six-bladed β propeller-structure (Buschiazzo et al. 2002), (ii) a globular C-domain with a lectin-like organization (blue in Fig. 8.3a) that is not required for TcTS activity, and (iii) a C-terminal and unfolded domain that is formed by 12-amino-acid repeats, known as a 'shed acute phase antigen' (SAPA) (Pollevick et al. 1991).

TcTS is linked to the surface of blood-derived trypomastigotes by a glycosylphosphatidylinositol-anchor; the lipid portion of this anchor consists of ceramide and hexadecylglycerol (Agusti et al. 1997), while in metacyclic trypomastigotes, ceramide is the sole constituent lipid (Agusti et al. 1998). The presence of ceramide in the glycosylphosphatidylinositol-anchor allows the enzyme to be actively cleaved from the surface of *T. cruzi* (Pollevick et al. 1991) by the action of a phospholipase C (Rosenberg et al. 1991). These data explain the presence of SAPA antigens (Parodi et al. 1992) and *trans*-sialidase activity in the serum of patients in the acute phase of Chagas' disease (De Titto and Araújo 1988, Mallimaci et al. 2010; Gil et al. 2011). The SAPA repeats cause enzyme oligomerization and induce the production of antibodies (Cazzulo and Frasch 1992). The insect-derived epimastigote forms express a monomeric and transmembrane TcTS lacking the SAPA portion (Chaves et al. 1993).

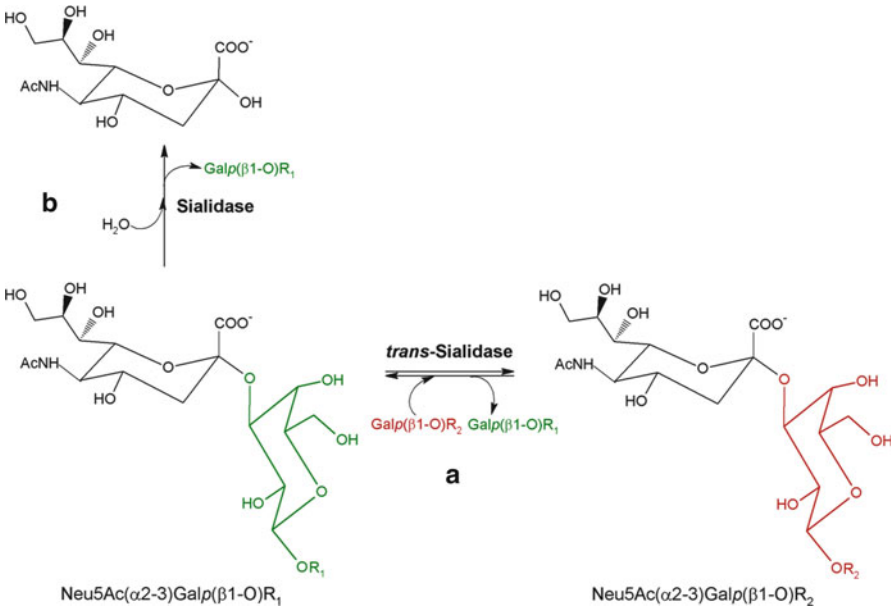

Fig. 8.2 (**a**) Transfer of Neu5Ac from Neu5Ac **α**-2-3Gal**β**1-x-containing linkage donors to terminal **β**-galactopyranosyl (**β**-Gal*p*) catalyzed by TcTS. (**b**) Sialoside hydrolysis catalyzed by TcTS

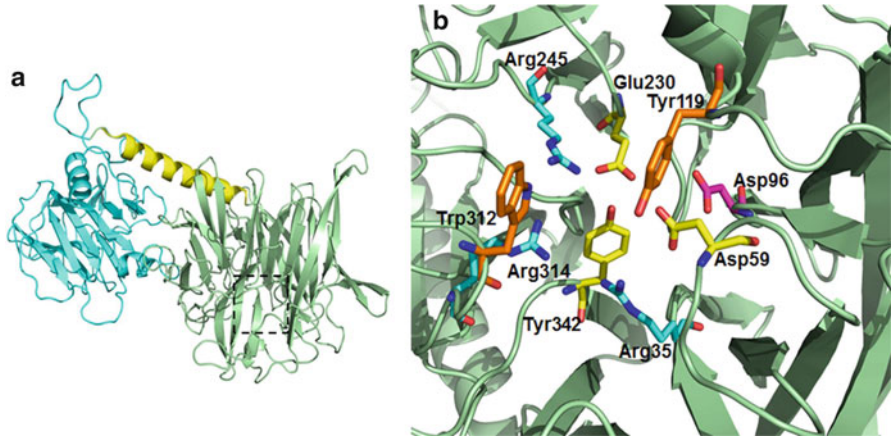

Fig. 8.3 (**a**) Overall structure of TcTS. The *square* shows the catalytic site position. (**b**) The active site of TcTS. The catalytic amino acid residues Tyr342, Glu230, and Asp59 (*yellow*), the Asp96 residue (*magenta*), the triad of arginines Arg35, Arg245, Arg314 (*blue*) and the gatekeepers Trp312 and Tyr119 (*orange*) are highlighted

The C-terminal globular domain is formed by two antiparallel β-sheets in a β-sandwich-like structure (Buschiazzo et al. 2002); this structure is linked to the N-terminal domain by a long α-helix (yellow in Fig. 8.2a), the largest of the few reported in the TcTS (Buschiazzo et al. 2002). The N-terminal domain comprises approximately 680 amino acids (Schenkman et al. 1992; Campetella et al. 1994) folded into a six-bladed β propeller, similar to the crooked β-barrel structure characteristic of microbial sialidases (Taylor 1996). This domain contains all the amino acid residues that are involved in sialic acid binding: (i) the motif S-x-D-x-G-x-T-W (also called Asp-box), repeated three to five times in the sequences of bacterial and mammalian sialidases (Roggentin et al. 1989), (ii) the x-R-x-P (or FRIP) region found at the N-terminal domain of the Asp-box, and (iii) three arginines known to bind the carboxylate group of sialic acid (Gaskell et al. 1995). Such a structure forms a deep catalytic pocket with hydrophobicity produced by residues such as Met95, Phe115, Trp120 and Val176 (Buschiazzo et al. 2000); these residues are suitable for the transfer reaction because they may contribute to water exclusion from the cleft, thus reducing the hydrolytic potential of the enzyme. The hydrophobic residues Trp312 and Tyr119 (Fig. 8.2b) are at the protein surface in close contact with water, thus acting as "gatekeepers" of the cleft (Carvalho et al. 2010). The loops carrying these residues move according to the incoming or outgoing of reagents (Demir and Roitberg 2009). The crystal structure of TcTS shows that Tyr119 adopts different positions in the absence or presence of sialoside (Buschiazzo et al. 2002). The loop carrying the Trp312 residue is at the opposite side of the catalytic cleft. This residue stabilizes the galactoside moiety of the substrate in the enzyme pocket through CH/π interactions (Nesmelova et al. 2010). The W312A mutation changes the substrate specificity, resulting in a mutant capable of hydrolyzing both α2-3- and α2-6-linked sialosides and leading to the loss of *trans*-sialidase activity (Paris et al. 2001).

The catalytic mechanism of TcTS represented in Fig. 8.4 shows that, as the sialoside approaches Tyr119 and Trp312 move away to allow the entry of the substrate into the catalytic cleft. Within the catalytic site, the carboxyl group of the sialoside binds to the arginine triad composed of Arg35, Arg245 and Arg314 (blue in Fig. 8.2b), while its acetamido group interacts with Asp96, forcing the ring to adopt a $^{4}H_{5}$ conformation. The hydroxyl group of Tyr342 reacts as a nucleophile, assisted by the nearby Glu230, and forms a covalent intermediate with the sialic acid ring. X-ray structures and experiments trapping the intermediate with fluorosugars followed by peptide mapping and crystallography support this mechanism (Watts et al. 2003; Amaya et al. 2004). The covalent intermediate assumes a $^{2}C_{5}$ conformation, and Asp59 donates its proton to the substrate aglycone. TcTS was the first enzyme described to undergo acid/base catalysis and to have a tyrosine (Tyr342) as the catalytic nucleophile (Watts et al. 2003). The use of a tyrosine as a nucleophile presents a distinct advantage over the use of a negatively charged carboxylate because the anomeric center of Sias is itself negatively charged and could therefore be subject to interfering charge repulsion. The single mutation from Tyr342 to His in the naturally occurring $TcTS_{Y342H}$ causes enzymatic inactivation (Cremona, et al. 1995) but conserves binding to Sia and β-Gal*p*-containing glycans (Todeschini et al. 2002a, 2004).

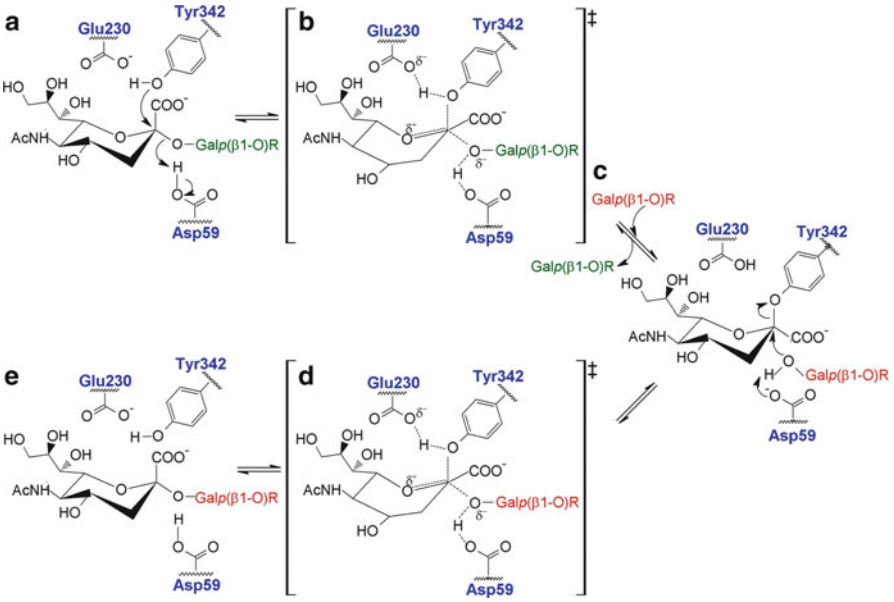

Fig. 8.4 Proposed reaction mechanism for TcTS. (**a**) Upon binding a sialic acid donor, the hydroxyl group of Tyr342 reacts as a nucleophile, assisted by the nearby Glu230 acting as a general base catalyst, and Asp59 protonates the leaving group. (**b**) Tyr342 attacks C2 from the sialyl moiety, and the reaction reaches its transition state. (**c**) The reaction proceeds with the complete breakdown of the glycosidic linkage, releasing the aglycone. At this time, the covalent intermediate of sialic acid bound to Tyr342 is formed. Such a state is perturbed by the entrance of an acceptor (*red*) in the active site of TS. (**d**) Asp59 acts as a basic catalyst by deprotonating the hydroxyl from C3 of terminal Gal, which, in turn, attacks the covalent intermediate, promoting a new transition state. (**e**) Gal completes the nucleophilic attack, and the reaction ends with the retention of the configuration of the sialic acid moiety

According to a ping-pong mechanism (Fig. 8.4), the aglycone leaves the pocket to enable the sialic acid acceptor substrate to bind to the enzyme. The transfer to the acceptor occurs through the attack of the 3-OH group of a lactose moiety or of water (as in other sialidases) deprotonated by the Asp59 residue acting as an acid/base catalyst (Damager et al. 2008) on the C2 of the sialyl-enzyme intermediate.

Further works have helped to elucidate the structural features that underlie efficient sugar transfer activity rather than simple hydrolysis by TcTS. Important evidences reveal that the $TcTS_{Y342H}$ binding site undergoes large conformational changes upon sialoside engagement, thus triggering the opening of a second binding pocket that accommodates a β-Gal*p* moiety in a ternary complex (Todeschini et al. 2004; Haselhorst et al. 2004). The incubation of $TcTS_{Y342H}$ with α2-6-sialyllactose in the presence of lacto-N-tetraose has shown that the incorrect positioning of sialoside into the binding site of TcTS does not trigger β-Gal*p* binding. Moreover, surface plasmon resonance results showed that lactose binds to an inactive mutant ($TcTS_{D59N}$) in the presence of α2-3-sialyllactose (Buschiazzo et al. 2002). Other key

residues that contribute to the plasticity of the binding site were identified by mutagenesis studies (Paris et al. 2001; Carvalho et al. 2010), by hybrid quantum mechanics/molecular mechanics simulations and by molecular dynamic simulation (Demir and Roitberg 2009; Mitchell et al. 2010; Pierdominici-Sottile and Roitberg 2011; Pierdominici-Sottile et al. 2011).

2.1 TcTS Substrate Specificity

TcTS catalyzes the transfer of Sias N-acetylneuraminic (Neu5Ac) and its derivative the N-glycolylneuraminic acid (Neu5Gc) from Siaα-2-3Galβ1-x-containing donors to terminal β-galactopyranosyl (β-Gal*p*)-containing acceptors and attaches them in α2-3-linkage (Vandekerckhove et al. 1992).

Natural sialic acid acceptors for TcTS on the surface of *T. cruzi* consist mainly of a family of highly *O*-glycosylated, threonine-rich mucin-like glycoproteins (Buscaglia et al. 2006; Mendonça-Previato et al. 2008) that are glycosylphosphatidylinositol-anchored to the parasite membrane (Previato et al. 1995). The Tc-mucins are the most expressed components of *T. cruzi* (2×10^6 copies per parasite) and compose the third most widely expanded gene family in the genome, comprising more than 1,000 genes (El-Sayed et al. 2005a, b; De Pablos and Osuna 2012). Carrying up to 60 % of their total mass in carbohydrates, mucins form an elaborate and highly decorated glycocalyx that allows the parasite to interact with and respond to its external environment. The structures of the sialic acid acceptors of non-infective epimastigote forms were described (Previato et al. 1994, 1995; Todeschini et al. 2001, 2009; Agrellos et al. 2003; Jones et al. 2004) as *O*-linked oligosaccharides attached to the peptide backbone through an *N*-acetylglucosamine (α-GlcNAc) residue (Previato et al. 1995, Mendonça-Previato et al. 2013) further substituted by β-Gal residues on O-4 and O-6. The major sialylated oligosaccharides reported thus far comprise a Neu5Acα2-3Gal*p*β1-4GlcNAc sialoside (Jones et al. 2004), a Gal*f*β1-4(Neu5Acα2-3Gal*p*β1-6) GlcNAc sialoside (Agrellos et al. 2003), a Gal*p*β1-4(Neu5Acα2-3Gal*p*β1-6)GlcNAc sialoside, and a Galpβ1-6(Neu5Acα2-3Gal*p*β1-4) GlcNAc sialoside (Previato et al. 1995; Todeschini et al. 2001). The Neu5Ac residue was distributed approximately equally between the digalactosylated species of the 4-arm and 6-arm. This observation suggests that the addition of the first Neu5Ac residue hinders the addition of a second residue (Previato et al. 1995), as disialylated forms were not observed. Neither the terminal β-galactofuranose- (β-Gal*f*)-linked residues found in *O*-glycans from G (Previato et al 1994), DM28c (Agrellos et al. 2003), Tulahuen (Jones et al. 2004) or Colombiana (Todeschini et al. 2009) strains nor the α-galactosyl residues found in the mucin glycans of infective trypomastigotes (Almeida et al. 1994) were found to be acceptors for TcTS.

A wide variety of molecules containing a terminal β-Gal*p*-unit are suitable acceptors for *trans*-sialidase activity *in vitro* (Vandekerckhove et al. 1992; Scudder et al. 1993). The natural acceptor N-acetyllactosamine (Galβ1-4GlcNAc) is a better substrate for the TcTS reaction than is lactose (Lac, Gal*p*β1-4Glc)

(Vandekerckhove et al. 1992). In addition, TcTS shows higher transfer rates for the Galβ1–4-linkage than for the Galβ1–3-linkage. The lactose open-chain derivatives lactitol and lactobionic acid and the products generated by the addition of Gal*p*, Gal*f* or benzyl residues to the lactitol molecule were found to be good acceptors of sialic acid (Agustí et al. 2004, 2007). Recently, the reactions of a series of octyl galactosides and octyl *N*-acetyllactosamines with TcTS were tested. The results showed that the TcTS acceptor binding site does not tolerate the substitution of Gal*p* at positions 2 and 4, while substitutions at position 6 of the Gal ring are well accepted (Harrison et al. 2011).

The tolerance of TcTS to modifications in the C-6 position of the acceptor Gal moiety makes the 6-deoxy-galactose (D-Fuc) derivative D-Fucβ1-6GlcNAc-α-benzyl an interesting acceptor. As the disaccharide is not a substrate for galactose oxidase, it was reported to be an acceptor substrate for TcTS activity in a quantification assay (Sartor et al. 2010).

In terms of donor substrates, neither thiosialosides (Harrison et al. 2001) nor 2-6-, 2-8-, or 2-9-linked sialic acids are substrates for TcTS and $TcTS_{Y342H}$ (Vandekerckhove et al. 1992). Both proteins recognize α2-3-linked sialic acid and its 7-carbon analog (Previato et al. 1985; Todeschini et al. 2002a). Binding can be abolished by either fucosylation or carboxyl reduction (Vandekerckhove et al. 1992; Todeschini et al. 2002a). Therefore, the ligands of the selectins sialyl $Lewis^x$ and sialyl $Lewis^a$ are not ligands for TcTS or $TcTS_{Y342H}$.

Given that the pocket around the glycerol moiety of sialoside (comprising the amino acids W120, T121, Q195, V203) is too small to accommodate bulky groups, acetylation at C7 and C8 prevents enzyme to bind, so does modification at the C4 position (Vandekerckhove et al. 1992). Interestingly, incorporation of aryl groups such as umbelliferyl and benzamide at the C9 position of the 2,3-difluorosialic acid produced selective and potent inhibitors of TcTS (Buchini et al. 2008).

The synthetic donors 4-methylumbelliferyl-*N*-acetyl neuraminic acid (4-MUNeu5Ac) and p-nitrophenyl-N-acetyl-neuraminic acid (pNPNeu5Ac) are poorer sialic acid donors to the enzyme than are α2-3sialyllactose (Neu5Acα2-3Galβ1-4Glc) or α2-3sialyllactosamine (Neu5Acα2-3Galβ1-4GlcNAc) (Ribeirão et al. 1997; Todeschini et al. 2000). In fact, natural sialosides would fit correctly in the TcTS catalytic pocket, thus inducing the acceptor donor to bind and increasing transfer rates, while synthetic donors such as 4-MUNeu5Ac and pNPNeu5Ac would not be able to trigger a sufficient shifting in the enzyme framework to allow acceptor binding and would thus be better substrates for hydrolysis reaction (Harrison et al. 2001).

Other sialosides such as 2-difluoromethyl-4-nitrophenyl-*N*-acetyl neuraminic acid and 5-acetamido-2-(4-N-5-dimethylaminonaphthalene-1-sulfonyl-2-difluoromethylphenyl)-*N*-acetyl neuraminic acid were suicide substrates for TcTS (Carvalho et al. 2010). The 2-difluoromethylphenyl aglycone released upon sialoside hydrolysis irreversibly inactivates TcTS. Recently, the synthesis of 1,2,3-triazole-linked sialic acid-6-O-galactose and the sialic acid-galactopyranoside were reported as a prototype for further design of new neoglycoconjugates as TcTS substrates (Campo et al. 2012).

Unlike free sialic acid, the synthetic monosaccharide 2,3-difluorosialic acid is recognized as a donor by the enzyme (Watts et al. 2003). The molecule α2,3-difluorosialic acid temporarily inactivates TcTS through covalent binding with the hydroxyl group of Tyr342, thus opening new avenues for the design of irreversible TcTS inhibitors (Watts et al. 2003).

Besides, recognition of a wide variety of substrates, glycoproteins, glycolipids, and oligosaccharides recognized by TcTS makes it an appropriate tool for enzymatic glycosylation of glycans (Šardzík et al. 2011).

3 *Trypanosoma cruzi Trans*-Sialidase Superfamily (TSs)

TcTS is part of the *trans*-sialidase-like superfamily (TSs), a large and highly polymorphic gene family comprising 1,430 gene members and 693 pseudogenes (Freitas et al. 2011) divided into eight groups. Group I contains active TcTS and inactive $TcTS_{Y342H}$ proteins, expressed in trypomastigote (tTS) and epimastigote (eTS) forms. Although the primary sequences of eTS and tTS are highly conserved (Chaves et al. 1993; Briones et al. 1995, Jäger et al. 2008), their 3′ untranslated regions (3′UTR) are entirely different (Jager et al. 2007). The 3′UTRs of the region regulate the expression of several genes in *T. cruzi* (Nozaki and Cross 1995; Weston et al. 1999; Di Noia et al. 2000) and are thought to play a role in the coordinated modulation of TSs stage-specific expression (Jager et al. 2007, 2008). Genes encoding $TcTS_{Y342H}$ members in the *T. cruzi* genome are found in the same number of copies (60–80 per haploid genome) as those encoding TcTS (Cremona et al. 1999). Group II comprises members of TSs proteins that have no *trans*-sialidase activity, including members of the family of gp85 surface glycoproteins gp82, TSA-1, SA85, gp90 and ASP-2. These proteins bind to β-galactose (Yoshida 2008), laminin (Giordano et al. 1994), fibronectin (Ouaissi et al. 1988), collagen (Velge et al. 1988; Santana et al. 1997), and cytokeratin (Magdesian et al. 2001) and are implicated in host cell attachment and invasion. Recently, it was demonstrated that regulatory elements in the 3′UTR of the GP82 are responsible for its stage-specific expression in *T. cruzi* metacyclic trypomastigotes (Bayer-Santos et al. 2012). FL-160, a representative of group III, is a regulatory protein that inhibits the alternative and classical complement pathways (Mathieu-Daudé et al. 2008). Tc13 is representative of group IV with an unknown function (García et al. 2008).

Various groups of the TSs family present motifs common to bacterial and mammalian sialidases, including FRIP (xRxP) and Asp box (Freitas et al. 2011), suggesting that other inactive members of TcTS might have lectinic properties. For instance, evidences have shown that the insect vector-derived metacyclic trypomastigote uses its stage-specific surface molecule gp82 to bind to gastric mucin and establish *T. cruzi* infection via an oral route (Neira et al. 2003; Yoshida 2008; Staquicini et al. 2010; Cortez et al. 2012a, b).

The significant sequence variability observed thus far suggests a strong selective pressure on the TSs gene family to diversify. This pressure may be provided in part

by the mammalian immune response because TSs proteins are targets of both humoral and cell-mediated immune responses (Frasch 2000). The TSs family is much smaller in *T. brucei* than in *T. cruzi*, and it is absent in *L. major* (El-Sayed et al. 2005a, b).

Studies show that all groups of the TSs are represented in the subtelomeric regions. Most of the sequences are members of group II (GP82, GP85, TC85), which includes 22 complete genes (Moraes Barros et al. 2012). To confirm that TSs and other subtelomeric genes were translated, the authors searched for peptides in the database of proteins expressed by *T. cruzi* (TriTrypDB). The results of this search suggest that *T. cruzi* subtelomeric regions can contain expression sites. The abundance of surface protein genes in the subtelomeric regions suggests that these regions may have acted as sites for DNA recombination and expansion and for the generation of new variants of surface proteins (Moraes Barros et al. 2012).

3.1 TcTS and Its Role in Host Parasite Interaction

Immune evasion is particularly important for organisms that target long-lived hosts. The sialylation of parasite glycoconjugates by TcTS activity plays a major role in protecting the infective agent from the host's innate immune response, thus favoring parasite survival. Evidence suggests that the sialylation of trypomastigote glycans confers resistance to killing by lytic antibodies (Almeida et al. 1994) directed to terminal α-galactosyl residues (Pereira-Chioccola et al. 2000). Indeed, the presence of Sia groups on the parasite mucins protects the blood forms of the parasite against complement-induced lysis and macrophage uptake (Tomlinson et al. 1994).

T. cruzi is an intracellular parasite and invasion of host cell is necessary to establish the infection. Infection of mammalian host cells by *T. cruzi* is a multi-step process that requires activation of multiple signal transduction pathways in both the host and the parasite that lead to parasite entry (Caradonna and Burleigh 2011). The host cells contain macromolecules such as laminin, thrombospondin, fibronectin and glycoconjugates that cover the surface of the host cells. The TSs family and sialic acids containing glycoproteins present on parasite surface assure an interface with host environment. In this scenario, the importance of Sia on the parasite surface during host cell infection is still not elucidated. While some studies have shown that the Sia-containing epitopes on parasite augment *T. cruzi* infection (Piras et al. 1987; Schenkman et al. 1991), other groups suggest that the presence of Sia is not required for the invasion of host cells (Araújo-Jorge and De Souza 1988; Yoshida et al. 1997). The finding that $TcTS_{Y342H}$, a natural mutant of TcTS, has two carbohydrate binding sites may explain these apparently ambiguous results. Although Schenkman et al. (1991) showed that the sialylation of the Ssp-3 epitope of mammalian cell-derived trypomastigotes is required for target cell recognition, Yoshida et al. (1997) reported that the removal of Sia from the surface of insect-derived metacyclic trypomastigotes enhances parasite-host interactions. The removal of Sia from *T. cruzi* glycoproteins and the concomitant

exposure of cryptic β-Gal*p* residues would favor $TcTS_{Y342H}$ interaction with both host sialoglycoconjugates and terminal β-Gal*p*-containing glycoproteins on the parasite surface, thus enhancing *T. cruzi*/host adhesion by the removal of Sia and the concomitant exposure of β-Gal*p* residues from host cell glycans. This phenomenon was well characterized for CD22, a mammalian Sia-binding lectin (Varki and Gagneux 2012). Thus, the removal of Sia and the concomitant exposure of β-Gal*p* residues from host cell glycans can be physiologically significant by promoting parasite adherence and penetration of host cells.

Another hypothesis is that TcTS sialylates the parasite glycomolecules, generating ligands for Sia-binding Ig-like lectin (Siglecs). Recent studies have shown that *T. cruzi* sialylglycoproteins binds to siglecs on the host cell surface (Erdmann et al. 2009; Jacobs et al. 2010). Siglecs are a family of sialic-acid-binding immunoglobulin-like lectins that promote cell–cell interactions and regulate the functions of cells in the innate and adaptive immune systems through glycan recognition (Varki and Gagneux 2012). *T. cruzi* mucin engagement with the Sia-binding protein Siglec-E promotes the immunosuppression of dendritic cells (Erdmann et al. 2009).

Concerning host cells it has being shown that TcTS promotes parasite attachment and entry into host cells through sialyl receptors (Souza et al. 2010). Experiments with Sia-deficient mutants of Chinese Hamster Ovaries (CHO) (Ciavaglia et al. 1993; Ming et al. 1993) first supported this premise. Sia-deficient cells were less infective than wild type cells, suggesting that the sialylation of glycoconjugates on CHO cell surfaces is necessary during *T. cruzi* invasion. The role of TcTS in parasite adhesion to and invasion of host cells is supported by results showing that the treatment of cells with modified Sia precursors N-acylmannosamines (Lieke et al. 2011) or with an irreversible inhibitor of the enzyme decreased cell invasion by *T. cruzi* (Carvalho et al. 2010). Studies with endothelial cells support the importance of TcTS and Sia-containing molecules on the first steps of parasite interaction and penetration of host cells. However, transfer reaction does not seem to be involved in this process as the inactive mutant $TcTS_{Y342H}$ also up regulates parasite entry into endothelial cells. The data show that $TcTS_{Y342H}$ binds α2-3-sialic acid containing molecules on endothelial cells resulting in NF-κB activation, expression of cell adhesion molecules E-selectin, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and rescue from apoptosis (Dias et al. 2008). Other evidences show that TcTS promotes trypanosome-host cell interaction independent of sialidase/*trans*-sialidase activities. TcTS mediates invasion of neural, epithelial, and phagocytic cells via nerve growth factor receptor (NGF) TrkA (Melo-Jorge and PereiraPerrin 2004, 2007). The effect is reproduced by TcTS mutants lacking catalytic activity (Chuenkova et al. 1999) and a ~22mer synthetic peptide (and thus without enzymatic activity) reproduces biological activities of the TcTS (Chuenkova and PereiraPerrin 2005).

Nevertheless, studies suggest that a major function of TcTS in host cell infection is to facilitate the escape of the trypomastigote forms from the parasitophorous vacuole into the cytosol and their subsequent differentiation into amastigotes (Hall et al. 1992; Hall 1993; Hall and Joiner 1993; Lopez et al. 2002; Rubin-de-Celis et al. 2006). The exit of trypomastigotes from the parasitophorous vacuole in sialic

acid-deficient cells occurs earlier than in wild-type cells suggesting that Sia may act as a barrier for parasite to escape (Hall et al. 1992; Hall and Joiner 1993; Rubin-de-Celis et al. 2006). Likewise, parasites over expressing TcTS on the surface escaped earlier from the vacuole than non-transfected parasites indicating that TcTS may help parasites to enter the cytosol (Rubin-de-Celis et al. 2006). TcTS seems to metacyclics over remove sialic acid from lysosomal membranes, which fuse with *T. cruzi* containing phagolysosomes after parasite invasion (Andrews 2002; Andrade and Andrews 2004). This appears to facilitate phagosomal membrane disruption by TcTox, the parasite pore forming molecule (Andrews et al. 1990).

In addition to its role in mammalian cell invasion, TcTS is involved in the pathogenicity of *T. cruzi*; indeed, the *in vivo* injection of small amounts of purified native TcTS activity increased parasitemia and mortality in *T. cruzi*-infected mice (Chuenkova and Pereira 1995; Freire-de-Lima et al. 2010). The effect observed was specific to the transfer activity of TcTS, as the same effect did not occur in mice primed with viral or bacterial sialidases. Nevertheless, the injection of TcTS into deficient SCID mice had no effect on parasitemia or mortality, suggesting that the mechanisms responsible for the observed effects involve host B and T lymphocytes (Chuenkova and Pereira 1995). These findings suggest that the soluble form of TcTS is a virulence determinant molecule with relevant biological effects on the host immune system. Consistent with TcTS functioning as a virulence factor, the heterologous expression of TcTS in *Leishmania major* enhances parasite virulence (Belen-Carrillo et al. 2000).

Multiple effects of TcTS on host T- and B-lymphocyte function were demonstrated (Fig. 8.1). The SAPA repeats induce the production of antibodies (Cazzulo and Frasch 1992; Buscaglia et al. 1998). The high immunogenicity of SAPA antigens might play a role *in vivo* by increasing the half-life of the protein in the blood and by delaying the formation of inhibitory antibodies against the catalytic site of TcTS (Buscaglia et al. 1999; Pitcovsky et al. 2002), which correlates with control of parasite levels (Risso et al. 2007). The crystal structure of an inhibitory antibody fragment in complex with the globular region of TcTS was recently determined (Buschiazzo et al. 2012). The structure showed that the antibody does not occlude the catalytic site enzyme, instead, the antibody performs a delicate action by inhibiting the movement of an assisting Tyr119, whose mobility is known to play a key role in the catalyze reaction. Moreover, the C-terminal region of TcTS activates B cells, inducing the production of nonspecific antibodies independent of the activity of T cells (Gao et al. 2002).

Furthermore, the engagement of TcTS and its inactive analog $TcTS_{Y342H}$ with epitopes containing α2-3-Sia on CD43 from host $CD4^+$ T cells, triggers a co-stimulatory response through the mitogen-activated protein kinase ERK1/2 cascade inducing mitogenesis (Todeschini et al. 2002b). These results suggest that TcTS is responsible for host polyclonal lymphocyte activation, a condition underlying the induction of immunopathology and preventing effective vaccination (Reina-San-Martin et al. 2000; Minoprio 2001) in the course of *T. cruzi* infection; these observations also corroborate the hypothesis that $TcTS_{Y342H}$ promotes glycan cross-linking.

Studies on the effect of TcTS on $CD8^+$ T cells show that TcTS resialylates $CD8^+$ T cell surface, thereby dampening the Ag-specific response and favoring parasite persistence in the mammalian host (Freire-de-Lima et al. 2010). TcTS-mediated resialylation *in vitro* and *in vivo* decreases the cytotoxic activity of antigen-experienced $CD8^+$ T cells against the immunodominant synthetic peptide IYNVGQVSI (Freire-de-Lima et al. 2010). These results demonstrate that *T. cruzi* subverts sialylation to attenuate $CD8^+$ T cell interactions with peptide/MHC class I complexes. $CD8^+$ T cell resialylation may represent a sophisticated strategy to ensure lifetime host parasitism. In an attempt to establish the nature of the Sia acceptor for TcTS on the $CD8^+$ T cell surface, $CD8^+$ T cells from mice lacking the ST3Gal-I sialyltransferase, an enzyme required for sialylation of core 1 *O*-glycans (Priatel et al. 2000), were infected with *T. cruzi*. The loss of ST3Gal-I sialyltransferase exposes the Gal1-3GalNAc-Ser/Thr moiety, creating an attractive model to establish CD43 as a natural receptor for native TcTS during *T. cruzi* infection. Indeed, the infection of mice lacking ST3-Gal-I sialyltransferase restores, at least in part, the binding of anti-CD43 S7 mAbs that recognize Sia-containing epitopes on CD43 of $CD8^+$ T cells. These findings indicate that CD43 is a target receptor for TS on the $CD8^+$ T cell surfaces. However, the resialylation by TcTS was also observed on $CD8^+$ T cells from CD43 KO mice, suggesting that, in the absence of CD43, other molecules are substrates for TcTS. Other studies using azido-modified unnatural Sia revealed that CD45 isoforms are Sia acceptors for TcTS activity as well (Muiá et al. 2010). Moreover, the sialylation of thymocytes by TcTS activity is crucial to deciding the outcome of the cells during interaction with thymic lectins. The alteration of the surface sialylation by TcTS (Mucci et al 2006) leads to *in vivo* depletion of the $CD4^+CD8^+$ double-positive thymocytes inside the "nurse cell complex" (Leguizamón et al. 1999).

TcTS activity can also compromises host cell homeostasis. Tribulatti and co-authors (2005) demonstrated that the administration of TcTS to uninfected mice reduces the Sia content of platelets (Fig. 8.1), thus exposing terminal galactose residues, which may explain the severe thrombocytopenia observed in *T. cruzi*-infected individuals. The recognition of the terminal galactose moiety exposed on the platelet surface accelerates platelet clearance by asialoglycoprotein receptor-expressing scavenger cells (Sørensen et al. 2009). The effect of TcTS on the lifetime of other cell types and plasma glycoproteins should be further verified.

Another interesting example of how TcTS activity can modulate host responses is the effect of the sialylation of host cell receptors. The desialylation of sialyl TOLL-like receptor 4 (TLR4) by TcTS induces receptor dimerization and facilitates MyD88/TLR4 complex formation and NF-kappaB activation in a manner similar to the responses observed with LPS (Amith et al. 2010). Likewise, TcTS alters the sialylation status of the tyrosine kinase receptor-A (TrkA) in PC12 cells, inducing receptor internalization, activation, neuronal differentiation and rescue from apoptosis (Woronowicz et al. 2004, 2007). The observed effects are triggered by the hydrolysis of Sia residues of TrkA by TcTS because a purified recombinant α2-3-neuraminidase, but not a catalytically inactive mutant of TcTS, induces the receptor phosphorylation.

Due its role in neuronal differentiation, neural repair and neuron protection against apoptosis upon Trk receptor binding, TcTS was termed a "parasitokine" or

"parasite-derived neurotrophic factor (PDNF)" (Chuenkova and Pereira 2000, 2003; Chuenkova and Pereiraperrin 2005, 2011). Trk receptors are a family of tyrosine kinases that regulate synaptic strength and plasticity in the mammalian nervous system (Fig. 8.1). TcTS binds to the TrK through the globular C-domain corresponding to the amino acid sequence 425–445 (Chuenkova and Pereiraperrin 2005), thus independently of any enzymatic activity. Following binding to Trk, TcTS causes receptor dimerization, the phosphorylation of tyrosine residues in the cytoplasmic domain and the generation of cell signals critical for neuronal survival (Chuenkova and Pereiraperrin 2011). More recently, it was demonstrated that TcTS-TrkA interaction in cardiac fibroblast induces an increased production of NGF, enabling, in a paracrine fashion, myocytes to resist oxidative stress (Aridgides et al. 2013). Thus, TcTS-elicited regenerative responses likely prolong parasite persistence in infected tissues.

4 Conclusions

On the basis of the above observations it would be reasonable to compare the apparently contradictory effects of TcTS during *T. cruzi* infection outlined in this review to the Chinese concept of yin-yang used to describe how seemingly opposite or contrary forces are interconnected, interacting to form a whole greater than either separate part. Thus, it would be more appropriate to say that TcTS effects are actually complementary, not opposing, effects, sometimes favoring the host, sometimes favoring the parasite ensuring a parasitism that last for host whole life.

Beyond the urgency of alternative drugs to treat the illness, to pursuit of TcTS inhibitors might clarify the role of TcTS in the pathogenesis of Chagas' disease. Although effective TcTS inhibitors have not yet been reached, efforts made in this area have found interesting lead compounds.

Furthermore, recognition of a wide variety of substrates recognized by TcTS makes it the proper tool for direct enzymatic glycosylation of sialyloligosaccharides in glycans synthesis. Finally the TcTS may be used as therapeutic agents to treat not only infectious diseases but also unrelated disorders.

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Chapter 9
Surface Topology Evolution of *Trypanosoma Trans*-Sialidase

Sergio Steven Cornejo Rubin De Celis

Abstract The *trans*-sialidase (TS) from *Trypanosoma cruzi* is a multifunctional protein given by its enzymatic activity and binding properties. The complex structure of TS promotes topology changes over the protozoa's surface with dramatic consequences for its biology. Detailed sequence analyses show that the evolution of TS in *T. cruzi* and other trypanosomes as well as its genomic organization is even more complex than it has been supposed before. All of these aspects are still neglected when TS is selected as a target for drug design and chemotherapy of Chagas' disease. Herein these aspects are discussed in the context of TS multifunctionality and dynamics drug design.

Abbreviations

GH-33	Glycoside hydrolase family number 33
GPI	Glycosylphosphatidylinositol
MDS	Multidimensional scaling plot
SAPA	Shed Acute Phase Antigen
TS	*Trans*-Siliadase
$TS^{342His/-}$	Inactive *Trans*-Siliadase without SAPA
$TS^{342His/SAPA}$	Inactive *Trans*-Siliadase with SAPA
$TS^{342Tyr/-}$	Active *Trans*-Siliadase without SAPA
$TS^{342Tyr/SAPA}$	Active *Trans*-Siliadase with SAPA

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1 Origin and Evolution of *Trypanosoma Trans*-Sialidase (TS)

Recent post-genomic phylogenetic analysis, including new sequences, indicates that the evolution of TS in *Trypanosoma cruzi* is monophyletic, while polyphyletic in others trypanosomes (Carvajal and Rubin in progress). Sialic acids have appeared in co-evolution, precisely from Echinoderms (Varki 1997; Lewis et al. 2009). The proposal of a sialidase origin in higher animals is based on the presence of apparently homologous enzymes in this kingdom, supporting the idea that some microorganisms may have acquired genetic information during association with their animal hosts. Furthermore, a sialidase gene was transferred via phages among bacteria (Roggentin et al. 1993). Acquisition in trypanosomes might have occurred from bacteria through the blood ingested by hematophogous insects. Interestingly, a sialidase activity was detected in the salivary gland of *Triatoma infestans* (Amino et al. 1998), supporting the notion that the origin of TS in *T. cruzi* could be through a parasite living within the insect vector.

Within trypanosomatids, *T. cruzi* TS probably evolved from a conventional sialidase of a common trypanosomatid ancestor. Genetically, the stage-specific expression of *T. cruzi* TS involves a highly conserved 3′ untranslated region (Jager et al. 2008), suggesting that all TS genes had a common ancestor. Initial phylogenetic reconstructions suggested that TS was derived from ancestral genes, present in the parasite forms growing in insects, before the development of parasitism of mammals by *T. cruzi* (Briones et al. 1995a). *Trypanosoma rangeli* has a sialidase enzyme that lacks TS activity, keeping the gene sequence closely to *T. cruzi* TS (Buschiazzo et al. 1993; Pontes de Carvalho et al. 1993). However, five mutations of *T. rangeli*-sialidase, Met96-Val, Ala98-Pro, Ser120-Tyr, Gly249-Tyr and Gln284-Pro, restored TS activity, revealing the formation of a TS-like binding site for the acceptor galactose, primarily defined by the phenol group of Tyr120 and the indole ring of Trp313, which adopts a new conformation, similar to that in *T. cruzi* TS, induced by the Gln284-Pro mutation (Paris et al. 2005).

The catalytic sites of *T. cruzi* TS and *T. brucei* TS are similar, but not identical. *T. brucei* has TS genes, coding enzymes with both sialidase and *trans*-sialidase activity. Curiously, *T. brucei* TS is about 10 times more efficient in transferring than in hydrolyzing sialic acid when compared to *T. cruzi* (Montagna et al. 2002). This could explain the pressure to increase the copy number of TS genes in *T. cruzi* genome compared to *T. brucei*, perhaps ensuring comparable levels of *trans*-sialylation in both organisms. *Trypanosoma congolense* conserves most of the identity and critical site residues keeping the *trans*-sialylation activity found in other *trans*-sialidases (Tiralongo et al. 2003; Koliwer-Brandl et al. 2011) as well as *Trypanosoma carassi*, a freshwater fish parasite (Aguero et al. 2002). In contrast, several other trypanosomatids lack TS or TS-like genes. The presence of TS enzymes in other trypanosomes raises the question as whether the enzyme role is similar, or it was adapted to perform multiple functions in the specific context of *T. cruzi* biology. It is therefore unknown how all these genes appeared and have evolved in some species and not in others.

2 Surface Diversity of *Trypanosoma* TS

According to structurally-related catalytic and carbohydrate-binding modules (or functional domains) of enzymes that degrade, modify, or create glycosidic bonds (Henrissat and Bairoch 1996), TS is a member of the glycoside hydrolase family number 33 (GH-33) (http://www.cazy.org/GH33.html) that preferentially transfers sialic acid units to β-galactopyranosyl (β-Gal*p*)-containing molecules, and exclusively synthesizing α2-3-linkages.

In *T. cruzi*, based on the protein alignment, TS belongs to the TS superfamily, which was divided into TS and TS-like family (Cross and Takle 1993; Schenkman et al. 1994b; Frasch 2000). However, based on multidimensional scaling (MDS) plot of the *T. cruzi* TS protein sequences, the TS superfamily cluster into eight TcS groups (Freitas et al. 2011). Altogether, the groups of the TS super family include 1,430 gene members, with 693 pseudogenes on the genome of *T. cruzi* (El-Sayed et al. 2005). All members of this heterogeneous superfamily are annotated in the *T. cruzi* genome database imprecisely as TS or putative TS (Aslett et al. 2010). Among the 223 proteins detected in the proteome with characteristics of TS, there are 15 genes predicted to encode enzymatically-active TS (Atwood et al. 2005) in the *T. cruzi* genome (El-Sayed et al. 2005), which are part of the TcS group I (Freitas et al. 2011).

Most of the TS found in the genomes of trypanosomes that express the protein on their surface belong to the TS family, based on its enzymatic activity and protein alignment (Carvajal and Rubin in progress).

2.1 TS Family

The TS family belongs to the TcS group I, based on the MDS analysis (Freitas et al. 2011). TcS group I is the only group that corresponds to the active and inactive enzyme, with and without the 12 amino acid repeats also called shed acute phase antigen (SAPA) (Affranchino et al. 1989). This group is characterized by the presence of eight conserved amino acid residues at the active site in the N-terminal domain involved into the catalytic activity (Fig. 9.1). The inactive form of the enzyme is a sialic acid-binding lectin protein (Todeschini et al. 2004). Near to the catalytic site, the N-terminal has cross-reacting epitopes (Pitcovsky et al. 2001). The lectin-like domain is the second region of TS, which contains the epitope Y21 capable to bind to a nerve growth factor receptor (TrkA binding domain) (Chuenkova and PereiraPerrin 2005) and also has cross-reacting epitopes (Pitcovsky et al. 2001). Only in trypomastigotes and metacyclics the enzyme is expressed with the SAPA repeats in the C-terminus domain (Chaves et al. 1993). SAPA also has cross-reacting epitopes (Pitcovsky et al. 2002). The TS family also shows the same conserved motifs (FRIP, Asp-box, and Fly) as sialidases (Fig. 9.1).

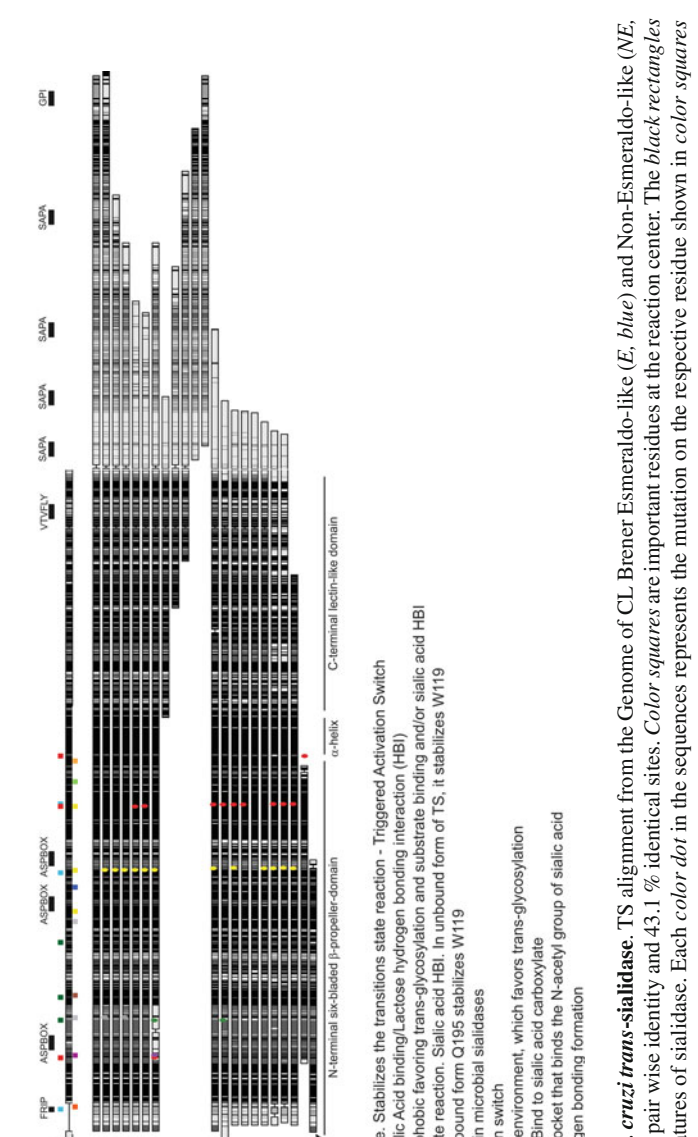

Fig. 9.1 **General alignment of *T. cruzi trans*-sialidase**. TS alignment from the Genome of CL Brener Esmeraldo-like (*E, blue*) and Non-Esmeraldo-like (*NE, red*). The Alignment has 76.6 % of pair wise identity and 43.1 % identical sites. *Color squares* are important residues at the reaction center. The *black rectangles* over the sequences are motif signatures of sialidase. Each *color dot* in the sequences represents the mutation on the respective residue shown in *color squares*

Table 9.1 Expression of *trans*-sialidase in Trypanosomes. The gene code $TS^{342Tyr/SAPA}$ means the active *Trans*-Siliadase with SAPA, $TS^{342His/SAPA}$ the inactive *Trans*-Siliadase with SAPA, $TS^{342Tyr/-}$ the active *Trans*-Siliadase without SAPA and $TS^{342His/-}$ the inactive *Trans*-Siliadase without SAPA

	$TS^{342Tyr/SAPA}$	$TS^{342His/SAPA}$	$TS^{342Tyr/-}$ [a]	$TS^{342His/-}$
T. cruzi Trypomastigote	+	+	+	+
T. cruzi Metacyclic	+	−	+	−
T. cruzi Epimastigote	−	−	+	−
T. brucei	−	−	+	−
T. rangeli	−	−	−	+
T. congolense	−	−	+	−
T. carassi	−	−	+	−

[a] In other trypanosomes was found only $TS^{342Tyr/-}$

In addition, the TcS group I may be divided into four types based on its structural features (Table 9.1). The first one corresponds to catalytically active enzymes, defined by the presence of Tyr342 in the β-propeller-N-catalytic domain (Cremona et al. 1995; Buschiazzo et al. 2002), with SAPA at the C-end ($TS^{342Tyr/SAPA}$). The second group is defined by the presence of Tyr342 in the β-propeller-N-catalytic domain without SAPA ($TS^{342Tyr/-}$). The third and the forth types correspond to the inactive enzyme with SAPA ($TS^{342His/SAPA}$) and without SAPA ($TS^{342His/-}$), however, it is necessary to prove the expression of the last one. The latter are 95 % identical to the first group, but are enzymatically inactive due to a single mutation, from a Tyr342 to His342 residue (Schenkman et al. 1994b; Cremona et al. 1995). Both groups of proteins were shown to bind to the substrates without catalyzing the sialic acid transfer (Cremona et al. 1999; Todeschini et al. 2004). The SAPA repeats increase the half-life of the protein in the blood (Buscaglia et al. 1999), but they are not essential for the enzymatic activity or stability (Cazzulo and Frasch 1992; Campetella et al. 1994; Schenkman et al. 1994a). $TS^{342Tyr/SAPA}$ and $TS^{342His/SAPA}$ are anchored to the parasite surface membrane by glycosylphosphatidylinositol (GPI), which is susceptible to the digestion by a parasite phosphatidylinositol-phospholipase C. The digestion of the GPI anchor releases the TS into the medium (Schenkman et al. 1992; Agusti et al. 1997, 1998). $TS^{342Tyr/-}$ has enzymatic activity, lacking the SAPA repeats at carboxy-terminal (Chaves et al. 1993) and it is not anchored to the membrane by GPI (Briones et al. 1995b). Whether TS released by the trypomastigotes are formed by TS^{342Tyr} or TS^{342His} polypeptides, or contain all the types (active, inactive with/without SAPA, shed, TrkA binding domain), it remains to be further studied.

2.2 TS-Like Family

The TS-like family is characterized by the TcS groups II, III, IV, V, VI, VII and VIII (Freitas et al. 2011). By the protein sequence analysis of TS-like family and TcS group I, they cluster in different branches of the phylogenetic tree (Carvajal and

Rubin in progress). The TS-like products lack enzymatic activity, have a 30–40 % amino acid sequence identity compared to TcS group I and do not align with the general pattern of TS^{342Tyr} gene and with the catalytic residues. They have one to four complete or degenerated Asp boxes, a FRIP motif and a sub-terminal FLY motif (Campetella et al. 1992; Schenkman et al. 1994b; Frasch 2000). However, Freitas et al. (2011) demonstrated that in addition to TcS group I, members of TcS group IV have both FRIP and Asp box motifs and consequently might present carbohydrate-binding properties. Thus, TcS group IV also might belong to glycoside hydrolase family GH-33. The TcS group IV is characterized by the *T. cruzi* antigen 13 or Tc13, which is a surface glycoprotein encoded by dozens of genes expressed in metacyclics and trypomastigotes (Bua et al. 1999). The TcS group II is known by the Tc-85, an 85-kDa surface glycoprotein (also called TSA, SA85, Tt34, ASP) present in amastigotes, metacylics and trypomastigotes (reviewed in (Alves and Colli 2008)). The TcS group III is characterized by FL160-FI/CEA/CRP, a flagellum-associated surface protein, chronic exoantigen and complement regulatory protein, respectively (Beucher and Norris 2008). The expression and biological function of the remaining groups TcS V, VI, VII and VIII still remain unknown.

3 Post-Genomic *T. cruzi* TS Gene Organization

The TS gene synteny and dispersion through chromosomes of *T. cruzi* genome is different from chromosome to chromosome and from strain to strain. A general dispersion of TS on the CL-Brener strain, a hybrid strain containing two types (Esmeraldo and non-Esmeraldo like sequences) is show in Fig. 9.2. The Esmeraldo-like sequences contain$TS^{342Tyr/SAPA}$ genes in chromosomes 11, 16, 17, 30, 33, 37 and the non-Esmeraldo-like sequences in chromosomes 6, 11, 16, 17, 30, 33 and there is 5TS genes not assigned to any chromosome in CL-Brener (Aslett et al. 2010).

Only four annotated proteins corresponding toTS^{342Tyr} possess the conserved Phe in the FLY residue. Also, in CL-Brener Esmeraldo and non-Esmeraldo like genome none of the annotated sequences contain the natural mutation of TS^{342His} (Fig. 9.2), which confers binding to the substrate without hydrolysis and/or *trans*-glycosylation. This should be taken into account at the time to analyze the infectivity of the strains and its expression as well. The TS genes in the chromosomes generally are near to genes codifying TS-like groups, and other abundant surface proteins known as mucins, masp (Buscaglia et al. 2006), as well as retrotransposons (Kim et al. 2005). Some of them are located near telomers, regions that undergo more frequently recombinative events (Kim et al. 2005). In Y-strain, tandem of 12 genes without interruptions are detected in one chromosomal band (Egima et al. 1996). Cosmid analysis also reveals that genes encoding catalytically active TS proteins ($TS^{342Tyr/SAPA}$ or $TS^{342Tyr/-}$) are interspaced with TS^{342His} (Egima et al. 1996), which strongly suggests a variable TS genome organization in *T. cruzi* strains.

A detailed study of the genomic organization of other TS *T. cruzi* strains and trypanosomatids as well as more TS sequence information would allow understanding

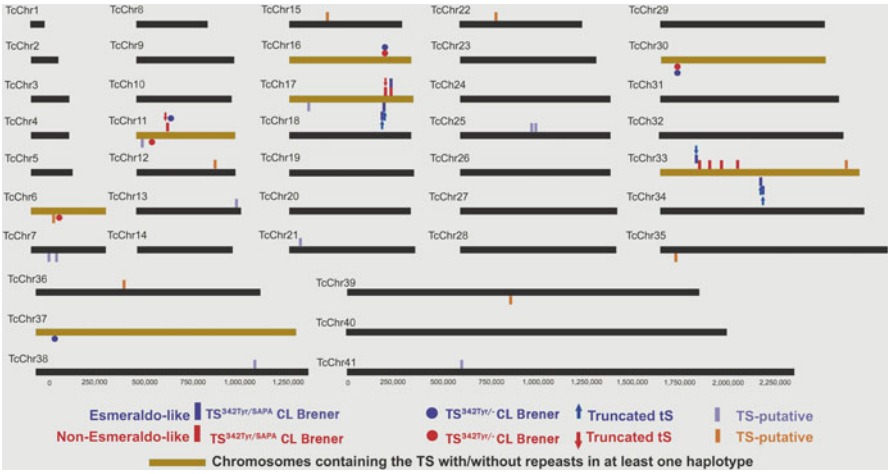

Fig. 9.2 ***T. cruzi trans*-sialidase genome organization**. The distribution of TS genes (*rectangle, dots, arrows*) in the genome of CL Brener chromosomes (*brown*) is indicated. The *blue* and *red rectangle* (SAPA containing genes) or *dots* (TS without SAPA) represent the position of the TS genes for Esmeraldo (*blue*) and non-Esmeraldo haplotypes (*red*). The genes above chromosomes indicate transcription in one direction, and bellow in the opposite direction. *Arrows* indicate truncated TS in the genome annotation. The *gold* and *light blue rectangles* correspond to putative enzymes, most likely members of TS-like family

the evolutionary process of the TS and the possible mechanism of acquisition through all trypanosomes. This may be important to characterize the strains infectivity and their differences.

4 Surface Expression Changes and TS Multifunctionality

The TS expression is highly repetitive, possibly variable in the different strains, with different expression among the cellular forms of *T. cruzi* (Rubin-de-Celis et al. 2006). TS has functions in the different stages of the parasite, from the stages growing in the insect vector to the forms present in mammalian hosts. The multifunctional roles of the TS in the parasite biology, the establishment of host infection and pathogenesis of Chagas' disease has been recently reviewed (Dc-Rubin and Schenkman 2012). However, here we will be briefly summarizing the relationship of the structure expression differences with the multifunctional roles of the TS.

In non-infective epimastigotes, the expression of $TS^{342Tyr/-}$ is post-transcriptionally regulated (Briones et al. 1995a). In contrast to $TS^{342Tyr/SAPA}$, which is expressed only in the infective forms of *T. cruzi*, $TS^{342Tyr/-}$ is not secreted into the medium, but it is associated to the membrane surface of trypomastigotes, metacyclics and epimastigotes (Rubin-de-Celis et al. 2006) (Table 9.1) (Fig. 9.3).

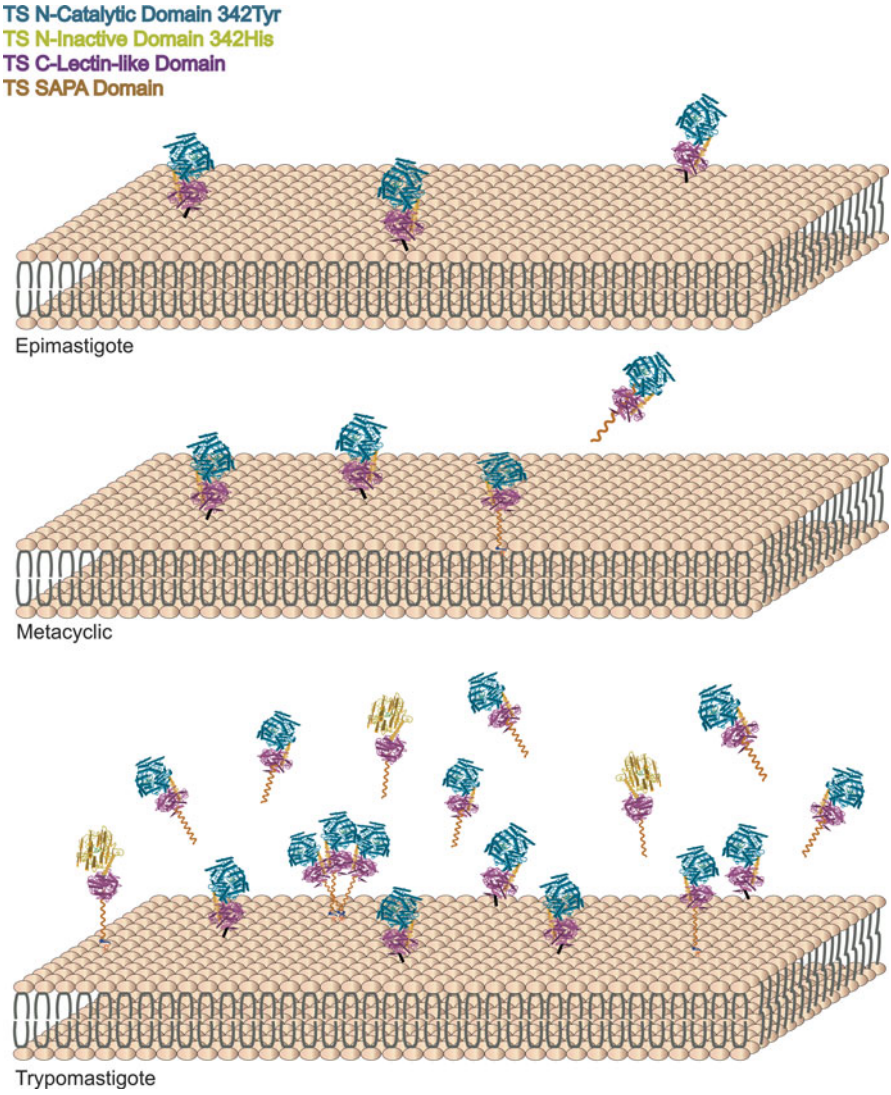

Fig. 9.3 Surface topology of *T. cruzi trans*-sialidase. The schematic model represents the TS surface topology of epimastigotes, metacyclics and trypomastigotes forms of *T. cruzi* based on experimental measures of TS activity (Rubin-de-Celis et al. 2006). However, it does not reflect the real values: for example, for each protein in metacyclics there should be 25 more proteins in trypomastigotes, either over the surface or secreted into the medium. The secretion of $TS^{342His/SAPA}$ in trypomastigotes is hypothetical and needs further investigation

Furthermore, in trypomastigotes the TS remain 2.5 times less anchored to the membrane than secreted into the medium (Rubin-de-Celis et al. 2006) (Fig. 9.3). The $TS^{342Tyr/-}$ expressed in epimastigotes transfer sialic acid to the 35–50 kDa

mucins (Schenkman et al. 1993). Sialylation protects the parasite from the action of glycolyticenzymes found in the gut of the insect vector (Garcia and Azambuja 1991), and influences the migration through the insect gut (Briones et al. 1994) by attachment/detachment of parasite to/from epithelial cells in the rectal ampoule of the insect (Garcia and Azambuja 1991; Frasch 1994). However, there is no evidence if this form expresses the inactive and/or lectin form of the protein (Fig. 9.3).

Metacyclics gain access to the bloodstream through mucosal surfaces mixed with excreted feces and urine of the vector. Small amounts of TS activity in metacyclis compared to trypomastigotes were detected in the extracellular medium (Rubin-de-Celis et al. 2006). However, it is suggested that even in metacyclis the $TS^{342Tyr/SAPA}$ is susceptible to the digestion by the phosphatidylinositol-phospholipase C (Schenkman et al. 1992; Agusti et al. 1997, 1998). The presence of $TS^{342Tyr/-}$ over the metacyclic and trypomastigotes surface and $TS^{342Tyr/SAPA}$ secreted into the medium influences the host by modulating the innate and acquired immune system, promoting signaling and therefore, change the target released TS-SAPA versus parasite (Dc-Rubin and Schenkman 2012). $TS^{342His/SAPA}$ is only expressed in trypomastigotes (Fig. 9.3). It is still unknown whether metacyclics express $TS^{342His/SAPA}$, however, it has been shown that the N-domain of the TS^{342His} binds to sialyl and beta-galactopyranosyl residues in a sequential ordered mechanism (Todeschini et al 2004), and cross-reacting epitopes at N-catalytic, lectin-like (TrkA binding) and SAPA domain (Pitcovsky et al. 2001, 2002; Chuenkova and PereiraPerrin 2005), suggest non-catalytic functions of the TS. The trypomastigote surface contains more diverse types of TS (Table 9.1) (Fig. 9.3), therefore much more possibilities of immune evasion (Dc-Rubin and Schenkman 2012). Trypomastigotes express high levels of TS compared with metacyclics, which have about 20-fold-less enzymatic activity (Rubin-de-Celis et al. 2006). Whether$TS^{342His/-}$ or $TS^{342His/SAPA}$ proteins are expressed and released by the metacyclics remains to be further studied.

5 Towards Surface Topology Drug Design of *T. cruzi* TS

The topological evolution and expression change of the TS in the parasite's surface is a key aspect for the multifunctional role of the TS in the life cycle of *T. cruzi*. The surface topology also includes the enzyme-protein secreted into the medium. The content of information of the TS diversity secreted and over the surface of each form of the protozoa not only has relation with the modulation of the immune system and the multifunctionality (Dc-Rubin and Schenkman 2012), but represents a potential role for dynamics drug design. Several drugs have been developed for inhibiting the TS activity, but surprisingly only a few of them have reached trial tests *in vitro* and none of them were tested *in vivo* (Dc-Rubin and Schenkman 2012). Among the different screenings, the new inhibiting compounds were found acting on the two binding sites: sialic acid and galactose.

5.1 Sialic Acid

Several inhibitors based on the TS crystal structure have been developed using the sialic acid binding site as a target. Among them, the following inhibitors are included: benzoic acid and pyridine derivatives (Neres et al. 2007), 3-benzothiazol-2-yl-4-phenyl-but-3-enoic acid (Neres et al. 2009), 3-fluorosyalil fluoride derivatives (Buchini et al. 2008), sulfonamide-containing hydroxylated chalcone, quinolinone derivatives, dihydroxylated (catechol) derivatives (Kim et al. 2009) and 2-difluoromethyl-4-nitrophenyl-3,5-dideoxy-*d*-glycero-α-*d*-galacto-2-nonulopyranosid acid (NeuNAcFNP) (Carvalho et al. 2010b). By click chemistry 1,2,3-triazole linked sialic acid-6-O-galactose and the sialic acid-galactopyranoside were synthesized with TS inhibitory activity at 1.0 mM (Campo et al. 2012) as well as the only one sialic acid-galactose target inhibitor C-sialosides with aromatic residues (Meinke et al. 2011). Up to now, the strongest inhibitor is the 6-chloro-9,10-dihydro-4,5,7-trihydroxy-9,10-dioxo-2-anthracenecarboxylic acid (Arioka et al. 2010). Novel sialic acid precursors, such as N-propionyl mannosamine and other N-acyl mannosamines, can change the transfer of the sugar and affect infection of *T. cruzi* (Lieke et al. 2011).

5.2 Galactose

Some lactose derivatives (especially lactitol) were shown to inhibit the sialic acid transfer to N-acetyllactosamine *in vitro* and *in vivo* (Agusti et al. 2004). Lactitol bioavailability was increased through covalent conjugation with polyethylene glycol (PEG), suggesting its potential use *in vivo* (Giorgi et al. 2010). Inhibitors directed to the galactose binding sites were also obtained with 1,4-di-substituted 1,2,3-triazole derivatives of galactose modified at either the C-1 or C-6 positions (Carvalho et al. 2010a).

However, even though some of those inhibitors acting on the sialic acid and galactose binding sites appear to be potential leads for Chagas' chemotherapy, it seems that they are not able to block the TS multifunctionality. The complexity of the TS structure makes the protozoa use it as an enzyme and/or binding protein. Here it is considered that the complex structure and expression changes of the TS drives its multifunctionality, which makes even more dynamic the surface topology of *T. cruzi* and its environment. This complexity has not been taken into account in the development of the actual TS inhibitors (Dc-Rubin and Schenkman 2012). They were developed using static biochemical and not dynamic biological proves, putting emphasis on its catalytic activity more than its binding properties, cross reactivity and biological functions.

6 Conclusion

Due the fact that TS is a complex molecule (enzyme/binding/cross-reactive protein) differentially expressed in the *T. cruzi* surface ($TS^{342Tyr/-}$, $TS^{342Tyr/SAPA}$, $TS^{342His/-}$ and $TS^{342His/SAPA}$) with biological implications (multifunctionality), it is

necessary to develop inhibitors (vaccines) acting on the catalytic and non-catalytic regions (cross-reacting epitopes and binding sites) (Pitcovsky et al. 2001, 2002; Chuenkova and PereiraPerrin 2005). Here this notion is called dynamics drug design. A quantitative/qualitative study of them will help to elucidate better its multifunctionality and the way to rational drug design, consequently to prevent *T. cruzi* infection and Chagas' disease. According to this notion, neutralizing TS antibodies have been proposed as inhibitors (Buschiazzo et al. 2012), however focused only on the catalytic activity. It will be more helpful if those neutralizing TS antibodies are developed to prevent the TS interactions through non-catalytic regions and use a strongest drug (Arioka et al. 2010) for the inhibition of the TS activity towards effective test against TS. Efforts in the development of TS dynamics drug design, considering its complex structure, different expression and multifunctionality will justify that more resources for biotechnology advancements aiming effective prevention of Chagas' disease are granted.

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Chapter 10
Ecto-nucleotidases and Ecto-phosphatases from *Leishmania* and *Trypanosoma* Parasites

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Abstract Ecto-enzymes can be defined as membrane-bound proteins that have their active site facing the extracellular millieu. In trypanosomatids, the physiological roles of these enzymes remain to be completed elucidated; however, many important events have already been related to them, such as the survival of parasites during their complex life cycle and the successful establishment of host infection. This chapter focuses on two remarkable classes of ecto-enzymes: ecto-nucleotidases and ecto-phosphatases, summarizing their occurrence and possible physiological roles in *Leishmania* and *Trypanosoma* genera. Ecto-nucleotidases are characterized by their ability to hydrolyze extracellular nucleotides, playing an important role in purinergic signaling. By the action of these ecto-enzymes, parasites are capable of modulating the host immune system, which leads to a successful parasite infection. Furthermore, ecto-nucleotidases are also involved in the purine salvage pathway, acting in the generation of nucleosides that are able to cross plasma membrane via specialized transporters. Another important ecto-enzyme present in a vast number of pathogenic organisms is the ecto-phosphatase. These enzymes are able to hydrolyze extracellular phosphorylated substrates, releasing free inorganic phosphate that can be internalized by the cell, crossing the plasma membrane through a Pi-transporter. Ecto-phosphatases are also involved in the invasion and survival of parasite in the host cells. Several alternative functions have been suggested for these enzymes in parasites, such as participation in their proliferation, differentiation, nutrition and protection. In this context, the present chapter provides an overview of recent discoveries related to the occurrence of ecto-nucleotidase and ecto-phosphatase activities in *Leishmania* and *Trypanosoma* parasites.

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Abbreviations

2′NMP	Nucleoside 2′monophosphate
3′AMP	Adenosine 3′monophosphate
3′NMP	Nucleoside 3′monophosphate
5′(deoxy)NMP	Deoxynucleoside 5′monophosphate
ACR	Apyrase conserved region
ADO	Adenosine
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CMP	Cytosine monophosphate
CTP	Cytosine triphosphate
DC	Dendritic cell
DIDS	4,4′-diisothiocyanostylbene 2′-2′-disulfonic acid
DNA	Deoxyribonucleic acid
Ecto-3′NT/NU	Ecto-3′nucleotidase/nuclease
Ecto-5′NT	Ecto-5′nucleotidase
E-NPP	Ecto-nucleotide pyrophosphatase/phosphodiesterase
E-NTPDase	Ectonucleoside triphosphate diphosphohydrolase
GMP	Guanosine monophosphate
GPI	Glycosyl phosphatidyl inositol
GTP	Guanosine triphosphate
IFN-γ	Interferon-γ
IL	Interleukine
IMP	Inosine monophosphate
ITP	Inosine triphosphate
NDP	Nucleoside diphosphate
NO	Nitric oxide
NTP	Nucleoside triphosphate
P1	Adenosine receptor
P2	ATP receptor
PFTM	Purine free trypanosome medium
PHO pathway	Phosphate signal-transduction pathway
PHP	Phosphohistidine phosphatase
Pi	Inorganic phosphate
PP	Phosphoserine/threonine phosphatase
PTP	Phosphotyrosine phosphatase
RNA	Ribonucleic acid
ROS	Reactive oxygen species
TNF-α	Tumor necrosis factor-α
TTP	Thymidine triphosphate
TYR	Tyrosine

UDP	Uridine diphosphate
UMP	Uridine monophosphate
UTP	Uridine triphosphate

1 Introduction

Although the concept of ecto-enzyme has been known for many years, interest in this class of membrane protein has increased recently. Ecto-enzymes can be defined as membrane-bound proteins that have their active site facing the extracellular millieu. They can be associated with plasma membrane by several forms, basically via transmembrane domains or glycosylphosphatidylinositol (GPI) anchor (Goding 2000; Zimmermann et al. 2012). Commonly, these enzymes are considered type II integral membrane proteins, with only one transmembrane domain, usually with a short N-terminal cytoplasmatic domain and a large extracellular C-terminal catalytic domain. In other cases, ecto-enzymes can have more than one transmembrane domain and a good example is the well-described mammalian ecto-ATPase (CD39) that has two transmembrane domains in both N-terminal and C-terminal regions, with an extracellular and catalytic region between them. With respect to GPI-anchored enzymes, alkaline phosphatases and ecto-5′nucleotidases can be cited as examples (Goding 2000). The GPI anchor can confer a rapid lateral motility to the anchored enzymes and also allows their cluster in lipid rafts regions of membrane (Zimmermann et al. 2012).

The products of ecto-enzymatic activities are released in the extracellular medium, since their catalytic sites act on extracellular substrates. Furthermore, these activities can be modified by impermeant reagents. As the cellular integrity is maintained during their action, it is possible to perform experiments using intact cells to determine and characterize a particular ecto-enzymatic activity. This approach allows the identification of many ecto-enzymes in several microorganisms. The physiological roles of these enzymes remain to be completed elucidated, however, many important events have already been related to them, such as the survival of parasites during their complex life cycle and the successful establishment of host infection (Meyer-Fernandes 2002; Gomes et al. 2011).

In this context, this chapter focuses on two remarkable classes of ecto-enzymes: the ecto-nucleotidases and the ecto-phosphatases, summarizing their occurrence and possible physiological roles in *Leishmania* and *Trypanosoma* genera.

2 Ecto-Nucleotidases

The discovery of a pathway for intercellular signaling through extracellular nucleotides contributed to the considerable progress in the study of ecto-nucleotidases during the past three decades (Gomes et al. 2011; Zimmermann et al. 2012).

The four major families of ecto-enzymes related to nucleotide hydrolysis include ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases), ecto-5′nucleotidases (ecto-5′NTs), ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs) and alkaline phosphatases. E-NPPs and alkaline phosphatases are capable of hydrolyzing not only nucleotides but also other substrates, and their roles in the control of purinergic signaling are still scarce (Zimmermann et al. 2012). In the case of trypanosomatids, another remarkable nucleotidase is the ecto-3′nucleotidase/nuclease (ecto-3′NT/NU), an important source of adenosine via 3′AMP hydrolysis (Paletta-Silva and Meyer-Fernandes 2012). This section will discuss the metabolism of extracellular nucleotides, emphasizing the following topics: the purine salvage pathway; purinergic signaling in host immune response; and occurrence of E-NTPDases, ecto-5′NTs and ecto-3′NT/NUs in *Leishmania* and *Trypanosoma* species.

2.1 Acquisition of Purine by Trypanosomatids: The Salvage Pathway

Purines are essential for all living organisms, since they are involved in several biological functions. ATP serves as the universal currency of cellular energy; cyclic AMP (cAMP) and cyclic GMP (cGMP) are key second messenger molecules; furthermore, purine nucleotides are required for the synthesis of nucleic acids, proteins and other metabolites (Kouni 2003; Carter et al. 2008).

Purine nucleotides can be synthesized by the *de novo* pathway, using as precursors aspartate, glutamate, glycine, formate and CO_2 to generate the purine ring of inosine monophosphate (IMP). As the purine bases are interconvertible, the IMP can be converted to other nucleotides via a set of reactions so called “salvage pathway”. Some organisms, including trypanosomatids, lack the machinery to synthesize purine *de novo* and are strictly dependent on the salvage pathway. These parasites are able to use preformed purines obtained from their hosts to supply their purine requirement (Hammond and Gutteridge 1984).

The first step in purine salvage is the translocation of preformed purine across the parasite membranes. However, passive diffusion of purines across the lipid bilayer of the plasma membrane is not possible, since they are hydrophilic molecules. For this reason, uptake of purine requires specialized translocation proteins or transporters. Nucleoside and nucleobase transporters have already been described in several *Leishmania* and *Trypanosoma* species (Hammond and Gutteridge 1984; Gottlieb and Cohn 1997). In this context, the expression of ecto-nucleotidases also plays important roles in purine acquisition, since they are capable of hydrolyzing nucleotides to nucleosides, which are then transported into cells. The involvement of these enzymes in purine acquisition was confirmed with studies that showed the up-regulation of ecto-nucleotidases under purine-depleted conditions (Gottlieb and Cohn 1997). Throughout this chapter, more examples of ecto-nucleotidases activities responsive to purine deprivation will be discussed.

2.2 *Extracellular Nucleotides: Purinergic Signaling in the Immune System*

2.2.1 The Discovery of a Purinergic Signaling Pathway

Purinergic signaling describes a system in which extracellular nucleotides act as signaling molecules, stimulating purinergic receptors (Sansom 2012). In the early 1970s, ATP was identified as non-adrenergic, non-cholinergic neurotransmitter in the gut and bladder. Nowadays, it is recognized as a co-transmitter in all nerves in the peripheral and central nervous system. However, the hypothesis of ATP acting as an extracellular messenger was at first refuted, since ATP was well established as an intracellular molecule, being the energy source of biochemical pathways. With the report that such a ubiquitous molecule would also act as an extracellular messenger, several researches about this novel signaling pathway have been started. Finally in early 1990s, cloning of the purinergic receptors was a turning point in the acceptance of the purinergic signaling hypothesis (Burnstock 2012).

2.2.2 Purinergic Receptors

There are two main families of purinergic receptors, the P1 and P2 receptors. The P1 receptors are activated by adenosine and can be divided into four subtypes: A1, A2A, A2B and A3. All of these subtypes are coupled to G proteins. They are differentiated basically by affinity to adenosine and alterations on cAMP concentration, reflecting the activation or inhibition of adenylate cyclase activity. The subtypes A1 and A2A are high-affinity adenosine receptors, activated in physiological ranges of adenosine, while A2B and A3 are low-affinity receptors that are activated when adenosine reaches higher concentrations, for example in inflammatory sites. Activation of A1 and A3 receptors generates inhibition of adenylate cyclase, whereas activation of A2A and A2B activates this enzyme, thereby increasing cAMP. With respect to P2 receptors, they can be activated by ATP, ADP, UTP and UDP and are divided into P2X and P2Y subtypes. The P2X receptor is also called ionotropic and functions as a non-selective cation ion channel. Meanwhile, the P2Y receptor is coupled to G protein and therefore is also called metabotropic (Burnstock and Ralevic 1998).

2.2.3 Purinergic Signaling and the Immune System

Under a stress condition, ATP can be released from cytoplasm to extracellular medium via lytic or, most likely, non-lytic pathways (Di Virgilio 2007), including release through stretch-activated channels, voltage-dependent anion channels, P2X7 receptors (a subtype involved in opening large pores in cell membrane), and connexin and pannexin hemichannels (Junger 2011). Not only ATP but also other nucleotides are released from cells at sites of inflammation of different

tissues undergoing different disease states. Once in the extracellular space, these nucleotides act as a "danger signal" that is recognized by the P2 purinergic receptors. Activation of P2 receptors has been related to important immunological events, such as cell migration, cytokine release, maturation of dendritic cells (DC) and the immune response during infections with intracellular pathogens (Coutinho-Silva and Ojcius 2012).

With respect to DC differentiation, chronic exposure to low concentrations of ATP drives DC maturation toward a phenotype favoring the development of Th2 response (Di Virgilio 2007). Massive discharge of ATP into the extracellular space can occur in acute situations, and the resulting activation of P2 receptors leads to proinflammatory effects by initiating a response characterized by the secretion of IFN-γ, IL-12, TNF-α and IL-1 (Di Virgilio 2007; Paletta-Silva and Meyer-Fernandes 2012).

The remarkable release of ATP in inflammatory site may culminate in the increase of extracellular adenosine levels. This occurs because of the action of ecto-nucleotidases that are responsible for the sequential dephosphorylation of ATP to adenosine. Unlike ATP, adenosine has been related to anti-inflammatory effects, by binding to A2 receptors and activating adenylate cyclase. The consequent increase of cAMP promotes the inhibition of proinflammatory cytokines production, as well as an increased production of the anti-inflammatory cytokine IL-10. Moreover, activation of A2 receptors is also involved in the inhibition of monocyte maturation and suppression of macrophage phagocytic function. It is noteworthy that adenosine can also inhibit adenylate cyclase if bound to A1 or A3 receptors; however, in purinergic signaling the final response depends on several factors, including receptor expression and sensitivity (Paletta-Silva and Meyer-Fernandes 2012).

The physiological balance between the extracellular nucleotide concentration, besides the expression of both purinergic receptors and ecto-nucleotidases are responsible for the extent to which purinergic signaling will contribute to the immune response (Coutinho-Silva and Ojcius 2012).

2.3 General Properties of Ecto-nucleotidases

2.3.1 Ecto-nucleoside Triphosphate Diphosphohydrolases (E-NTPDases)

The E-NTPDases are ecto-nucleotidases capable of hydrolyzing extracellular purine or pyrimidine nucleotides tri- and diphosphates, in the presence of millimolar concentrations of divalent cations, generating nucleosides monophosphate as the final product. Individual members of E-NTPDase family may differ considerably regarding sequence identity, however, all of them share five highly conserved domains called "apyrase conserved regions (ACRs)" (Zimmermann 2001). The ACRs are conserved short stretches of amino acids containing residues essential for enzyme function (Sansom 2012). A variety of deletion and mutation experiments demonstrated that ACRs play an important role in forming the catalytic site of E-NTPDases.

This active site is formed by a complex folding process of the protein and it is also possible that the catalytic properties are affected by the enzyme quaternary structure (Zimmermann 2001). NTPDase 1, a well-characterized member of E-NTPDases, presents two transmembrane domains at the C-terminal and N-terminal regions and is constitutively palmitoylated at a cysteine within the N-terminal region. This covalent lipid modification allows insertion of the protein in caveolae domains of plasma membrane. In addition to lipid modifications, members of the E-NTPDase family can also be N-glycosylated and have the ability to form oligomers, making them potential candidates for channel formation. The apparent molecular mass of the glycosylated monomers is in the order of 70–80 kDa (Zimmermann et al. 2012).

In mammals, eight members of the E-NTPDase family have been identified: NTPDase 1, NTPDase 2, NTPDase 3 and NTPDase 8 are cell surface-located forms; NTPDase 4 and NTPDase 7 are intracellular forms, located in organelles membranes with active site facing their lumens; NTPDase 5 and NTPDase 6 are intracellularly located and probably secreted forms, since secretion was observed after heterologous expression (Zimmermann 2001; Robson et al. 2006; Zimmermann et al. 2012).

The cell surface-located forms hydrolyze both nucleoside triphosphates and diphosphates but with different substrate specificity. E-NTPDase 2 is also called ecto-ATPase because its activity is almost restricted to NTP, presenting poorly NDP hydrolysis. On the other hand, E-NTPDases 1, 3 and 8 are also called ecto-ATP-diphosphohydrolase (or even ecto-ATPDase and ecto-apyrases) because they are able to hydrolyze both NTP and NDP (Knowles and Li 2006). For historical reasons and mainly in immunological viewpoint, E-NTPDase 1 is also called CD39 (Sansom 2012).

The genes for E-NTPDases family also have relatives in invertebrates, plants, yeasts, and protozoans (Zimmermann 2001). Although the nomenclature of E-NTPDases in the mammalian field has already been standardized (Zimmermann et al. 2000), in protozoans there is a wide range of nomenclature used for NTPDases (Sansom 2012). The terms "ecto-ATPases" and "ecto-ATP diphosphohydrolase" are the most employed, as a means of emphasizing the substrate preference of the enzyme in question (Meyer-Fernandes 2002).

2.3.2 Ecto-5′Nucleotidase (Ecto-5′NT)

Ecto-5′NT is an ecto-enzyme involved in the availability of adenosine by dephosphorylation of 5′AMP. Vertebrate ecto-5′NT (also known as CD73) can hydrolyze not only 5′AMP but also 5′CMP, 5′UMP, 5′IMP and 5′GMP, whereby 5′AMP generally is the most effectively hydrolyzed nucleotide (Zimmermann et al. 2000). To date, seven human 5′NTs have been characterized, but just one is attached to the outer leaflet of the plasma membrane. The soluble forms are located in the cytosol or in the mitochondrial matrix and differ from ecto-5′NT in affinity for 5′AMP and substrate specificity. Ecto-5′NT hydrolyses 5′-AMP with K_m of 3–50 μM and shows no activity towards nucleoside 2′- and 3′-monophosphates. On the other hand, soluble 5′NTs dephosphorylate 5′AMP with low affinity (K_m > 1 mM), share an

ability to hydrolyse 5′-(deoxy)NMP, 2′NMP and 3′NMP, and mainly participate in salvage pathways and/or *de novo* nucleotide synthesis (Yegutkin 2008).

Molecular cloning revealed phylogenetic relationships between the vertebrate ecto-5′NT and functionally related enzymes in invertebrates, yeasts/fungi, bacteria, and archaea but seem to be lacking in plants (Zimmermann et al. 2000). This activity was also reported in protozoans, including *Leishmania* and *Trypanosoma* species (Gbenle and Dwyer 1992). The substrate specificity may be differentiated among ecto-5′NT from different organisms (Zimmermann et al. 2000).

Structural studies reveal that vertebrate ecto-5′NT consists of two glycoprotein subunits with apparent molecular mass of 60–70 kDa (~160 kDa for the dimer), tethered by non-covalent bonds. Results obtained from mouse ecto-5′NT showed that deglycosylation of the protein did not alter its catalytic activity. Ecto-5′NT is attached to the plasma membrane by a GPI anchor, and consists of two domains that are linked by a long α-helix. The N-terminal domain binds zinc and other different metal ions and confers the phosphohydrolase activity to the enzyme. The C-terminal domain has a unique structure, responsible for the substrate specificity by binding the base and ribose moieties of the nucleotide substrates. The active site of ecto-5′NT is located in a cleft between the two domains (Zimmermann et al. 2000; Yegutkin 2008).

Trypanosomatids are incapable of synthesizing purine *de novo*, therefore ecto-5′NT may play an important role on purine metabolism, since it is the last ecto-enzyme involved in the sequential hydrolysis of ATP to adenosine. As nucleotides usually cannot be transported into cells, their conversion to nucleoside is critical to purine acquisition (Gottlieb 1989). Another remarkable role from ecto-5′NT is observed in immunological events. The proinflammatory role provided by extracellular ATP is well-established, as well as the anti-inflammatory roles of adenosine. By the action of ecto-ATPases followed by ecto-5′NT, ATP is converted to adenosine and consequently to the inhibition of the immune response, which leads to a successful parasite infection (Paletta-Silva and Meyer-Fernandes 2012).

2.3.3 Ecto-3′Nucleotidase/Nuclease (Ecto-3′NT/NU)

Hydrolysis of 3′nucleotides have been reported in bacteria (Shimada and Sugino 1969; Rodden and Scocca 1972; Felicioli et al. 1973), plants (Kowalske et al. 1976; Olson et al. 1982; Brown and Ho 1986), fungi (Shishido and Ando 1985), and several trypanosomatid species (Gottlieb and Dwyer 1983; Gbenle and Dwyer 1992). In trypanosomatids, this unique, externally-oriented, surface membrane enzyme was first identified, purified, and biochemically characterized in *Leishmania donovani* (Debrabant et al. 2001). This bifunctional ecto-enzyme is involved in hydrolysis of both extracellular 3′nucleotides and nucleic acids (RNA and single-stranded DNA). The dephosphorylation of 3′nucleotides generates nucleosides that can be transported across plasma membranes by nucleoside transporters. Moreover, nucleic acid hydrolysis results in accumulation of 5′nucleotides. Thereby, considering the action of an ecto-5′NT, those 5′nucleotides would also be converted in nucleoside

and inorganic phosphate (Pi). Therefore, organisms that express both ecto-3′NT/NU and ecto-5′NT have the capacity to generate nucleosides and Pi from either exogenously supplied nucleotides or nucleic acids (Gottlieb 1989).

The gene encoding *L. donovani* 3′NT/NU (*Ld*3′NT/NU) was isolated and characterized as a member of the class I nuclease family, since it showed significant sequence homology with S1 and P1nucleases, two secreted fungal nucleases from *Arpergillus* and *Penicillium*, respectively, that are archetype members of this class of enzyme (Debrabant et al. 1995, 2000). Among this class I nuclease family, only 3′NT/NU has been characterized as a cell-surface membrane protein (Debrabant et al. 1995).

Using techniques of homologous expression with *Ld*3′NT/NU gene, the functional domains of this ecto-enzyme was elucidated. It has a molecular mass of about 40 kDa and presents the following domains: the N-terminal signal peptide for targeting this enzyme to endoplasmic reticulum; the C-terminal transmembrane domain that anchors this enzyme to the parasite surface; and the *N*-linked glycosylation site. Although the C-terminal domain plays an important role anchoring this protein to the cell surface, removal of *Ld*3′NT/NU C-terminal domain resulted in the release/secretion of a fully active enzyme, which indicated that the C-terminal domain is not required for enzymatic activity of this protein. Likewise, deletion of its single *N*-linked glycosylation site showed that such glycosylation was not required for the enzymatic functions of *Ld*3′NT/NU. However, *N*-linked glycosylation is probably related to the folding and trafficking events, playing an important role on targeting this protein to parasite cell surface membrane (Debrabant et al. 2000).

Ecto-3′NT/NU is generally not found in mammalian cells, even though 3′nucleotides are available in several mammalian tissues. The major concentration of 3′AMP in mammals was reported in spleen, an organ commonly susceptible to *Leishmania* colonization (Gottlieb and Dwyer 1983; Bushfield et al. 1990). Since 5′NTs are abundant in mammalian cells not only as plasma membrane proteins but also as soluble enzymes, the occurrence of 3′NT/NU in pathogenic parasites may has been evolutionarily selected as it allows parasites to compete with their hosts for 3′nucleotides rather than the corresponding 5′nucleotides (Gottlieb and Dwyer 1983).

2.4 *Evidence of Ecto-nucleotidases in* Leishmania *and* Trypanosoma *Species*

2.4.1 Molecular Evidences for Occurrence of E-NTPDase

In 2000s, advances in sequencing of parasite genomes allowed the description of regions that encode putative NTPDases. Among trypanosomatid parasites, only the NTPDase from *Trypanosoma cruzi* has been molecularly characterized. The genome of *T. cruzi* encodes a single predicted NTPDase and substrate specificity indicates that it belongs to NTPDase 1 family. A recombinant form of the enzyme

that has been expressed and purified from bacteria presented activity against both ATP and ADP (Santos et al. 2009). The genomes of *Trypanosoma brucei* and of the five species of *Leishmania* for which genome sequence information is available possess two predicted NTPDases (Berriman et al 2005; Peacock et al 2007; Sansom 2012).

The *in silico* analysis has identified a conserved domain in NTPDase isoforms of plants and pathogenic organisms from distinct phylogenetic lineages. This domain, named B domain, is not related to ACRs, but seems to be conserved during host and parasite co-evolution (Faria-Pinto et al. 2008; Maia et al. 2013). Studies using antibodies against potato apyrase allowed the identification of B domain in *Leishmania infantum* (Maia et al. 2013), *Leishmania braziliensis* (Rezende-Soares et al. 2010), and possibly in *Leishmania amazonensis* (Coimbra et al. 2008). Recently, mouse polyclonal antibodies were produced against two synthetic peptides derived from B domain. Using these antibodies, ultrastructural immunocytochemical microscopy has been performed and NTPDase 1 was identified on the parasite surface and in its subcellular cytoplasmic vesicles, mitochondrion, kinetoplast and nucleus. Furthermore, both antibodies were able to inhibit ecto-ATPase activity of *L. braziliensis* and significantly reduce the promastigotes growth *in vitro*. Interestingly, the antibody that showed to be more effective in inhibition of ecto-ATPase activity was also more cytotoxic, suggesting that this activity may be involved in cell survival (Porcino et al. 2012).

2.4.2 Reports and Biochemical Characterization of E-NTPDase Activities

Although few studies were performed under molecular levels, several ecto-ATPase activities, related to the presence of NTPDases, have been characterized (Sansom 2012). The sequential hydrolysis of extracellular ATP to adenosine suggests that not only E-NTPDases but also ecto-5′NTs are present in plasma membrane of several parasites, such as *Leishmania tropica* (Meyer-Fernandes et al. 1997), *L. amazonensis* (Berrêdo-Pinho et al. 2001; Pinheiro et al. 2006), *L. braziliensis*, *Leishmania major* (Marques-da-Silva et al. 2008), *L. infantum* (Maia et al. 2013), *T. cruzi* (Bisaggio et al. 2003; Fietto et al. 2004; Meyer-Fernandes et al. 2004), *Trypanosoma rangeli* (Fonseca et al. 2006), and *T. brucei* (de Souza et al. 2007).

NTPDase activity requires divalent cations; however, the responses to metals are different among the described parasites. Usually, NTPDases are stimulated by either magnesium or calcium (Sansom 2012), however, in *L. tropica* (Meyer-Fernandes et al. 1997), *L. amazonensis* (Berrêdo-Pinho et al. 2001), *T. cruzi* (Meyer-Fernandes et al. 2004) and *T. rangeli* (Fonseca et al. 2006), the ecto-ATPase activities are stimulated by magnesium and manganese, but not by calcium. Ecto-ATPase activities from *L. infantum* (Maia et al. 2013) and *T. brucei* showed a distinct profile, being stimulated by calcium. However, in *T. brucei* the stimulation by magnesium was still more efficient and stimulation by manganese and zinc was also observed (de Souza et al. 2007).

E-NTPDase activities from *L. infantum*, *L. braziliensis* and *L. major* have not been fully characterized; however, their abilities to hydrolyze ATP and ADP were evaluated. Both *L. braziliensis* and *L. major* were able to hydrolyze ATP and ADP virtually in the same ratio (Marques-da-Silva et al. 2008; Maia et al. 2013).

In *T. cruzi* the broad hydrolysis of nucleoside di- and triphosphate suggests the presence of an E-NTPDase. The ratio for ATP and ADP hydrolysis is higher in infective trypomastigotes when compared to non-infective epimastigotes (Fietto et al. 2004). The presence of an E-NTPDase isoform with high ATP/ADP hydrolysis ratio has already been described in virulent strains of *Toxoplasma gondii* and such characteristic was related to the pathogenicity of the parasite (Asai et al. 1995; Nakaas et al. 1998). The results with *T. cruzi* suggest a possible involvement of the NTPDase in the infectious process, as well as a potential modulatory effect of ATP (Bisaggio et al. 2003). A Mg-dependent ecto-ATPase activity has also been identified and characterized in *T. cruzi*. The substrate of this activity is the complex Mg-ATP. Unlike the basal ecto-ATPase activity, determined in the absence of divalent metals, the Mg-dependent ecto-ATPase activity is not able to hydrolyze ADP (Meyer-Fernandes et al. 2004).

The complex Mg-ATP is also substrate to ecto-ATPase activities described in *T. rangeli* (Fonseca et al. 2006) and *T. brucei* (de Souza et al. 2007). The Mg-dependent ecto-ATPase of *T. rangeli* presented high specificity for ATP and ITP, although it was also able to hydrolyze other nucleosides triphosphate and ADP (Fonseca et al. 2006). With respect to Mg-dependent ecto-ATPase activity from *T. brucei*, ITP, ATP, CTP, GTP and UTP were the preferred substrates, but TTP and ADP hydrolysis is also observed (de Souza et al. 2007).

Finally, Mg-dependent ecto-ATPase activities have also been described in *L. tropica* (Meyer-Fernandes et al. 1997) and *L. amazonensis* (Berrêdo-Pinho et al. 2001). As observed in *T. rangeli* (Fonseca et al. 2006), ATP was the best substrate for Mg-dependent ecto-ATPase of *L. tropica*, although ADP and other nucleosides triphosphate produced lower reaction rates (Meyer-Fernandes et al. 1997). The Mg-dependent ecto-ATPase activity from *L. amazonensis* has also a high specificity for ATP, however, it is not able to hydrolyze ADP (Berrêdo-Pinho et al. 2001), as observed in *T. cruzi* (Meyer-Fernandes et al. 2004).

In order to confirm that the observed activities were really ecto-ATPases, classical ATPase inhibitors were tested. Apart from E-type ATPases, these trypanosomatids may present F-type ATPases, which are located in the mitochondrion and inhibited by both oligomycin and sodium azide (Meyer-Fernandes et al. 1997); V-type ATPases, which are located in vacuoles and inhibited by bafilomicyn A_1 (Bowman et al. 1988); and P-type ATPases, located in organelles or in plasma membrane, such as H^+-ATPases, Na^+/K^+-ATPase and Na^+-ATPase that are inhibited by, respectively, sodium orthovanadate, ouabain and furosemide (Zilberstein and Dwyer 1985; Caruso-Neves et al. 1998a, b). The ATPase activities described in *L. tropica* (Meyer-Fernandes et al. 1997), *L. amazonensis* (Berrêdo-Pinho et al. 2001), *T. cruzi* (Meyer-Fernandes et al. 2004), *T. rangeli* (Fonseca et al. 2006) and *T. brucei* (de Souza et al. 2007) were insensitive to all those classical ATPase inhibitors, which excludes the action of other types of ATPases instead of an ecto-ATPase.

Furthermore, these activities were also insensitive to ammonium molybdate, a potent inhibitor of 5′nucleotidase activity (Gottlieb and Dwyer 1983), suggesting that this enzyme is not involved in ATP hydrolysis.

To support external localization of the ATP-hydrolyzing site, the impermeant inhibitor 4,4′-diisothiocyanostylbene 2′-2′-disulfonic acid (DIDS) was tested (Meyer-Fernandes 2002), and all the ecto-ATPase activities described above were inhibited in a dose-dependent manner (Meyer-Fernandes et al. 1997; Berrêdo-Pinho et al. 2001; Meyer-Fernandes et al. 2004; Fonseca et al. 2006; de Souza et al. 2007). The same profile of inhibition was observed when suramin was tested (Meyer-Fernandes et al. 2004; Fonseca et al. 2006; de Souza et al. 2007). Suramin is a noncompetitive inhibitor of ecto-ATPases and an antagonist of P2 purinoreceptors (Hourani and Chown 1989; Ziganshin et al. 1995).

As dephosphorylation of ATP could also be performed by non-specific phosphatases, other inhibitory assays were performed. Inhibitors of acid phosphatases, such as sodium fluoride (Gottlieb and Dwyer 1981), sodium orthovanadate (Mikalsen and Kaalhus 1998) and ammonium molybdate (Gottlieb and Dwyer 1983), as well as the alkaline phosphatase inhibitor levamizole (Van Belle 1976), were unable to modulate the described ecto-ATPase activities (Meyer-Fernandes et al. 1997; Berrêdo-Pinho et al. 2001; Meyer-Fernandes et al. 2004; Fonseca et al. 2006; de Souza et al. 2007). Moreover, the response to pH variation is another parameter that distinguishes ecto-ATPase activity from ecto-phosphatase activity of these parasites. Although most of them presented ecto-phosphatase activities with acid profiles (Meyer-Fernandes et al. 1997; Berrêdo-Pinho et al. 2001; Meyer-Fernandes et al. 2004), the respective ecto-ATPase activities either displayed alkaline profiles (Meyer-Fernandes et al. 1997; Berrêdo-Pinho et al. 2001; Fonseca et al. 2006), or were not modulated by the pH variations over the range tested (Meyer-Fernandes et al. 2004; de Souza et al. 2007).

2.4.3 The Contribution of Ecto-nucleotidases in Purine Acquisition

Trypanosomatids cannot synthesize purines *de novo*, therefore they resort to the host purines to perform the salvage pathway and supply their purine requirement (Gottlieb and Cohn 1997). It has been postulated that the ecto-nucleotidases could play a role in the salvage of purines by converting extracellular nucleotides to nucleosides, which are able to cross plasma membrane via specialized transporters (Hammond and Gutteridge 1984; Gottlieb and Cohn 1997; Meyer-Fernandes 2002). In Fig. 10.1 it can be observed that the conversion of ATP to adenosine, promoted by E-NTPDases and ecto-5′NTs, is essential to parasites for various reasons. Among them, the generation of adenosine that supplies the requirement of purines for the parasites. Furthermore, it is noteworthy the contribution of ecto-3′NT/NU, a bi-functional enzyme that acts in both nucleic acids and 3′nucleotides. The occurrence of both ecto-5′NT and ecto-3′NT/NU allows the parasite to obtain adenosine from nucleic acids.

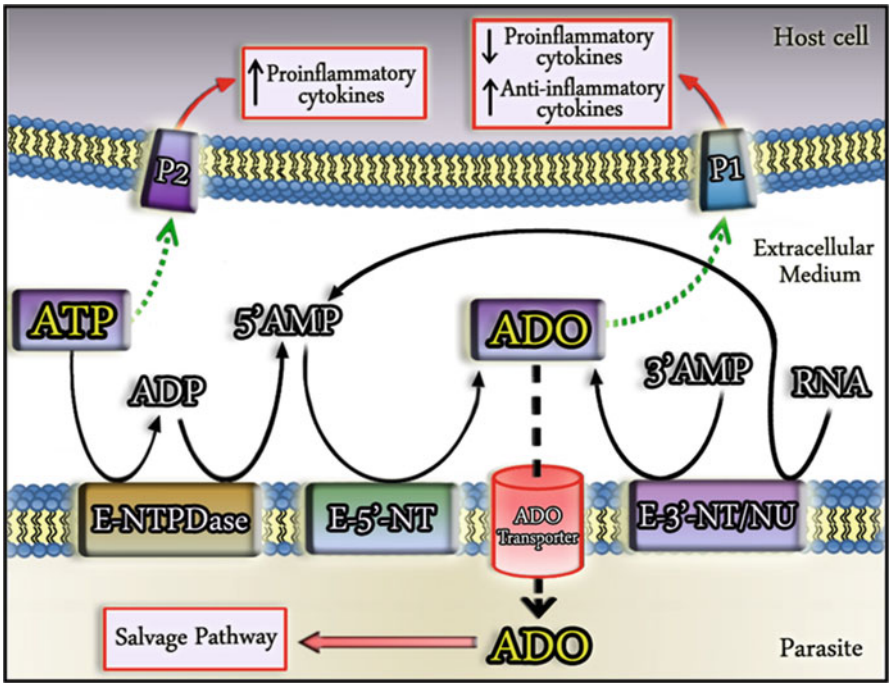

Fig. 10.1 The action of ecto-nucleotidases in parasite cell surface. Schematic representation of the reactions catalyzed by the ecto-nucleotidases, emphasizing their substrates and products. The sequential hydrolysis of ATP to adenosine plays a role in both purine acquisition and suppression of host-immune response. *E-NTPDase* ecto-nucleoside triphosphate diphosphohydrolase, *E-5′-NT* ecto-5′nucleotidase, *E-3′-NT/NU* ecto-3′nucleotidase/nuclease, *ADO* adenosine, *P2* P2 receptor, *P1* P1 receptor

The hydrolysis of 5′AMP was described in *L. tropica* (Meyer-Fernandes et al. 1997), *L. amazonensis* (Berrêdo-Pinho et al. 2001; Pinheiro et al. 2006), *L. braziliensis*, *L. major* (Marques-da-Silva et al. 2008), *T. cruzi* (Bisaggio et al. 2003; Fietto et al. 2004; Meyer-Fernandes et al. 2004), *T. rangeli* (Fonseca et al. 2006), and *T. brucei* (de Souza et al. 2007), suggesting that the action of both E-NTPDases and ecto-5′nucleotidases enables the generation of adenosine from ATP. In *L. amazonensis* (Pinheiro et al. 2006) and *T. brucei* (de Souza et al. 2007), the release of adenosine from ATP hydrolysis was confirmed and quantified by HPLC analysis.

The Mg-dependent ecto-ATPase activities from *T. rangeli* (Fonseca et al. 2006) and *L. amazonensis* (Berrêdo-Pinho et al. 2001) decreased almost 70 % and 50 %, respectively, when parasites were grown in the presence of 5 mM of adenosine. In *L. amazonensis*, this negative modulation has been shown to occur via down-regulation of the enzyme expression, as confirmed by flow cytometry analysis of parasites incubated with different anti-NTPDase antibodies (Pinheiro et al. 2006). Similar results were obtained with *T. brucei*: the parasites grown in

purine free trypanosome medium (PFTM) supplemented with 100 μM ATP showed a reduction of more than 50 % on their Mg-dependent ecto-ATPase activity when compared to the control group that was grown in PFTM without any supplement (de Souza et al. 2007).

It is known that 3′AMP is available to parasites in several vertebrate host tissues (Bushfield et al. 1990), thereby the ecto-3′NT activity can play an important role in salvage pathway by generating adenosine. A very low ecto-3′nucleotidase activity has been described in *Trypanosoma* species such as *T. brucei* (Gbenle et al. 1986) and *Trypanosoma rhodesiense* (Gottlieb et al. 1986), however, *T. cruzi* lacks any detectable activity. It is probable that in *Trypanosoma* group this enzyme is only a vestigial protein (Gottlieb 1989). Meanwhile, ecto-3′NT/NU was reported in several *Leishmania* species, such as *L. donovani* (Gottlieb and Dwyer 1983), *L. major* (Lakhal-Naouar et al. 2008), *Leishmania mexicana* (Sopwith et al. 2002), *L. amazonensis* (Paletta-Silva et al. 2011), *L. infantum*, *L. tropica* and *L. braziliensis* (Vieira et al. 2011).

A great evidence of the involvement of 3′NT/NU in purine acquisition is the modulatory effect promoted by purine deprivation in culture medium. This modulation was observed in several parasites (Gottlieb and Cohn 1997). Among *Leishmania* species, *L. mexicana* parasites grown in limiting condition of purines showed an increase in ecto-3′NT/NU activity as well as in the distribution of the enzyme at the cell surface (Sopwith et al. 2002). On the other hand, the ecto-3′NT/NU from *L. major* did not respond to purine starvation (Lakhal-Naouar et al. 2008).

2.4.4 Possible Roles of Ecto-nucleotidases in Virulence

Ecto-enzymes are located in the plasma membrane with their active sites facing the external environment, and could be one of the parasite proteins that initially come into contact with the host cells in the invasion process. This peculiar location suggests that these enzymes may be involved in virulence and infection. Several studies corroborate this hypothesis, such as the fact that exogenous carbohydrates involved with cellular recognition and adhesion of the parasites to their hosts are able to stimulate ecto-ATPase activities in different trypanosomatids (Meyer-Fernandes et al. 2010). Different carbohydrates involved in the attachment/invasion of salivary glands of the invertebrate host *Rhodnius prolixus* stimulated the Mg-dependent ecto-ATPase from *T. rangeli*. Among the carbohydrates tested, D-glucosamine and D-fructose have increased this activity more than twofold (Fonseca et al. 2006). Similar to *T. rangeli*, carbohydrates also stimulates the *L. tropica* (Peres-Sampaio et al. 2001) and *T. cruzi* ecto-ATPase activity. In *T. cruzi*, D-galactose stimulated Mg-dependent ecto-ATPase activity in a dose-dependent manner (Meyer-Fernandes et al. 2004).

The ecto-ATPase activity from *L. amazonensis* was increased when the parasite was submitted to heat shock (Peres-Sampaio et al. 2008). During its life cycle, these parasites undergo a similar situation when leaving the sand fly vector and they are injected into the warmer mammalian host (Sansom 2012). The increased

ecto-ATPase activity in *L. amazonensis* suggests that this activity may play a fundamental role in parasites during infection (Pinheiro et al. 2006; Sansom et al. 2008; Meyer-Fernandes et al. 2010).

Recently, a study has evaluated several *L. braziliensis* isolates that differ in their ability to hydrolyze adenine nucleotides. A positive correlation was observed between the time for peak of lesion development in C57BL/6 J mice and ecto-nucleotidase activity and clinical manifestation of the isolate (Leite et al. 2012). Similar results were obtained in *L. amazonensis*: mice inoculated with an isolate that presents higher ecto-ADPase and 5′nucleotidase activities have developed necrotic and ulcerated lesions displaying an infiltrate rich in parasitized macrophages. On the other hand, the inoculation of an isolate that presents lower activities induced smaller lesions and a mixed inflammatory infiltrate, without necrosis or ulceration (Souza et al. 2011).

The parasites *T. cruzi* and *T. brucei* have bloodstream life cycle stages and, in particular, *T. brucei* remains extracellular and replicates in the bloodstream. Furthermore, it is known that ADP can activate platelets by binding P2Y receptors (Sansom 2012). Both *T. cruzi* (Meyer-Fernandes et al. 2004) and *T. brucei* (de Souza et al. 2007) present ecto-ATPase activities which means that they can generate ADP from ATP hydrolysis. However, the described activities are also able to hydrolyze ADP, almost in the same ratio. Then, those ecto-ADPase activities may be involved in the inhibition of platelet aggregation, allowing entry of the parasite in the vertebrate host (Sansom 2012).

Another indicative for the participation of ecto-nucleotidases in parasite infection is their differential expression depending on the developmental stage of the parasites. In *L. amazonensis*, the ecto-ATPase activity was measured in both promastigote and amastigote forms. Interestingly, the activity from amastigotes was much higher than that observed in promastigotes, with an increase of almost 200 % (Pinheiro et al. 2006). Furthermore, the Mg-dependent ecto-ATPase activity was also compared in virulent and avirulent promastigotes. The parasites cultured for long periods (more than 600 passages) in axenic medium are termed avirulent because are unable to establish disease. The virulent promastigotes were obtained from culturing amastigotes isolated from hamster lesions, for up to five subcultures. Virulent promastigotes showed a very high Mg-dependent ecto-ATPase activity, with an increase of about 50 % when compared to avirulent forms (Berrêdo-Pinho et al. 2001). Similarly, ecto-ATPase activity is up to 20-fold higher in the infective trypomastigotes than in noninfective epimastigotes of *T. cruzi* (Fietto et al. 2004; Meyer-Fernandes et al. 2004). Moreover, the ratios of ATP: ADP hydrolysis was also different comparing the two stages. For trypomastigotes, the ATP: ADP ratio was 2:1, while for epimastigotes it was 1:1. This result can suggest the occurrence of different NTPDases isoforms with stage-specific expression (Fietto et al. 2004).

In addition to E-NTPDases, ecto-3′nucleotidase activities have been reported as stage-specific enzymes. In *Leishmania*, their expression and activity are related to promastigotes forms, as observed in *L. donovani* (Debrabant et al. 1995), *L. mexicana* (Sopwith et al. 2002) and *L. major* (Lakhal-Naouar et al. 2008). In *L. amazonensis*, the ecto-3′nucleotidase activity of virulent promastigotes was twofold

higher than that observed in avirulent ones (Paletta-Silva et al. 2011). Similar results were obtained with *L. infantum* when non-metacyclic and metacyclic promastigotes forms were compared and the last one showed higher ecto-3′nucleotidase activity. Furthermore, the visceral species *L. chagasi* and *L. donovani* have shown higher activities than the cutaneous species *L. amazonensis*, *L. braziliensis*, *L. tropica* and *L. major* (Vieira et al. 2011).

2.4.5 Ecto-nucleotidases Modulating the Host Immune Response

In mammalian host, the production of proinflammatory cytokines is upregulated in the presence of ATP via P2 receptors. On the other hand, a decrease in proinflammatory cytokines and also an increase in anti-inflammatory ones are performed by adenosine binding in P1 receptors (Di Virgilio 2007). As observed in Fig. 10.1, the ecto-nucleotidases present in parasite surface are directly involved in the balance ATP/adenosine. By the action of E-NTPDase and ecto-5′NT, ATP is sequentially converted to adenosine, leading to a decrease in the inflammatory response. Moreover, adenosine can also be generated from other sources by the action of ecto-3′NT/NU.

The modulation of inflammatory response by ecto-nucleotidases was confirmed by several studies involving *in vitro* interaction assays with parasites and macrophages. The number of *T. cruzi* parasites attaching to mouse peritoneal macrophages was reduced in the presence of suramin and DIDS, two potent inhibitors of ecto-ATPase activity. Interestingly, ATP, the substrate for this enzyme, was able to protect from those inhibitory effects, increasing the number of parasite-infected macrophages (Bisaggio et al. 2003; Meyer-Fernandes et al. 2004). Furthermore, suramin, as well as ARL67156 and gadolinium, were able to decrease the trypomastigotes infectivity *in vitro* and also *in vivo*, since mice infected with those pretreated trypomastigotes had lower levels of parasitemia and higher host survival than non-inhibited control parasites (Santos et al. 2009).

In *L. amazonensis*, the pretreatment of the parasites with anti-NTPDase antibodies reduced the interaction of promastigotes with mouse peritoneal macrophages. This result confirmed that at least part of the characterized ecto-ATPase activity is really related to the occurrence of an NTPDase, and furthermore that this activity plays an important role on the interaction between parasites and macrophages (Pinheiro et al. 2006). Another study with *L. amazonensis* showed that chromium (III) adenosine 5′triphosphate complex (CrATP) is able to inhibit both ecto-ATPase activity and parasite interaction with mouse peritoneal macrophages (Ennes-Vidal et al. 2011). It is noteworthy that the inhibition of ecto-ATPase activity by CrATP has also been observed in *T. cruzi* and *T. rangeli* (Moreira et al. 2009).

The participation of 5′- and 3′NTs on the generation of adenosine and consequently in the increase in parasite-macrophage interaction was also described in *L. amazonensis*. The addition of 1 mM 5′AMP significantly increased the

interaction of parasites and peritoneal macrophages; however, addition of the same concentration of 3′AMP generated a more pronounced effect, increasing the interaction to the same levels obtained with the addition of adenosine. This occurs because ecto-3′NT activity is 60-fold higher than ecto-5′NT activity in *L. amazonensis*, thereby treatment with 3′AMP can generate much more adenosine. In summary, although ecto-3′NT may be the most potent source of adenosine to parasite, the participation of ecto-5′NT has also a relevant contribution in this process (Paletta-Silva et al. 2011).

Similar results were obtained with *L. infantum* in which the ecto-3′NT activity was tenfold higher than the ecto-5′NT activity. Moreover, its ecto-3′NT activity was increased when parasites were grown in low phosphate concentrations conditions. The same response was not observed with ecto-5′NT activity. Interestingly, the interaction between parasites and peritoneal macrophages was higher when the parasites were obtained from phosphate-depleted medium (Vieira et al. 2011).

The involvement of ecto-nucleotidases in the establishment of infection was also evidenced with an *in vivo* study that performed the inoculation of C57BL/6 mice with metacyclic forms of *L. amazonensis* or *L. braziliensis*. The animals were capable of controlling the infection by *L. braziliensis*, however those infected with *L. amazonensis* developed chronic lesions with elevated number of parasites. This response was related to the fact that *L. amazonensis* showed higher capacity of hydrolyzing ATP, ADP and AMP and, consequently, of adenosine generation (Maioli et al. 2004). The treatment with the ecto-5'NT inhibitor ammonium molybdate has increased the ability of *L. braziliensis* to hydrolyze AMP. Interestingly, these parasites were also able to increase the parasitism and the size of footpad lesion of infected mice. The same responses were obtained when adenosine was directly added in the moment of infection. Probably, the effects promoted by adenosine are via A2B receptors since the use of an antagonist led to a decrease in lesion size and parasitism (Marques-da-Silva et al. 2008).

Another study performed with *L. braziliensis* has observed that mice inoculated with parasite isolates presenting high or low ecto-nucleotidase activities differ in their immunological response. The inoculation with PPS6m parasites, the isolate with higher ecto-nucleotidase activity, has led to the development of delayed lesions, presenting larger parasite loads than animals inoculated with SSF, the isolate that presented lower activities. Moreover, PPS6m parasites were able to modulate the host immune response by inhibiting DC activation and NO production by activates J774 macrophages. These results suggest that ecto-nucleotidase activities present on the promastigote forms of *L. braziliensis*, may interfere with the establishment of the immune response, decreasing the host control of parasite dissemination (Leite et al. 2012).

As previously mentioned, *L. amazonensis* promastigotes kept in culture for extended numbers of passages are consider avirulent because of the lost of ability to cause disease. Recently, this phenomenon was related to the concomitant reduction of ecto-nucleotidase activities, since these enzymes have an important role in infection by *L. amazonensis* by acting directly in its adhesion to target cells and by modulating host cell chemokine production (de Souza et al. 2010).

3 Ecto-phosphatases

Protozoan parasites are exposed to diverse environmental stimuli throughout their life cycles in all biological systems. Protein phosphorylation and dephosphorylation are central events in cell recognition of external and internal signals, leading to specific responses. In this context, ecto-phosphatases are ecto-enzymes that presumably hydrolyze extracellular phosphorylated substrates, releasing free inorganic phosphate. Because of its extracellular active site and ability to hydrolyze phosphomonoesters, ecto-phosphatases may enable organisms to obtain necessary nutrients from organic phosphates in their environment. Several alternative functions have been suggested for these enzymes, such as participation in proliferation, differentiation, adhesion, virulence and infection (Cosentino-Gomes and Meyer-Fernandes 2011; Gomes et al. 2011). The focus of this section will be the extracellular metabolism of Pi, emphasizing the occurrence of membrane-bound and secreted phosphatase activities in *Leishmania* and *Trypanosoma* parasites. Furthermore, the following topics will also be discussed: the imbalance of phosphorylation-dephosphorylation of protein residues; the different classification criteria proposed to phosphatases; and the mechanisms of uptake of inorganic phosphate.

3.1 The Role of Phosphatases and Kinases in Regulation by Phosphorylation

Protein phosphorylation is undoubtedly the most common and also the best studied post-translational modification. It has been estimated that about 30 % of all proteins can be regulated by phosphorylation (Andreeva and Kutuzov 2008). Indeed, most proteins in the cell can be regulated, directly or indirectly, via this mechanism (Kutuzov and Andreeva 2008). The phosphorylation status of any protein is controlled by both protein kinases and phosphatases (Andreeva and Kutuzov 2008). Traditionally, kinases had been studied more intensively than phosphatases, considering that kinases are responsible for phosphorylation, while phosphatases merely act to remove phosphate. However, in the mid of the 90s, protein phosphatases started to be more studied; it was realized that they are also regulated by a variety of mechanisms and are of no less importance in cellular physiology than protein kinases (Kutuzov and Andreeva 2008).

Protein phosphatases remove phosphate groups from various phosphorylated amino acids. The most predominant phosphorylation sites in eukaryotic cells are detected on serine, threonine and tyrosine residues. The sequencing of several protozoan parasites genomes has allowed the first phosphoproteome analysis. In the *T. brucei* bloodstream forms, 491 phosphoproteins were identified, which means that 5.5 % of the proteins were phosphorylated in this life cycle stage. The majority of the identified proteins were phosphorylated on serine or threonine (75 % and 21.5 % respectively) and only 3.5 % were tyrosine-phosphorylated. In vertebrates

(HeLa cells) an even lower percentage of tyrosine-phosphorylated proteins have been described (86.4/11.8/1.8 % Ser/Thr/Tyr) (Szöör 2010). Although phosphorylation on tyrosine residues comprises only a small fraction of all protein phosphorylation events, it plays a disproportionately important role in such aspects of signaling as cell-cycle control or differentiation. For this, several studies involving phosphotyrosine phosphatases were performed in protozoan parasites. Indeed, interfering with protein phosphorylation represents a potentially powerful pharmacological approach (Nascimento et al. 2006; Andreeva and Kutuzov 2008).

3.2 Classification of Phosphatases

The classification of phosphatases is based on their biophysical and biochemical properties, such as specificity, optimum pH, and its cellular localization (Camici et al. 1989). Hereafter, these different classifications will be discussed.

3.2.1 Substrate Specificity

The phosphorylation of serine, threonine and tyrosine residues results in the formation of a phosphoester linkage. Apart from them, the phosphorylation of histidine residues occurs on nitrogen atoms, producing a phosphoramidate bond. A class of phosphatases, namely phosphohistidine phosphatases (PHPs), is involved in dephosphorylation of phosphohistidine residue. PHPs have already been described in prokaryotes and eukaryotes, including yeast, amoeba, fungi and plants (Klumpp and Krieglstein 2002); however, their occurrence in trypanosomatids has not been established yet.

Phosphatases that act specifically in phosphoserine and phosphothreonine are known as phospho-serine/threonine phosphatases (PPs). Basically, their mechanism of catalysis involves a nucleophilic attack from a water molecule to the phosphorylated residue, which occurs in a single step, without transference of phosphate to the enzyme. The activation of the water molecule depends on the presence of metallic ions (Egloff et al. 1995).

On the other hand, enzymes that hydrolyze specifically phosphotyrosine are known as phosphotyrosine phosphatases (PTPs). Unlike PPs, PTPs do not require metal ions for catalysis. They are characterized by the active-site sequence motif HCxxGxxRS(T) within the catalytic domain of approximately 200–300 residues. Basically, the mechanism of catalysis involves the formation of a phosphorylated intermediate of cysteine, due to nucleophilic attack of its thiol group (Denu et al. 1996).

There is also a third group, namely dual-specific phosphatases, which are capable of hydrolyzing phosphotyrosine as well as phosphoserine and phosphothreonine residues. However, these enzymes are commonly classified as a subfamily of PTPs, because of their similarities in the active site and mechanism of catalysis (Denu et al. 1996).

3.2.2 Optimum pH

Phosphatases that are able to hydrolyze a wide variety of organic esters by releasing a phosphate ion in a pH range that extends from 4 to 7 are known as acid phosphatases. These enzymes present a ubiquitous distribution. Acid phosphatases are mostly glycoproteins, and some of which are metallo-hydrolases (Anand and Srivastava 2012). Meanwhile, most of acid phosphatases present a metal ion-independent catalytic mechanism and forms a phosphorylated intermediate that probably involves histidine residues (Vincent et al. 1992).

On the other hand, alkaline phosphatases are typically formed by two subunits of about 94 kDa that are capable of hydrolyzing a wide variety of phosphate monoesters in an optimum pH that belongs to the alkaline range (Vincent et al. 1992). Generally, their catalytic mechanism involves the transfer of a transient phosphate group to a serine residue present at the active site, which also contains the bimetallic nucleus Zn/Zn (Jedrzejas 2000). However, metal-independent alkaline phosphatase activities have already been described (Scheibe et al. 2000).

3.2.3 Subcellular Localization

Phosphatases can also be classified according to their subcellular location, which can be intracellular (Andrews and Stark 2000; Zhan et al. 2000); secreted from the cell to extracellular medium (Rodrigues et al. 1999; Dutra et al. 2001; Fernandes et al. 2013); or even membrane-bound enzymes, which may be associated to plasma membranes (Fernandes et al. 1997). In this context, the ecto-phosphatases are surface membrane-bound proteins, whose active site faces the extracellular medium (Furuya et al. 1998). It is noteworthy that both ecto- and secreted phosphatases are involved in extracellular metabolism of phosphate.

3.3 Uptake of Inorganic Phosphate

Pi is an essential nutrient for all organisms, due to its requirement not only for the biosynthesis of nucleic acids, proteins, lipids, and sugars, but also for energy metabolism, and signal transduction. Therefore, the organisms have evolved regulatory mechanisms for acquisition, storage and release of this molecule (Ogawa et al. 2000).

Transport of Pi across the plasma membrane is the first step for its utilization in the cell. In all cells, the uptake of essential nutrients, ions and metabolites is mediated by a specialized class of integral membrane transport proteins that allows the water-soluble solutes to cross the hydrophobic barrier of the phospholipid bilayer. The variations in concentration of extracellular nutrients as well as the uptake events result in cellular responses such as alteration in gene expression and protein activity (Persson et al. 1999).

The phosphate signal-transduction pathway, known as PHO pathway, has been described in different organisms, such as plants (Ticconi et al. 2001), bacteria (Torriani 1990) and fungi (Oshima 1997). In *Saccharomyces cerevisiae*, PHO pathway regulates the expression of several genes involved with the availability and absorption of Pi from extracellular sources (Auesukaree et al. 2004). Basically, transcription of genes encoding acid and alkaline phosphatases and the Pi transporters are coordinately repressed and derepressed depending on the Pi concentration in the culture medium (Oshima et al. 1996).

In trypanosomatids, there is no description mentioning the occurrence of the PHO system; however it is already known that Pi starvation modulates ecto-phosphatase activities (Fonseca-de-Souza et al. 2008; Dick et al. 2010) and, recently, it was described that parasites can transport Pi across their plasma membranes (Dick et al. 2012; Russo-Abrahão et al. 2013). The first study providing the description of a mechanism for Pi transport across plasma membrane of trypanosomatids was performed with *T. rangeli*. Two modes of Pi transport were described, one coupled to Na^+-ATPase and other coupled to H^+-ATPase. They seem to be responsible for Pi acquisition during *T. rangeli* life cycle (Dick et al. 2012).

Another recent study has identified an H^+/Pi contransporter of high-affinity in *L. infantum*. This transporter seems to be modulated by the Pi concentration in the culture medium of parasites. An increase in mRNA levels of transporters and in Pi uptake was observed when parasites grew in a culture medium with 2 mM compared to the ones that grew with 80 mM Pi (Russo-Abrahão et al. 2013).

It is known that the ecto-phosphatases can generate Pi from a wide variety of phosphorylated substrates (Gomes et al. 2011). Given that parasites are able to transport this nutrient from the extracellular medium, it is likely that ecto-phosphatases play an important role in Pi acquisition, as shown in Fig. 10.2a. The action of an ecto-phosphatase leads to the generation of Pi that can be internalized by the cells, crossing the plasma membranes through a co-transport with Na^+ or H^+. Once inside the cell, inorganic phosphate is routed to the synthesis of biomolecules, energetic metabolism and signal transduction.

3.4 Evidence of Ecto-phosphatases in Leishmania *and* Trypanosoma *Species*

3.4.1 Identification and Localization of Membrane-Bound or Secreted Phosphatase Activities

Although the first demonstration of phosphatase activity in *T. brucei* and *T. cruzi* took place in 1972, their physiological roles are still not well-established (Jadin and Creemers 1972). Evidences suggest that they may be involved with nutrition, protection, virulence, cellular differentiation and proliferation (Cosentino-Gomes and Meyer-Fernandes 2011). To describe these evidences, Table 10.1 summarizes

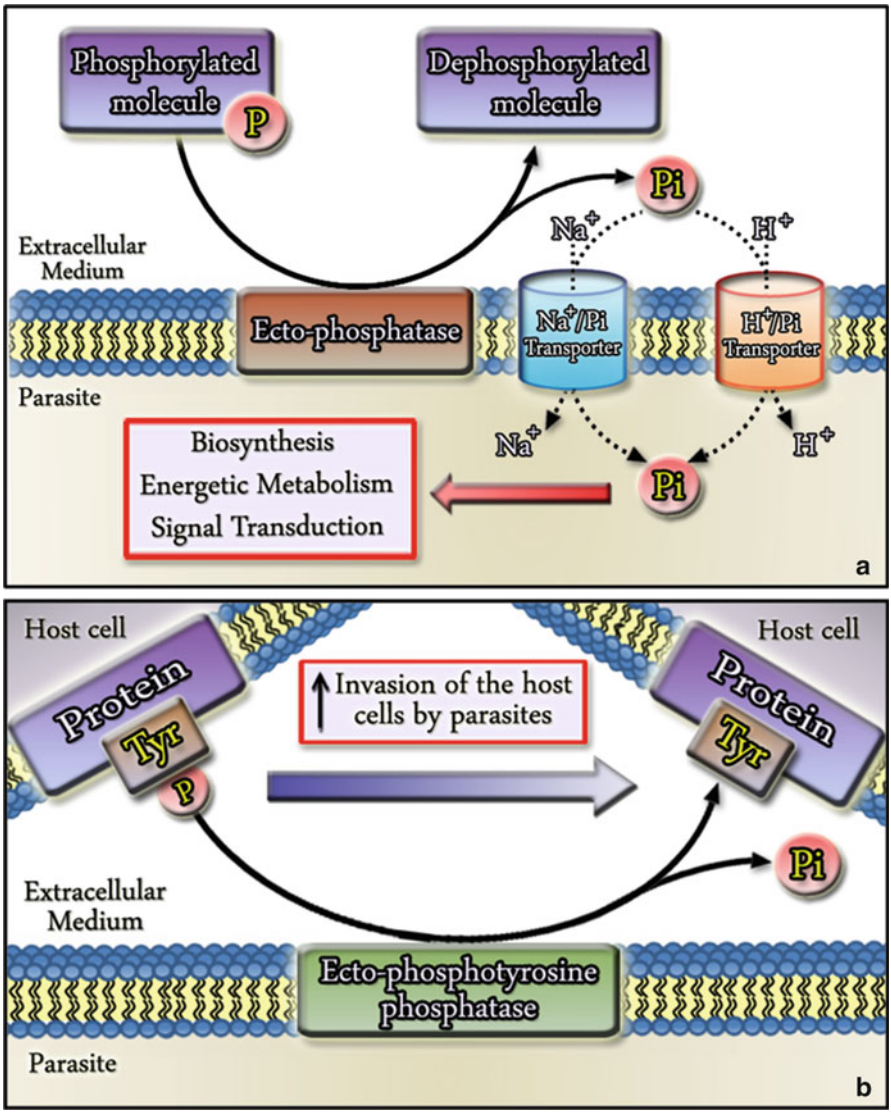

Fig. 10.2 The action of ecto-phosphatases in parasite cell surface. Ecto-phosphatases are able to hydrolyze a wide variety of phosphorylated substrates and the released extracellular Pi can be transported into cell by co-transporters of Na^+ or H^+. Once inside the cell, Pi is routed to the biosynthesis of nucleic acids, proteins, lipids, and sugars; energetic metabolism; and also signal transduction (**a**). Moreover, the dephosphorylation of phosphotyrosine residues present in plasma membrane of host cells may facilitate the parasite invasion (**b**). *Tyr* tyrosine residue, *Pi* inorganic phosphate

Table 10.1 Possible functions of ecto-phosphatases in *Trypanosoma* and *Leishmania* parasites

Functions	Evidences
Nutrition	The hydrolysis of a phosphorylated substrate by the ecto-phosphatase from *T. rangeli* was able to supply Pi requirement in culture medium (Fonseca-de-Souza et al. 2008)
Protection	A purified acid phosphatase from the external surface of *L. donovani* inhibits superoxide anion production by human neutrophils (Remaley et al. 1984)
	Ecto-phosphatase from *T. rangeli* is modulated by ROS (Cosentino-Gomes et al. 2009)
Virulence	Ecto-phosphatase from *L. donovani* is higher in virulent strains than in avirulent strains (Singla et al. 1992)
	Tyrosine dephosphorylation facilitates host-cell invasion in *T. cruzi* (Zhong et al. 1998) and *T. rangeli* (Dos Santos et al. 2012)
Cellular differentiation	Stage-specific regulation in expression and/or activity of ecto-phosphatases occurs in: *L. major* (Aguirre-García et al. 2006), *T. brucei* (Bakalara et al. 2000), *T. cruzi* (Nakagura et al. 1985)
Cellular proliferation	Ecto-phosphatase activity from *T. rangeli* is increased along the first days of parasites growth in culture medium (Fonseca-de-Souza et al. 2008)
	The growth of *L. amazonensis* is completely abolished in the presence of its ecto-phosphatase inhibitor sodium orthovanadate (De Almeida-Amaral et al. 2006)

several studies that correlate the occurrence of ecto-phosphatases in *Trypanosoma* and *Leishmania* parasites to each function mentioned above.

Membrane-bound phosphatases or even secreted enzymes have been described in several protozoan parasites, such as: *Trypansoma rhodesiense* (McLaughlin 1986), *Trypanosoma congolense* (Tosomba et al. 1996), *T. brucei* (Fernandes et al. 1997; Bakalara et al. 2000), *T. cruzi* (Furuya et al. 1998; Meyer-Fernandes et al. 1999; Dutra et al. 2006), *T. rangeli* (Gomes et al. 2006; Fonseca-de-Souza et al. 2008; Cosentino-Gomes et al. 2009; Dick et al. 2010), *L. mexicana* (Menz et al. 1991; Wiese et al. 1996), *L. donovani* (Gottlieb and Dwyer 1981; Remaley et al. 1984, 1985), *L. amazonensis* (De Almeida-Amaral et al. 2006) and *L. major* (Aguirre-García et al. 2006). Electron microscopic studies were performed in *T. congolense* (Tosomba et al. 1996), *T. rangeli* (Gomes et al. 2006), *L. mexicana* (Menz et al. 1991) and *L. donovani* (Gottlieb and Dwyer 1981) and confirmed the occurrence of membrane-bound phosphatases. These approaches have confirmed the wide distribution of phosphatases on the cell surface, possibly reflecting some physiological adaptation for parasite survival within the host (Cosentino-Gomes and Meyer-Fernandes 2011).

Studies of cloning and purification of membrane-bound phosphatases have been performed in *L. mexicana* (Wiese et al. 1996), *L. donovani* (Remaley et al. 1984) and *T. brucei* (Bakalara et al. 2000). In *L. donovani*, an acid phosphatase insensitive to tartrate was characterized as an integral membrane glycoprotein that accounts for more than 70 % of the total acid phosphatase activity of the parasite (Remaley et al. 1984).

The occurrence of this membrane-bound acid phosphatase was confirmed later, by the identification of the gene that encodes this enzyme (Shakarian et al. 2002) Differently, a phosphatase cloned and purified in *L. mexicana* were located in the endosomal/lysosomal compartment between the flagellar pocket and the nucleus in wild-type promastigotes. However, the overexpression of the enzyme led to its abundant exposure on the cell surface (Menz et al. 1991; Wiese et al. 1996). A new phosphatase class, lacking homology to known phosphatases, was described in *T. brucei*, by the cloning and purification of an acid phosphatase that seems to be regulated by the parasite life cycle stage (Bakalara et al. 2000).

The localization of the observed activity can also be determined by using classical phosphatase inhibitors, since secreted phosphatases are commonly inhibited by tartrate (Santos et al. 2002). In a study comparing two strains of *T. cruzi*, the addition of tartrate inhibited about 90 % of the phosphatase activity from Colombiana strain, suggesting that it involved secreted enzymes (Dutra et al. 2006). Interestingly, another strain of *T. cruzi* (Dm28c) was shown to secret an acid phosphatase activity after stimulation by platelet-activating factor (Rodrigues et al. 1999). The occurrence of secreted phosphatase activity was also described in *Leishmania*, such as *L. tropica* (Jaffe et al. 1990), *L. mexicana* (Menz et al. 1991) *L. donovani* (Gottilieb and Dwyer 1982) and *L. amazonensis* (Martiny et al. 1996). Recently, a study involving the use of two antibodies and specific inhibitors showed that *L. mexicana* secreted phosphotyrosine phosphatases (Escalona-Montaño et al. 2010).

It is noteworthy that some authors designated secreted activities as ecto-phosphatases regarding that both activities are involved with extracellular metabolism. However, the term "ecto-phosphatases" usually refers to membrane-bound phosphatases whose catalytic site faces the extracellular medium. To identify ecto-phosphatases, the phosphatase activity assays can be performed using intact cells. This approach has already been performed in *T. brucei* (Fernandes et al. 1997, 2003a, b), *T. cruzi* (Furuya et al. 1998; Meyer-Fernandes et al. 1999), *T. rangeli* (Gomes et al. 2006; Fonseca-de-Souza et al. 2008; Cosentino-Gomes et al. 2009; Fonseca-de-Souza et al. 2009; Dick et al. 2010) and *L. amazonensis* (De Almeida-Amaral 2006), confirming the existence of ecto-phosphatase activity in these parasites.

3.4.2 Modulators of Ecto-phosphatase Activities: Classical Inhibitors, Divalent Metals and Pi

In general, the ecto-phosphatase activities described in parasites are acid phosphatases that present high affinity for phosphotyrosine analog substrates, such as *p*-nitrophenyl phosphate, an artificial substrate commonly used to characterize acid phosphatases. Sodium fluoride, an acid phosphatases inhibitor (Dutra et al. 1998), was able to inhibit both *T. cruzi* (Furuya et al. 1998; Meyer-Fernandes et al. 1999) and *T. rangeli* (Fonseca-de-Souza et al. 2008) ecto-phosphatase activities. Ammonium molybdate and sodium orthovanadate are commonly used as phosphotyrosine phosphatase inhibitors (Dutra et al. 1998). Both inhibitors were

capable of inhibiting *T. rangeli* ecto-phosphatase activity (Fonseca-de-Souza et al. 2008; Dick et. al.2010). In *T. cruzi* (Furuya et al. 1998; Meyer-Fernandes et al. 1999) and *T. brucei* (Fernandes et al. 2003a, b), only orthovanadate was tested and also showed an inhibitory effect. Interestingly, orthovanadate inhibited only phosphortyrosine activity of *L. amazonensis*, while no modulation was observed in parasite phosphoserine and phosphothreonine phosphatases (De Almeida-Amaral et al. 2006).

Okadaic acid and microcystin are two classical phosphoserine/threonine phosphatase inhibitors that were tested in *T. rangeli* ecto-activities. Parasites grown in culture medium with low Pi concentration expressed an ecto-phosphatase activity that had preference for phosphoserine and phosphothreonine substrates. Consequently, this activity is inhibited by okadaic acid. The ecto-phosphatase activity from parasites grown at high Pi was insensitive to both okadaic acid and microcystin (Dick et al. 2010).

Moreover, divalent metals showed to be able to modulate ecto-phosphatase activities. The inhibition promoted by zinc was observed in several parasites, including *T. brucei* (Fernandes et al. 1997, 2003a), *T. cruzi* (Furuya et al. 1998; Meyer-Fernandes et al. 1999), *T. rangeli* (Dick et al. 2010) and *L. amazonensis*. This effect is probably due to its redox properties and its tight binding to sulfhydryl groups. The inhibition of ecto-phophatase activity of *L. amazonensis* by zinc was protected dose-dependently by co-incubation with reduced glutathione and cysteine, two zinc-binding thiol compounds. However, serine, an amino acid that does not present the thiol group, did not exert such effect. This result suggests that, at least in *L. amazonensis*, the inhibitory effect exerted by zinc may be through the coordination of some residues in a zinc-binding site (De Almeida-Amaral et al. 2006). On the other hand, magnesium, manganese, cobalt and cooper were able to increase the ecto-phosphatase of *T. brucei* (Fernandes et al. 2003a). Furthermore, the stimulation by magnesium was also observed in *T. cruzi* (Meyer-Fernandes et al. 1999) and *T. rangeli* (Fonseca-de-Souza et al. 2008; Dick et al. 2010).

Different ecto-phosphatase activities have already been described in the external surface of *T. rangeli* (Gomes et al. 2006; Fonseca-de-Souza et al. 2008; Dick et al. 2010). An ecto-phosphatase activity showed to be differentially expressed in short and long epimastigotes forms of *T. rangeli* H14 strain (Gomes et al. 2006), while a Mg-dependent activity was characterized in Macias strain (Fonseca-de-Souza et al. 2008). The Mg-dependent ecto-phosphatase activity from *T. rangeli* showed to be activated at alkaline pH ranges while the basal activity did not respond to pH variation. Moreover, basal ecto-phophatase activity, measured in the absence of any metals, was able to hydrolyze phosphoserine, phosphothreonine and phosphotyrosine at almost the same ratio; however, phosphotyrosine activity was not stimulated by magnesium (Fonseca-de-Souza et al. 2008; Dick et al. 2010). Interestingly, calcium was not able to inhibit basal activity; meanwhile, it inhibited Mg-dependent ecto-phosphatase activity dose-dependently (Fonseca-de-Souza et al. 2008).

In *T. rangeli* Macias strain it was also demonstrated that the concentration of Pi in culture medium can modulate ecto-phosphatase activity, probably inducing the expression of a different enzyme that has an increased affinity for the

phosphorylated substrate. The ecto-phosphatase activities from parasites grown in low Pi (~2 mM) or high Pi (50 mM) culture media were compared with respect to several biochemical parameters. A different profile of inhibitory response was observed when the classical phosphatase inhibitors were tested. Furthermore, the variation of pH could not modulate the ecto-phosphatase activity from parasites grown at high Pi; however, the activity from parasites grown at low Pi was increased in the acid range of pH curve. The modulation by metals was also different in both activities. While activity from parasites grown at high Pi was stimulated by magnesium and insensitive to zinc, the activity from parasites grown at low Pi was inhibited by zinc and no metal was capable of increasing the same. Finally, while ecto-phosphatase activity from parasites grown at high Pi was able to hydrolyze phosphoserine, phosphothreonine and phosphotyrosine in the same ratio, the activity from parasite grown at low Pi showed a preference to phosphoserine and phosphothreonine substrates (Dick et al. 2010).

3.4.3 Ecto-phosphatases as Virulence Markers: Possible Roles in Adhesion and Survival of Parasites in Host Cells

Several studies have demonstrated that ecto-phosphatase activities are related to parasite growth and survival. In *T. rangeli*, the presence of Pi in culture medium is essential for its growth. However, the parasites showed to be able to achieve their maximal growth when the inorganic Pi of culture was replaced by β-glycerophosphate, a substrate for phosphatases. Therefore, ecto-phosphatase activity may play an important role on parasite nutrition by generating extracellular Pi from phosphorylated substrates (Fonseca-de-Souza et al. 2009). Furthermore, Mg-dependent ecto-phosphatase activity from *T. rangeli* showed to be increased along the first days of parasites growth in culture medium. This result suggests that this activity should play a role in proliferation events (Fonseca-de-Souza et al. 2008). Indeed, the growth of *L. amazonensis* is completely abolished in the presence of its ecto-phosphatase inhibitor sodium orthovanadate (De Almeida-Amaral et al. 2006).

The occurrence of a stage-specific regulation in expression and activity of these enzymes have also been observed. A membrane-bound phosphotyrosine phosphatase identified in the plasma membrane of *L. major* showed to be stage-specific. Ultrastructural localization of the enzyme showed that in procyclic promastigote forms it was scattered throughout the cytoplasm, while in metacyclic promastigotes the expression is restricted to the plasma membrane. Interestingly this phosphotyrosine phosphatase is more expressed in metacyclic forms; however, the specific activity is increased in procyclic promastigotes (Aguirre-García et al. 2006).

In bloodstream forms of *T. brucei*, the ecto-phosphatase located in the flagellar pocket, the sole site for endocytosis in trypanosomes, showed to be modified by ubiquitination. These results suggest that this ecto-phosphatase may be involved in endocytosis process, considering that ubiquitination modification of plasma membrane proteins serves as an internalization signal (Steverding 2005). The same activity was not observed in procyclic forms; however, other different ecto-phosphatase

activities have already been identified in these parasites (Fernandes et al. 2003a). Similar results were obtained with *T. cruzi* ecto-phosphatase activities. Ecto-phosphatase activity from amastigote forms showed to be increased when compared to epimastigotes and trypomastigotes forms, suggesting that an elevated activity is probably required for the intracellular parasitization (Nakagura et al 1985).

Membrane-bound and secreted kinase and phosphatase activities are directly involved with the degree of virulence in pathogenic microorganisms (Cunningham 2002; Fernandes et al. 2013). In *L. donovani*, it was observed that ecto-phosphatase activity was significantly higher in virulent strains than in avirulent strains, suggesting that ecto-phosphatase may be considered as virulence marker (Singla et al. 1992).

Several studies have demonstrated that ecto-phosphatase activities are involved in cell adhesion and invasion of distinct microorganisms, including bacteria (Ivanov et al. 2005), fungal cells (Kneipp et al. 2004; Collopy-Junior et al. 2006; Kiffer-Moreira et al. 2007; Portela et al. 2010; Kneipp et al. 2012; Cosentino-Gomes et al. 2013) and protozoa (Zhong et al. 1998; Aguirre-García et al. 2002; Anaya-Ruiz et al. 2003; Dos Santos et al. 2012). Protein tyrosine phosphatases are increasingly recognized as important effectors of host-pathogen interactions (Heneberg 2012). Regarding trypanosomatids, invasion of cultured myoblasts by *T. cruzi* induced tyrosine dephosphorylation of myoblast proteins. Moreover, the process of invasion was greatly reduced in the presence of sodium orthovanadate, a phosphotyrosine phosphatase inhibitor. These results suggest an important role for protein tyrosine dephosphorylation in the invasion of host cells by *T. cruzi* (Zhong et al. 1998).

Recently, a study with *T. rangeli* emphasized the importance of parasite phosphotyrosine ecto-phosphatase activity to the successful parasite colonization in the invertebrate host *R. prolixus*. Immunoassays revealed a large number of phosphotyrosine proteins in extracts of *R. prolixus* salivary glands, which could be potentially targeted by *T. rangeli* during adhesion. The adhesion of parasites to salivary glands of the insect was inhibited by sodium orthovanadate, ammonium molybdate and zinc chloride, three known phosphotyrosine phosphatase inhibitors. Inhibition of both activities and adhesion were assayed concomitantly, suggesting a correlation between them. As the inhibitory effect of sodium orthovanadate on ecto-phosphatase activity showed to be irreversible, *T. rangeli* epimastigotes were pretreated with this inhibitor washed and incubated with *R. prolixus* salivary glands. Differential interference contrast microscopy demonstrated that the pretreatment with sodium orthovanadate greatly inhibited or even suppressed the adhesion of the parasites to salivary gland. These results suggest that dephosphorylation of structural phosphotyrosine residues on the gland cell surfaces is a key event in the interaction between *T. rangeli* and *R. prolixus* salivary glands, confirming the importance of the parasite ecto-phosphatase activity (Dos Santos et al. 2012).

Figure 10.2b summarizes the action of ecto-phosphatases in phosphotyrosine residues present in plasma membrane of the host cell. As dephosphorylation of these residues is related to a successful parasite invasion, the occurrence of ecto-phosphatases in plasma membrane of parasites may facilitate this process, increasing the capacity of parasite to invade its hosts.

In addition to improving the process of invasion, ecto-phosphatase activities may also be involved on survival and proliferation of the parasites in both invertebrate and mammalian hosts. Reactive oxygen species (ROS) sensing is likely to be an important mechanism for the adaptation and interaction of trypanosomatids with the different environments to which they are subjected during their life cycles (Steenkamp 2002). Recently, H_2O_2 was shown to be a ubiquitous intracellular messenger at subtoxic concentrations, playing a key role in intracellular signal transduction through the reversible inactivation of the active site of several enzymes, including phosphatases (Hertog et al. 2005; Cosentino-Gomes et al. 2009).

Both the external addiction and the endogenous mitochondrial production of H_2O_2 have been able to inhibit ecto-phosphatase activity from *T. rangeli*. Meanwhile, this inhibition can be reversed by the addition of enzymatic or nonenzymatic antioxidants (Cosentino-Gomes et al. 2009). Interestingly, ecto-phosphatase activities from *L. donovani* and *T. cruzi* seem to be more resistant to H_2O_2 than that from *T. rangeli*, suggesting that different strategies might have been developed by pathogenic or non-pathogenic parasites of mammalian hosts (Saha et al. 1985; Cosentino-Gomes et al. 2009).

In *L. donovani*, the isolated ecto-phosphatase activity was able to inhibit the production of superoxide anions in intact human neutrophils. As *L. donovani* are obligate intracellular parasites, a resistance to oxidative bursts from the host's immune system may be essential for their survival within the hydrolytic milieu of mammalian macrophage phagolysossomes (Remaley et al. 1984).

4 Conclusions

Throughout this chapter, several studies were reported describing the occurrence of ecto-enzymatic activities in plasma membranes of *Leishmania* and *Trypanosoma* parasites. Many of these studies have suggested that ecto-nucleotidases and ecto-phosphatases may play important roles from both physiological and immunological viewpoints. Some events promoted by the action of the described ecto-nucleotidases are summarized in Fig. 10.1. The described reactions show the consumption of ATP and also the different sources of adenosine generation, reflecting the ability of the parasite in attenuate the inflammatory response from mammalian host.

The generation of adenosine is also involved in purine salvage pathway. As trypanosomatids are unable to perform the *de novo* pathway, they are strictly dependent of exogenous source of purines. These parasites present transporters with a high affinity for nucleosides, when compared to mammalian ones, therefore they can obtain purine from their hosts. In this context, the generation of nucleosides from nucleotides by the action of E-NTPDases and ecto-5′NTs is of great importance. Besides them, another remarkable enzyme involved in nucleoside generation is ecto-3′NT/NU. This ecto-enzyme is able to hydrolyze 3′nucleotides to nucleosides and also nucleic acids to 5′nucleotides that can be converted to nucleosides by the action of an ecto-5′NT. It is noteworthy that ecto-3′NT/NU has not been described in mammals.

With respect to ecto-phosphatases, Fig. 10.2 summarizes some important roles of these enzymes in parasite nutrition and host cell invasion. Different phosphorylated molecules, present in extracellular millieu, can be dephosphorylated by the action of an ecto-phosphatase. This hydrolysis generates Pi that can be internalized by the cells. Ecto-phosphatases are also involved in the invasion and survival of parasite inside host cells. Recent studies have demonstrated that the dephosphorylation of phosphotyrosine residues, present on plasma membrane of host cells, may contribute to a successful parasite invasion. Moreover, ecto-phosphatases seem to increase the resistance to oxidative bursts from the host immune system, ensuring the parasite survival inside the host cell.

With the increased interest about ecto-enzymes, many studies were performed in order to determine the different roles for ecto-nucleotidases and ecto-phosphatases in trypanosomatids. Certainly, more studies are still required to determine the extension of their participation in the physiology of the parasite as well as in host-pathogen interactions. However, based on the results discussed throughout this chapter, it is possible to affirm that these ecto-enzymes are involved in important events, such as: nutrition and differentiation of the parasite; invasion and survival of the parasite in the host cell; modulation of the host immune response and establishment of the infection.

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Chapter 11
GP63 Function in the Interaction of Trypanosomatids with the Invertebrate Host: Facts and Prospects

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Abstract The GP63 of the protozoan parasite *Leishmania* is a highly abundant zinc metallopeptidase, mainly glycosylphosphatidylinositol-anchored to the parasite surface, which contributes to a myriad of well-established functions for *Leishmania* in the interaction with the mammalian host. However, the role of GP63 in the *Leishmania*-insect vector interplay is still a matter of controversy. Data from GP63 homologues in insect and plant trypanosomatids strongly suggest a participation of GP63 in this interface, either through nutrient acquisition or through binding to the insect gut receptors. GP63 has also been described in the developmental forms of *Trypanosoma cruzi*, *Trypanosoma brucei* and *Trypanosoma rangeli* that deal with the vector. Here, the available data from GP63 will be analyzed from the perspective of the interaction of trypanosomatids with the invertebrate host.

Abbreviations

EC	Enzyme class
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
GIPLs	Glycoinositolphospholipids
GPI	Glycosylphosphatidylinositol

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GPI-PLC	Glycosylphosphatidylinositol-phospholipase C
HIV	Human immunodeficiency virus
LPG	Lipophosphoglycan
MSP	Major surface peptidase
PARP	Procyclic acidic repetitive protein
PSP	Promastigote surface peptidase
VSG	Glycosylphosphatidylinositol-anchored variant surface protein

1 GP63 in *Leishmania,* Phytomonads and Monoxenic Trypanosomatids

In the mid-1980s, it was identified and characterized a protein in promastigotes of different species of the genera *Leishmania* (Fong and Chang 1982; Lepay et al. 1983; Bouvier et al. 1985; Etges et al. 1986) that presented immunological cross-reactivity with sera from hosts infected with *Leishmania* spp. This protein presents a molecular mass around 63 kDa, is a zinc metallopeptidase, accounts for about 1 % of the total proteins in promastigotes and is mainly glycosylphosphatidylinositol-(GPI)-anchored to the plasma membrane, while hydrophilic and secreted isoforms are also described. This protein was termed leishmanolysin, glycoprotein of 63 kDa (GP63), surface acid peptidase, promastigote surface peptidase (PSP) and lately as major surface peptidase (MSP). It belongs to the enzyme class EC 3.4.24.36 (clan MA, family M8 of endopeptidases), with several common characteristics with mammalian matrix metallopeptidases (Fig. 11.1). There are a number of excellent reviews on GP63 in *Leishmania* (Yao et al. 2003; Yao 2010; Olivier et al. 2012).

Since its discovery, due to its potential relevant functions during the life cycle of leishmania and its therapeutic potential, this molecule has been extensively investigated and a myriad of functions have been described for GP63 from *Leishmania* spp. in the mammalian host. The asserted functions of GP63 include: (i) evasion of complement-mediated lysis, (ii) facilitation of promastigotes phagocytosis by macrophages, (iii) migration through the extracellular matrix, (iv) inhibition of natural killer cellular functions, (v) resistance to antimicrobial peptide killing, (vi) degradation of macrophage and fibroblast cytosolic proteins with implications in cellular transduction signals, and (vii) promotion of survival of intracellular amastigotes. These functions were extensively reviewed and will not be further explored here (Yao et al. 2003; Yao 2010; Olivier et al. 2012).

Intriguingly, GP63 is predominantly expressed on the surface of promastigotes of *Leishmania* rather than in amastigotes, the former are the developmental forms found in the insect vector. In spite of this, little is known about the functions performed by this molecule in the phlebotomine sandfly. Up to now, there are few reports that tried to assess GP63 role in the invertebrate host; however, there is a huge amount of information that indirectly links GP63 to a function in this part of the life cycle of the parasites. Here, we will try to systemize this information. Particularly, monoxenic and plant trypanosomatids have contributed considerably

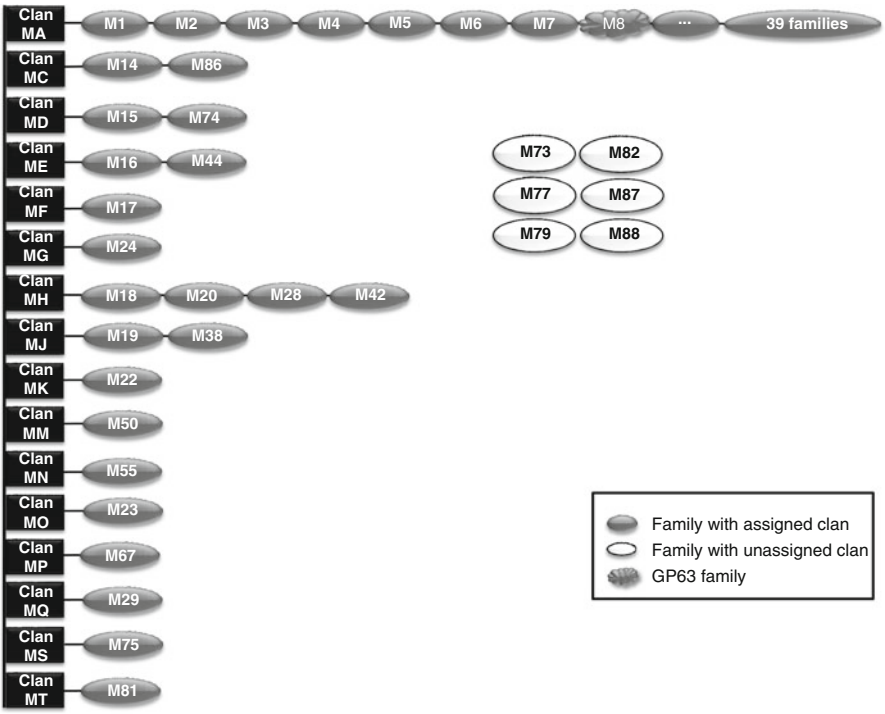

Fig. 11.1 Overview of the metallopeptidase clans and families according to the MEROPS Database (Rawlings et al. 2012). The clan (*black* boxes) contains enzymes that have arisen from a common evolutionary origin of peptidases and represents one or more families (*dark grey* circles) that show evidence of their evolutionary relationship. The white circles represent six families with unassigned clans. GP63 belongs to clan MA, family M8, as highlighted. This clan possesses 39 families, of which only eight are represented

for improving GP63 knowledge on the interaction with the insect. Monoxenic trypanosomatids are thought to develop its life cycle restricted to the invertebrate host presenting specific developmental forms, while plant trypanosomatids, the phytomonads, alternate between a plant and an insect host (Figs. 11.2 and 11.3). However, host specificity is not that stringent, as discussed below.

A pioneer work published by Etges in 1992 showed, in parasites of the monoxenic genera *Crithidia* and *Herpetomonas* (Fig. 11.3), the presence of a surface metallopeptidase with biochemical characteristics similar to GP63. One year later, Inverso et al. (1993) demonstrated that the parasite *Crithidia fasciculata* contains homologues of the leishmanial *GP63* genes, which are transcribed and contain potential GPI anchor addition sites. Later, Schneider and Glaser (1993) also demonstrated similarities between *Leishmania* GP63 and a surface metallopeptidase of *Herpetomonas samuelpessoai*, such as surface location, including a GPI anchor, inhibition by 1,10-phenanthroline and cleavage of a non-peptide substrate previously shown to be hydrolyzed only once by leishmanial GP63 (Bouvier et al. 1990).

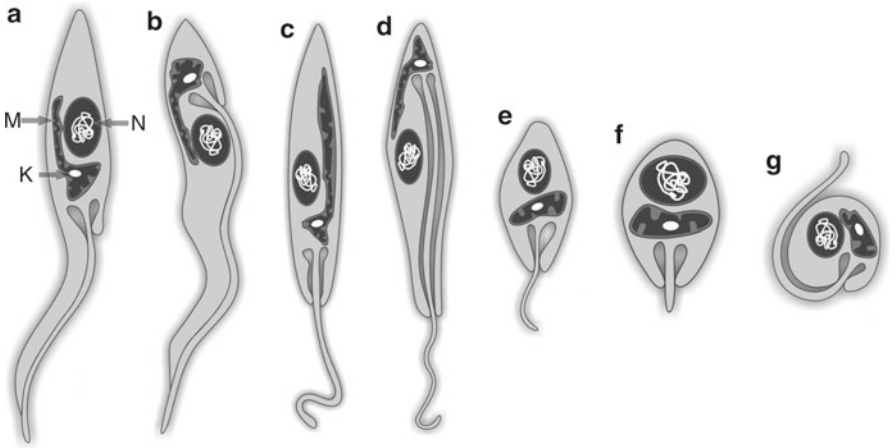

Fig. 11.2 Basic morphotypes of trypanosomatids that serve as genus-defining characteristics in the current taxonomy. Trypanosomatids forms are defined by cell shape, point of flagellum emergence and position of the kinetoplast (*K*) in relation to the nucleus (*N*). For epimastigote (**a**), promastigote (**c**), choanomastigote (**e**) and amastigote (**f**) morphotypes, the kinetoplast is located in an anterior position relative to the nucleus, while for trypomastigote (**b**) and opisthomastigote (**d**), the kinetoplast is located in a posterior position, and for spheromastigote (**g**), it is laterally located. Epimastigote and trypomastigote forms present an undulating membrane connecting to their flagellum, once the flagellum is attached to the cell membrane, while other forms have a free flagellum, with the exception of amastigote forms, which presents a very small flagellum. In all morphotypes, only one large mitochondrion (*M*) is observed, which is ramified through the cell body, being the region where the kinetoplast is located

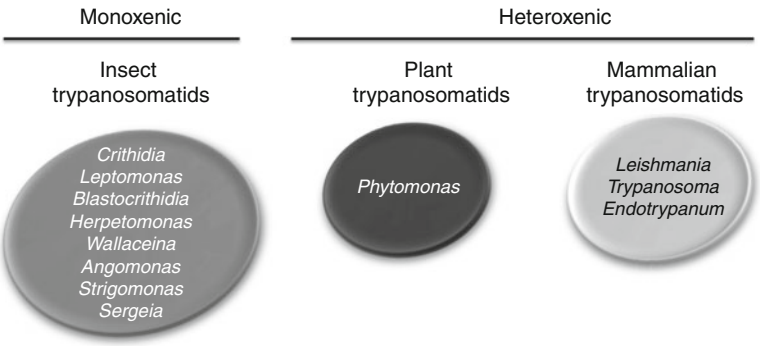

Fig. 11.3 The recognized genera in the Trypanosomatidae family. The development of monoxenic genera occurs in a single invertebrate host, although insect trypanosomatids can be found in plants and there are some reports that describe the presence of these trypanosomatids in mammalian hosts. The heteroxenic trypanosomatids alternate between an insect vector and a plant or mammalian host, and the latter are responsible for important human disease, such as leishmaniasis, Chagas disease and African trypanosomiasis

After these reports, it was generally accepted that GP63 should perform a function in the insect host, considering that this is the only shared part of the life cycle among *Leishmania* and these monoxenic trypanosomatids (Fig. 11.3). However, this hypothesis was tested only years later either in monoxenic trypanosomatids or in *Leishmania* spp.

Up to now, there are only two reports that tried to assess GP63 role in the interaction of *Leishmania* spp. with the invertebrate host. *L. major GP63* knockouts presented survival rates comparable to the wild type parasites when the anterior gut of *Phlebotomus dubosqui* was analyzed (Joshi et al. 2002). On the other hand, Hajmová et al. (2004) reported that the down-regulation of *GP63* in a *L. amazonensis* clone adversely affected its early development in the neotropical *Lutzomyia longipalpis* sand fly. The possibility exists that GP63 may function differently for these two distinct *Leishmania* species in their interactions with different invertebrate vector species, which could be correlated to vector competence (Santos et al. 2006). Also, studies using parasites with genetic alterations (knockout, down-regulation or RNA silencing, for instance) should be interpreted with caution, since the parasites can overexpress other molecules to overcome the function of the target gene. Indeed, another GPI-anchored molecule, lipophosphoglycan (LPG), is the dominant molecule on the surface of *Leishmania* spp. promastigotes, and overlaps functions with GP63, such as resistance to complement-mediated lysis and facilitation of phagocytosis by macrophages. One of the best characterized roles of LPG is in the interaction with the insect host, being responsible for parasite binding and release from the midgut of the sand fly vector, accounting for retrograde migration of the metacyclic promastigote to the sand fly proboscis (reviewed by de Assis et al. 2012). It is conceivable that GP63 and LPG, or even other molecules not considered yet, can act synergistically or redundantly, with the failure of one being compensated by the other (Yao 2010).

In monoxenic trypanosomatids and phytomonads (Fig. 11.3), our research group has contributed with a bulk of information either on the occurrence, distribution and biochemical characteristics of these molecules or on the function performed by GP63 homologues in these parasites (Table 11.1) (see Santos et al. 2006 for an extensive review). All the monoxenic trypanosomatids and the phytomonads examined up to now possess GP63 homologues with proteolytic activity (Table 11.1), as well as all *Leishmania* spp. assessed so far. It was somewhat surprising that no proteolytic activity was reported in *Leishmania tarentolae* by means of gelatin zymography (Raymond et al. 2012). Details on the methodological approach used to assess proteolytic activity were not described; nevertheless proteolytic activity was detected in *L. mexicana*, *L. major*, *L. donovani*, *L. infantum* and *L. amazonensis*, which ensures methodological accuracy. Interestingly, in the lizard parasite *L. tarentolae*, *GP63* is highly expanded with 49 putative genes as compared to 29 in *L. braziliensis*, 7 in *L. infantum* and 5 in *L. major* (Raymond et al. 2012). Since no metallopeptidase activity was detected in *L. tarentolae*, the authors hypothesized that high sequence variability of the *L. tarentolae GP63* genes may affect GP63 peptidase activity (Raymond et al. 2012). In spite of this, it should be pointed out that several factors influence peptidase detection through zymography

Table 11.1 Presence of GP63 homologues in monoxenic and plant trypanosomatids

Trypanosomatids	Molecular mass of the gp63 homologue protein(s)[a]	Presence of a GPI anchor[b]	Cell-associated metallopeptidase activity(ies)[d]	Role in invertebrate adhesion	Reference(s)
Strigomonas culicis	50 (c, s)	+	50, 55, 76	Not determined	d'Avila-Levy et al. 2005
Angomonas deanei	62 (c, s), 67 (c)	+	51, 58	*Aedes aegypti* (gut)	d'Avila-Levy et al. 2003, 2006a, 2008
Crithidia fasciculata	54 (s)[c]	+ [c]	59, 63	Not determined	Etges 1992, Inverso et al. 1993, Branquinha et al. 1996
Crithidia guilhermei	62 (c, s), 67 (c)	Not determined	57, 61	*Aedes aegypti* (gut)	Branquinha et al. 1996, Nogueira de Melo et al. 2001, d'Avila-Levy et al. 2006a
Crithidia lucilae	75 (s), 97 (s), >100 (s)	Not determined	55, 61	Not determined	Branquinha et al. 1996, Jaffe and Dwyer 2003
Herpetomonas megaseliae	52 (c, s), 60 (c), 63 (c)	+	50, 60, 70	*Aedes aegypti* (gut) and *Megaselia scalaris* (gut)	Nogueira de Melo et al. 2006
Herpetomonas samuelpessoai	63 (c, s)	+	55–66	*Aedes aegypti* (gut)	Schneider and Glaser 1993, Branquinha et al. 1996, Santos et al. 2003, Elias et al. 2006, Pereira et al. 2010b

Leptomonas colossoma	52 (c, s), 63 (c, s)	Not determined	not detected	*Aedes albopictus* (insect cell line)	Pereira et al. 2009
Leptomonas samueli	52 (c, s), 63 (c, s)	Not determined	60, 70	*Aedes albopictus* (insect cell line)	Pereira et al. 2009
Leptomonas wallacei	52 (c, s), 63 (c, s)	Not determined	55	*Aedes albopictus* (insect cell line)	Pereira et al. 2009
Leptomonas seymouri	97 (s)	Not determined	65, 70, 78, 80	not determined	Jaffe and Dwyer 2003, Santos et al. 2005
Phytomonas françai	62 (c), 67 (c, s)	Not determined	Not determined	*Aedes aegypti* (gut)	Almeida et al. 2003, d'Avila-Levy et al. 2006a
Phytomonas serpens	52 (c), 60 (c, s), 63 (c)	+	Not detected	*Oncopeltus fasciatus* (salivary gland)	Vermelho et al. 2003, d'Avila-Levy et al. 2006b, Santos et al. 2007

[a] The molecular masses of the GP63 homologues (in kDa) were determined by comparison with protein standards after SDS-PAGE and Western blotting using anti-gp63 polyclonal antibodies. The letters (c) and (s) refer to cell-associated and secretory polypeptides, respectively

[b] Presence of a GPI anchor (+) was assessed by treating parasites or purified polypeptides with phospholipase C and probing the polypeptides with anti-CRD antibody

[c] Gene with high homology to the gp63 was cloned and sequenced in *C. fasciculata*, the gene contains a potential site for addition of a glycosylphosphatidylinositol anchor

[d] The numbers indicate the relative molecular masses in kilodaltons of metallopeptidases detected by gelatin-SDS-PAGE

(d'avila-Levy et al. 2012), and further efforts must be done in order to completely exclude GP63 activity in *L. tarentolae*, which was isolated from the lizard *Tarentola mauritanica* (Elwasila 1988), and is probably the most widely studied *Leishmania* (*Sauroleishmania*) species. In lizards, the parasites live predominantly as promastigotes in the lumen of the cloacae and intestine or in the bloodstream (Wilson and Southern 1979).

Collectively, the occurrence of GP63 homologues with proteolytic activity in all monoxenic trypanosomatids and phytomonads analyzed so far (Table 11.1) reinforced the hypothesis previously raised on GP63 function in the insect vector. However, it must be pointed out that there is growing evidence that presumed monoxenic trypanosomatids are associated with diffuse cutaneous infection in patients infected with the human immunodeficiency virus (HIV). These patients developed a diffuse "leishmanias is-like" syndrome with numerous amastigotes in the skin nodules, indeed even immunocompetent persons can be infected with monoxenic trypanosomatids (for a comprehensive review, see Chicharro and Alvar 2003). Corroborating reports with patients, it has been shown *in vitro* the ability of several monoxenic trypanosomatids to invade and survive in either phagocytic or non-phagocytic mammalian host cells (Matteoli et al. 2009; Pereira et al. 2010a). Indeed, GP63 plays a crucial role in this process (Matteoli et al. 2009; Pereira et al. 2010a). Taking it into consideration, the assumption of GP63 function based on the intersection of the life cycle between monoxenic trypanosomatids and leishmaniais a matter of speculation, which requires experimental support. It can simply represent an evolutionary vestige.

Nevertheless, one function conserved among various trypanosomatids appears to be nutrient acquisition in the gut of various insects. GP63 has been found wherever it has been searched: all *Leishmania* species, monoxenic trypanosomatids, phytomonads, as well as *Trypanosoma brucei*, *Trypanosoma cruzi* and *Trypanosoma rangeli* (Table 11.1) (Bangs et al. 1997; El-Sayed and Donelson 1997; Grandgenett et al. 2000; Bangs et al. 2001; Cuevas et al. 2003; LaCount et al. 2003; Santos et al. 2006; Grandgenett et al. 2007; Kulkarni et al. 2009; Ferreira et al. 2010; Yao 2010). GP63 is an endopeptidase with a wide substrate specificity and optimum pH, therefore it can fulfill a nutritional role for the parasite. However, since GP63 does not act as an exopeptidase, the coordinated action of other enzymes is necessary to provide free amino acids from the peptides generated by GP63 hydrolysis of proteins (Fig. 11.4). Alternatively, it could act in the degradation of proteinaceous components in the insect providing room for parasite access to cellular receptors in the insect gut epithelium, or protection against the insect defenses (Fig. 11.4). Afterwards, each parasite would continue its life cycle, which can involve parasite binding and replication, parasite invasion to gain access to the hemocoel, or parasite migration to the salivary gland. Alternatively, peptidases can also act as adhesins or ligands. Indeed, GP63 action as a ligand on the interaction with macrophages has been reported: it contains the sequence SRYD that is antigenically related to the RGDS sequence of fibronectin, suggesting a potential interaction of GP63 with macrophage fibronectin receptors (Soteriadou et al. 1992) (Fig. 11.4).

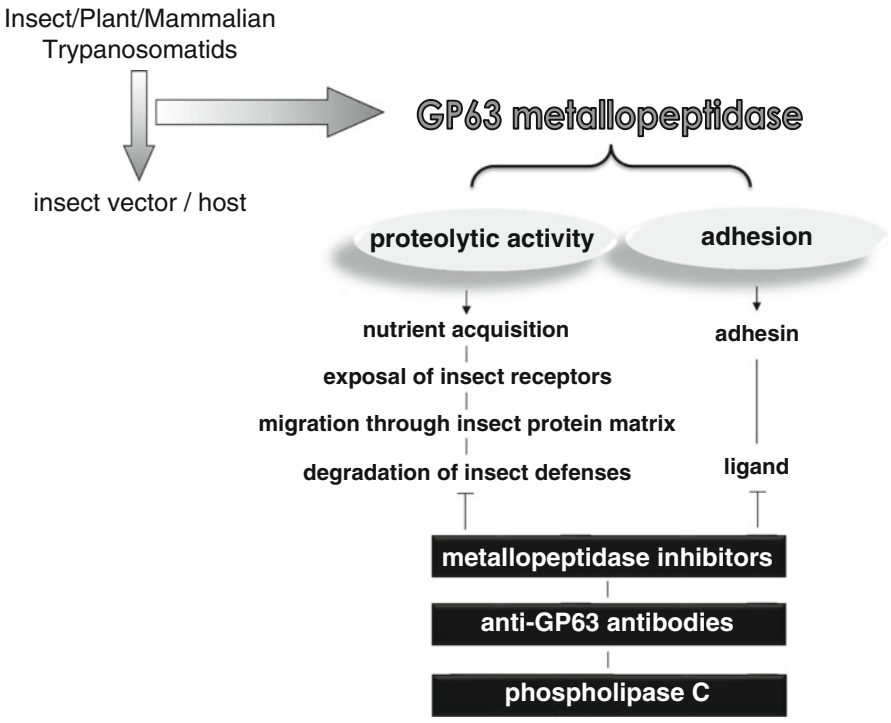

Fig. 11.4 Possible roles played by GP63 metallopeptidase produced by insect, plant and mammalian trypanosomatids (Table 11.1 and Fig. 11.1). The interplay between trypanosomatids and the insect vector or host can be mediated through GP63 molecules, which can act either as a peptidase or in the adhesion. Consequently, metallopeptidase inhibitors, anti-GP63 antibodies or phospholipase C can be used to block one or several of these fundamental events, which help to assess GP63 function

Keeping in mind this last possibility, our research group started to investigate GP63 actual involvement in the interaction of monoxenic trypanosomatids and phytomonads with the insect host (Fig. 11.3). For this purpose, we used *Aedes aegypti* as an experimental model for studies regarding trypanosomatids-insects interaction. As a matter of fact, *Strigomonas culicis* (previously known as *Blastocrithidia culicis*, Teixeira et al. 2011) and *Herpetomonas samuelpessoai* can successfully colonize *A. aegypti* gut (Corrêa-da-Silva et al. 2006; Pereira et al. 2010b). Also, *Herpetomonas* sp. is found repeatedly in this insect (Weinman and Cheong 1978), which validates this insect as a suitable model. At first, we have pre-treated parasites with metallopeptidase inhibitors or anti-GP63 antibodies. Both treatments provoked a pronounced effect on the binding ability of several trypanosomatids to the insect gut *in vitro* (Table 11.1). Phospholipase C (PLC) treatment of the parasites also caused a drastic reduction on parasite binding, reinforcing the participation of GPI-anchored molecules (Table 11.1). Finally, the pre-treatment of the dissected guts with purified GP63 from *Angomonas deanei* (previously known

as *Crithidia deanei*, Teixeira et al. 2011) also induced a marked decrease in parasite adhesion in a dose-dependent manner, which strongly suggested the saturation of insect gut cell receptors by the GP63-like polypeptide (d'Avila-Levy et al. 2006a). This effect is independent of GP63 proteolytic activity or its tertiary structure, since similar inhibitory patterns were observed among proteolytically active GP63, heat-inactivated enzyme, or the apoenzyme, which maintains the tertiary structure but lacks enzymatic activity due to ions chelation (d'Avila-Levy et al. 2006a). The possibility of the presence of an insect gut receptor for GP63 was further supported by the demonstration that a yet unidentified 50 kDa protein on the *A. aegypti* gut extract can directly bind GP63 molecules (d'Avila-Levy et al. 2006a).

Another interesting insect to study trypanosomatids interaction, particularly the phytomonads, is *Oncopeltus fasciatus*. The hemipteran *O. fasciatus* is the natural and experimental host for several species of trypanosomatids, including *Phytomonas* spp. (Dias et al. 2012). In the biological cycle of *Phytomonas*, the parasites are ingested when a phytophagous insect feeds on an infected plant, then the flagellates invade the intestinal epithelium and reach the hemolymph. After travelling throughout the hemocoel, the protozoa reach the external face of the salivary glands, where they bind and invade, and finally gaining access to the salivary gland lumen. When the infected insect feeds on another plant, the flagellates are then transmitted via saliva (for an extensive review on phytomonads, see Camargo 1999). In the tomato parasite *Phytomonas serpens* GP63 molecules are also implicated in the interaction of these parasites with *O. fasciatus* salivary glands (d'Avila-Levy et al. 2006b). Also, cruzipain homologues in *P. serpens* are relevant for the interaction of this phytomonad with the insect salivary gland, illustrating the multifaceted molecular interactions that may occur (Santos et al. 2006).

One may argue that interaction studies employing insect models instead of the natural hosts of the trypanosomatids provide meaningless data, because they represent an artificial situation induced in laboratory, which have only marginal correlation and cannot be extrapolated to what happens in nature. It is an interesting and critical point to be considered when interpreting results employing insect host models. However, experimental studies with insects deal with the difficulties in establishing a successful laboratory insect colony or the difficulties in obtaining insect field samples. Therefore, this critical point should not hamper efforts to study the intricate and sophisticate interplay between trypanosomatids and insects. In addition, the presence of trypanosomatids in Hemiptera is considered conspicuous, since the literature data of the flagellate-harboring hemipteran species are not the product of a systematic study on the prevalence of flagellates in insects, but the result of incidental observations by various researchers in diverse circumstances (Camargo 1999). The bulk of information that our research group has provided on GP63 participation on the insect host interaction has been obtained using insect host models. However, we have validated one of the insect models employed so far, by showing that GP63-like proteins play a role in the attachment of *Herpetomonas megaseliae* to the gut of its original host, *Megaselia scalaris* (Nogueira de Melo et al. 2006). Curiously, *H. megaseliae* showed an enhanced binding ability to *A. aegypti*, in comparison to its original host (Nogueira de Melo et al. 2006), which reinforces the observation made by Camargo (1999) that there is no

systematic study to determine the exact prevalence and distribution of monoxenic trypanosomatids in the insects. Actually, we call *M. scalaris* the original host of *H. megaseliae* and not the natural host, exactly because it is not known which hemipteran species would frequently present this trypanosomatid.

2 GP63 in *T. cruzi*, *T. brucei* and *T. rangeli*

GP63 homologues have also been described in *T. cruzi*, *T. brucei* and *T. rangeli* (Bangs et al. 1997; El-Sayed and Donelson 1997; Grandgenett et al. 2000; Bangs et al. 2001; Cuevas et al. 2003; LaCount et al. 2003; Grandgenett et al. 2007; Kulkarni et al. 2009; Ferreira et al. 2010). *T. cruzi* possesses a complex proteolytic pattern that displays different peptidases belonging to virtually all peptidases clans, which were either biochemically characterized or identified in the genome (see Alvarez et al. 2012 for an excellent updated review on *T. cruzi* peptidases). Genes encoding GP63 have been extensively amplified in the *T. cruzi* genome (more than 420 genes and pseudogenes) when compared to *T. brucei* and *Leishmania infantum* (13 and 7, respectively). The biological meaning of the distinct repertoire of genes is still unknown. However, it is interesting to note that there is an indirect correlation between gene expansion and proteolytic activity. For instance, *T. cruzi* is by far the protozoan with the largest *GP63* gene expansion, while metallopeptidase activity is fairly low. On the contrary, *Leishmania* spp. presents abundant detectable GP63 activity and a low number of *GP63* genes.

Although metallopeptidase is not the most abundant and readily detected activity in *T. cruzi,* metallopeptidases were detected biochemically, and presented a distinct pattern during metacyclogenesis (Bonaldo et al. 1991; Lowndes et al. 1996). In the recent review by Alvarez et al. (2012), the authors depicted that out of the 425 *GP63* genes in *T. cruzi*, 251 are pseudogenes; out of the 174 remaining true genes, there is proteomic evidence for the expression of 29, and mRNAs corresponding to 31 genes have been identified in the TritrypDataBase, but there is very little overlapping with the proteins previously identified by mass spectrometry (Alvarez et al. 2012). The high heterogeneity of *T. cruzi GP63* genes creates a challenge to the study of this gene family. The first challenge in studying multigenic families is to classify the genes under study. Cuevas et al. (2003) analyzed *T. cruzi* genome and identified several groups of genes that belong to the *GP63* family, with multiple members in each. In this sense, *GP63* genes were grouped in *Tcgp63-I* and *II*, which are present as high-copy-number genes, as well as *Tcgp63-III*, which are pseudogenes (Cuevas et al. 2003). At least four *GP63* mRNA, which belong to *Tcgp63-I*, are developmentally regulated in the different parasite stages, being more abundant in amastigotes than in epimastigotes or trypomastigotes (Grandgenett et al. 2000). Although *Tcgp63-II* genes are more abundant in *T. cruzi* genome than *Tcgp63-I*, only the latter is detectable at protein level and presents expressive mRNA levels in all developmental stages of *T. cruzi* CL Brener strain. Tcgp63-I is a proteolytically active enzyme bound to the membrane by a GPI anchor and with a possible role in the

infection of host cells, since antibodies raised against a synthetic peptide derived from Tcgp63-I sequence partially blocked invasion of Vero cells by trypomastigotes (Cuevas et al. 2003).

Later, a distinct research group produced a polyclonal antiserum against a recombinant Tcgp63. This antibody reacted with a 55-kDa protein present in metacyclic trypomastigotes, and a 61-kDa form, present at epimastigotes, amastigotes and culture-derived trypomastigotes (Kulkarni et al. 2009). It seems that this antiserum recognizes a distinct subset of *T. cruzi* GP63, since Cuevas et al. (2003) described reactive bands of 75 and 78 kDa in epimastigotes, 75 kDa in amastigotes, while in metacyclic trypomastigotes only the 78-kDa band appeared (Cuevas et al. 2003). The treatment of trypomastigotes with this polyclonal antiserum also reduced the infection of myoblasts (Kulkarni et al. 2009). Competition experiments of pre-incubation of myoblasts with a 29-kDa TcGP63 fragment also revealed a marked decrease in infection, suggesting that the 29-kDa C-terminal fragment possesses a binding site(s) for the surface of host cells (Kulkarni et al. 2009).

Recently, Ma et al. (2011) reanalyzed *GP63* genes and classified *T. cruzi* GP63 peptidases into four groups according to sequence features. The authors also compared the similarity between trypanosomatids GP63 and vectors (insects) and hosts (human and rodents), and found that GP63 from trypanosomatids are more similar to those of their vectors (Ma et al. 2011). The possible role of Tcgp63 on the interaction of *T. cruzi* with the midgut of triatomines has still not been assessed, but it is an open possibility, due to its surface location and expression in epimastigotes, as well as metacyclic trypomastigotes. In this sense, our research group assayed the effect of metallopeptidase inhibitors as well as the effect of the antibody raised by Cuevas et al. (2003), and both treatments considerably reduced the adhesion of *T. cruzi* to *R. prolixus* explanted midguts (Uehara et al. unpublished data). In *T. cruzi*, one of the best studied and abundant molecule is cruzipain. This cysteine peptidase is expressed in large amounts on the *T. cruzi* surface of epimastigotes, and recently, our research group demonstrated its participation in *T. cruzi* interaction with the insect vector (Uehara et al. 2012). Also, glycoinositolphospholipids (GIPLs) molecules, GP72 and calpains are involved in *T. cruzi* interaction with the invertebrate host (De Jesus et al. 1993; Basombrío et al. 2002; Nogueira et al. 2007; Ennes-Vidal et al. 2011). The molecular interactions that take place in the insect midgut that allows *T. cruzi* epimastigote binding and multiplication with further migration to the rectum and metacyclogenesisis certainly dependent on multiple molecules, both in the vector and on the parasite. The study of isolated molecules is insufficient to fully elucidate the functional impact of the complex structures that can be formed and are upon influence of the microenvironment of the insect midgut (Uehara et al. 2012).

Another protozoan parasite that harbors *GP63* genes is *T. brucei*, the causative agent of African sleeping sickness in humans, which is transmitted by the bite of infected tsetse flies. This protozoan parasite alternates between the bloodstream of mammalian hosts and the insect vector and are exclusively extracellular throughout the life cycle. When a tsetse fly ingests a trypanosome-infected blood meal, both slender and stumpy bloodstream forms of the parasites enter the tsetse midgut. Although subject to some controversy (Vickerman 1965), it is generally thought

that stumpy forms are pre-adapted to life in the tsetse midgut, and only they differentiate efficiently to the tsetse midgut procyclic form, which develops in tsetse gut and migrates to the salivary glands, being transmitted to the mammalian host during another blood meal, where they differentiate into the bloodstream form (Bass and Wang 1991; MacGregor and Matthews 2010). The surface of the bloodstream forms from *T. brucei* is covered by the abundant GPI-anchored variant surface protein (VSG). During differentiation of bloodstream forms to the procyclic forms, the VSG is replaced by another glycoprotein, designated procyclic acidic repetitive protein (PARP) or procyclin (Gruszynski et al. 2006). The first report that a peptidase mediates *T. brucei* VSG release from bloodstream forms during transformation into procyclic forms was done by Ziegelbauer et al. (1993). Later, it was demonstrated that this peptidase is a zinc-metallopeptidase (Bangs et al. 1997), which is present throughout the procyclic stage. In this sense, besides acting in VSG release, it could also provide some essential function related to the survival of the parasite in the midgut of the tsetse (Bangs et al. 1997). In the same year, El-Sayed and Donelson showed for the first time the presence of genes homologues to the leishmanial *GP63* in *T. brucei*. These genes were shown to be transcribed equally in procyclic and bloodstream trypanosomes, but their mRNAs accumulate to a 50-fold higher level in bloodstream trypanosomes (El-Sayed and Donelson 1997). The predominant expression of GP63 in bloodstream forms leads the authors to hypothesize that it could act protecting bloodstream trypanosomes against complement-mediated lysis (El-Sayed and Donelson 1997). However, no protein characterization was performed, nor these genes were correlated with the metallopeptidase activity previously described. Later, Bangs et al. (2001) tried to reinforce the idea that a metallopeptidase was responsible for VSG release, in this sense they assayed a panel of metallopeptidase inhibitors, including some used to treat human diseases. The authors showed the anti-proliferative effect of several compounds together with their ability to inhibit VSG release; these compounds were also able to inhibit purified leishmanial GP63 (Bangs et al. 2001). Although no direct evidence has been demonstrated, these data strongly suggested that a metallopeptidase is responsible for VSG release, although a correlation with the GP63 homologues was still requiring confirmation.

Finally, in 2003, *T. brucei GP63* homologues were well characterized, they belong to at least three gene families (*TbMSP-A*, *–B*,and *–C*) based on their different untranslated regions (UTRs) and their differential expression during the *T. brucei* life cycle. All the gene families contain approximately 30 % amino acid identity with the leishmanial GP63. Bloodstream forms have mRNAs from all three gene families, whereas procyclic cells have detectable mRNA only from *TbMSP-B* (LaCount et al. 2003). RNA interference of *TbMSP-B* revealed that this molecule can function to release the transgenic VSG from the surface of procyclic trypanosomes. Thus, TbMSP-B imparts a protein-processing function to the surface of African trypanosomes (LaCount et al. 2003). No effect on the kinetics of cultured trypanosome growth in either procyclic or bloodstream stages were observed (LaCount et al. 2003). VSGs are actively released by two modes: GPI hydrolysis and proteolysis. TbMSP-B is present in both bloodstream and procyclic form and together with phospholipase

C (GPI-PLC) acts in removal (60 %) of the VSG during differentiation from bloodstream to procyclic form, as demonstrated by gene deletion experiments (Grandgenett et al. 2007). When double mutant bloodstream trypomastigotes were generated (*TbMSPB-/– PLC-/-*), these cells were incapable of differentiation, and were defective to remove most of cell surface VSGs (Grandgenett et al. 2007). Interestingly, when *TbMSPB-/–* or *PLC-/-* were individually generated, parasites could still differentiate and release part of the VSG (Grandgenett et al. 2007). Thus, GPI-PLC and TbMSP-B act synergistically in VSG release during *T. brucei* differentiation. Accordingly, *TbMSP-B* and *GPI-PLC* expression are coordinately and inversely regulated, most likely under the negative control of labile *trans*-acting factors (Gruszynski et al. 2006). TbMSP-B is a surface-localized zinc metallopeptidase that is expressed predominantly in differentiating bloodstream-into-procyclic forms and in established procyclic cells (Grandgenett et al. 2007). TbMSP-B expression pattern and cellular localization together with the change in the surface coat of these differentiating cells (from a peptidase-sensitive VSG to a peptidase-resistant procyclin) is consistent with TbMSP-B involvement in VSG loss during differentiation. TbMSP-B maintenance in procyclics may serve for some other important function, perhaps to ensure that deleterious effects of any lingering or leaky VSG transcription are squelched at the protein level (LaCount et al. 2003); alternatively, TbMSP-B could act in the surface of proliferating procyclics for physiological purposes in the fly midgut (Bangs et al. 2001). This last hypothesis has never been explored.

In spite of the data on GP63 functional role in *T. brucei*; the enzyme has never been isolated and biochemically characterized. Recently, a peptidase sensitive to metallopeptidase inhibitors (1,10-phenanthroline, EDTA, EGTA) with neutral optimum pH and capable of degrading casein and gelatin was isolated from crude bloodstream forms from *T. brucei*. The peptidase presents a molecular mass of approximately 40 kDa, but unfortunately the enzyme identity was not further explored, and cannot be directly linked to any of the gene families previously described (de Sousa et al. 2010).

In *T. rangeli*, 13 sequences were identified corresponding to *GP63* genes described in other trypanosomatids (Ferreira et al. 2010). Some of the *T. rangeli GP63* identified present the HE*XX*H motif, which is characteristic of the catalytic site of metallopeptidases; the presence of two histidines and one glutamic acid residues is conserved among all trypanosomatid *GP63* sequences studied and are essential for proteolytic activity (McGwire and Chang 1996). *GP63* transcripts were detected in the epimastigote forms of *T. rangeli*, which opens new possibilities to study the function of this gene family in a nonpathogenic trypanosome (Ferreira et al. 2010), including in the interaction of *T. rangeli* with its insect host.

3 Concluding Remarks

GP63 homologues have been observed in all monoxenic trypanosomatids examined to date, as well as in the phytomonads and heteroxenic mammalian parasites – *T. cruzi*, *T. brucei* and *T. rangeli*. The identification of GP63 homologues among

trypanosomatids with different life cycles may help to improve the knowledge on GP63 function and evolution. Two interesting questions arise from the critical interpretation of the data available: (1) is there an indirect correlation between gene expansion and proteolytic activity, as data from *T. cruzi* and *L. tarentolae* suggest? and (2) is the ubiquitous presence of GP63 on the surface of the trypanosomatids developmental forms that face the invertebrate host environment an evolutionary vestige or does GP63 play a role in this interface? There is substantial data suggesting that GP63 homologues found in monoxenic trypanosomatids and phytomonads play essential roles in the parasite nutrition through degradation of gut content, as well as in the binding to the insect epithelial cells (Fig. 11.4). In *Leishmania*, the actual role of GP63 on the interaction with the vector is still a matter of controversy, while in *T. cruzi* only preliminary data is available suggesting the participation of GP63 in *T. cruzi* binding to *R. prolixus* gut. In *T. brucei* and *T. rangeli*, GP63 possible role in the insect interaction has never been explored. Certainly, in the forthcoming years, more data generated by distinct research groups will fulfill these gaps on GP63 knowledge.

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Chapter 12
Highlights on Trypanosomatid Aminoacyl-tRNA Synthesis

Carla Polycarpo

Abstract Aminoacyl-tRNA synthetases aaRSs are responsible for the aminoacylation of tRNAs in the first step of protein synthesis. They comprise a group of enzymes that catalyze the formation of each possible aminoacyl-tRNA necessary for messenger RNA decoding in a cell. These enzymes have been divided into two classes according to structural features of their active sites and, although each class shares a common active site core, they present an assorted array of appended domains that makes them sufficiently diverse among the different living organisms. Here we will explore what is known about the diversity encountered among trypanosomatids' aaRSs that has helped us not only to understand better the biology of these parasites but can be used rationally for the design of drugs against these protozoa.

Abbreviations

A	Adenine
aa-AMP	aaRS, 4, 5 aminoacyl-adenylate complexed with aminoacyl-tRNA synthetase
aaRS	Aminoacyl-tRNA synthetase
aa-tRNA	Aminoacyl-tRNA
AC1	Anticodon binding motif

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AC2	Anticodon binding motif
AIDQ	TrpRS and TyrRS motif present in the catalytic site (alanine, isoleucine, aspartate, glutamine)
AlaRS	Alanyl-tRNA synthetase
AMP	Adenosine-5′-monophosphate
Arg	Arginine
ArgRS	Arginyl-tRNA synthetase
Asn	Asparagine
AsnRS	Asparaginyl-tRNA synthetase
Asp	Aspartate
AspRS	Aspartyl-tRNA synthetase
ATP	Adenosine-5′-triphosphate
AUA	Isoleucine codon
AUG	Methionine codon
C	Cytosine
CPK	Coloring convention designed by Robert Corey and Linus Pauling, and improved by Walter Koltun.
Cys	Cysteine
CysRS	Cysteinyl-tRNA synthetase
E	Glutamate
E. coli	*Escherichia coli*
eEFSec	Selenocysteine elongation factor
EMAP II-like domain	Endothelial monocyte-activating polypeptide II-like domain
ESE	Eukaryotic specific extension present in Tryptophanyl-tRNA synthetases
fmet	Formylmethionine
FTHF	N10-formyltetrahydrofolate
G	Guanine
GatCAB	tRNA dependent amidotransferase of Archaea, bacteria and eukaryotic organelles
GatDE	Archaeal specific tRNA dependent amidotransferase
GatFAB	Yeast specific tRNA dependent amidotransferase
GLDQ	Trypanosomatid TyrRS motif present in the catalytic site (glycine, leucine, aspartate, glutamine)
Gln	Glutamine
GlnRS	Glutaminyl-tRNA synthetase
Glu	Glutamate
GluRS	Glutamyl-tRNA synthetase
Gly	Glycine
GlyRS	Glycyl-tRNA synthetase
GTP	Guanosine-5′-triphosphate
GXDQ	TrpRS motif present in bacteria (glycine, any amino acid, aspartate, glutamine)
H	Histidine
HAM	Histidyl-adenosinemonophosphate

HARS1	Human histidyl-tRNA synthetase 1
HARS2	Human histidyl-tRNA synthetase 2
HIAQ	Motif present in the active site of the trypanosomal tyrosyl-tRNA synthetase in place of motif
HIGH	(Histidine, isoleucine, alanine, glutamine)
HIGH	Motif presents in the active site of aaRS class I (histidine, isoleucine, glycine, histidine)
His	Histidine
HisRS	Histidyl-tRNA synthetase
Ile	Isoleucyl
IleRS	Isoleucyl-tRNA synthetase
K_{cat}	Catalytic constant
K_i	Dissociation constant for inhibitor binding
KISKS	Motif present in the active site of trypanosomatid methyl-tRNA synthetase (lysine, isoleucine, serine, lysine, serine)
K_m	Michaelis Menten constant
KMSKS	Motif present in the active site of aminoacyl-tRNA synthetases class I (lysine, methionine, serine, lysine, serine)
L30	Ribosomal protein
Leu	Leucine
LeuRS	Leucyl-tRNA synthetase
Lys	Lysine
LysRS	Lysyl-tRNA synthetase
MAMP	Methionyl adenylate
Met	Methionine
MetRS	Methionyl-tRNA synthetase
Mg^{+2}	Magnesium ion
mRNA	Messenger ribonucleic acid
MTF	Methionyl-tRNA formyltransferase
PDB	Protein data bank
Phe	Phenylalanine
PheRS	Phenylalanyl-tRNA synthetase
PLP	Pyridoxal phosphate
PP_i	Pyrophosphate
Pro	Proline
ProRS	Prolyl-tRNA synthetase
PSTK	O-phosphoseryl-tRNA kinase
Pyl	Pyrrolysine
PylRS	Pyrrolysyl-tRNA synthetase
SBP2	SECIS binding protein
SCF	Stem contact fold
Sec	Selenocysteine
SECIS	22, 23, 26, 27, 31, 40
Sel1	Kinetoplastid specific selenoprotein
selA	Selenocysteine synthase (bacterial)

SelK	Selenoprotein K
SelT	Selenoprotein T
SelTryp	Kinetoplastid specific selenoprotein
Sep	Phosphoserine
SepRS	Phosphoseryl-tRNA synthetase
SepSecS	O-phospho-L-seryl-tRNASec: L-selenocysteinyl-tRNA synthase
Ser	Serine
SerRS	Seryl-tRNA synthetase
SPS2	Selenophosphate synthase
ThrRS	Threonyl-tRNA synthetase
Tpr	Tryptophan
tRNA	Transfer ribonucleic acid
$tRNA^{fMet}$	Formylmethionine tRNA isoacceptor
$tRNA^{Met\text{-}e}$	Methionine elongator tRNA isoacceptor
$tRNA^{met\text{-}i}$	Methionine initiator tRNA isoacceptor
TrpRS	Tryptophanyl-tRNA synthetase
Tyr	Tyrosine
TyrRS	Tyrosine tRNA-synthetase
U	Uracyl
UAA	Termination codon (uracyl, adenine, adenine)
UAG	Termination codon (uracyl, adenine, guanine)
UGA	Termination codon (uracyl, guanine, adenine)
V	Valine
Val	Valine
ValRS	Valy-tRNA synthetase
W	Tryptophan
WHEP	Helix-turn-helix domain found in some aminoacyl-tRNA synthetasese
Ψ	Pseudouridine

1 Introduction

The faithful translation of the genetic code contained in the messenger RNA (mRNA) is usually described in three main steps: Initiation, Elongation and Termination. The aminoacylation of the transfer RNA (tRNA) with the correct amino acid, which results in an aminoacylated tRNA, the main substrate used by the ribosome, while not considered part of the translation cycle, is an integral and often overlooked step in the overall process of translation. A full complement of aminoacyl-tRNAs (aa-tRNAs) is necessary to translate all the existing codons in an mRNA molecule and the aminoacyl-tRNA synthetases (aaRSs) are the enzymes responsible for producing aa-tRNAs. For each natural amino acid cotranslationally inserted into a polypeptide chain there is at least one specific aaRS able to single out the correct pair of molecules among a set of different tRNA isoacceptors and amino acids. The overall error rate in translation is approximately 10^{-4}, indicating that

protein synthesis is accomplished through a high-fidelity process, necessary for the cell's life maintenance (Loftfield and Vanderjagt 1972; Ibba and Soll 1999; Crain et al. 2002; Charrière et al. 2006; Bruske et al. 2009). Although previously believed that in all organisms there were 20 aaRSs (one for each amino acid known at the time) and only one route for tRNA aminoacylation, we now know it is not the case, and indirect pathways of aa-tRNA formation as well as new amino acids have been described (Hao et al. 2002; Sheppard et al. 2008; Merritt et al. 2011). Nevertheless, in the eukaryotic nucleus there are at least 20 of these enzymes (encoded by one or more genes) and the tRNAs are charged through a direct route, with the exception of the selenocysteinyl-$tRNA^{Sec}$. In some mitochondria there is another exception to this rule, as is the case of glutaminyl-$tRNA^{Gln}$ synthesis.

The need to develop new drugs against trypanosomatids is well known, as drug-resistance is becoming a real challenge for disease control (Yaremchuk et al. 2002; Tsunoda et al. 2007; Fidalgo and Gille 2011). Considering that some aaRSs have already been studied in these organisms, the findings of these studies can be invaluable in assisting us in the search for specific inhibitors for trypanosomatids, particularly in those cases where the aminoacylation pathway is distinct from that of the host. In trypanosomatids there are two places where tRNA aminoacylation occurs, the nucleus and the mitochondria; aaRSs, amino acids and tRNAs should all be available in both compartments for translation to begin. One of the oddities in these organisms is that they have lost all tRNA genes in their mitochondria and both aaRSs and tRNAs have to be imported into the mitochondria (Simpson et al. 1989; Hancock and Hajduk 1990; Schneider and Maréchal-Drouard 2000). Thus, not only the structural diversity of aaRSs can be explored in trypanosomatids, but the aminoacyl-tRNA synthesis system as whole.

In this chapter, we will explore the aminoacyl-tRNA synthesis in trypanosomatids. Unique aspects of aminoacylation in these organisms, that may be relevant for directed drug discovery, will be highlighted. We shall look specifically to the enzymes for which scientific publications are available up to now.

2 Aminoacyl-tRNA Synthetases

For the 20 universal amino acids cotranslationally inserted into polypeptide chains, 21 aaRSs have been described, as a functional duplication of the Lysyl-tRNA synthetase (LysRS) is encountered (Perona and Hou 2007; Arnez and Moras 2009). A 22nd aaRS is present in some methanogenic organisms as the means to get cysteine (Cys) inserted into their proteins. The enzyme charges phosphoserine (Sep) onto $tRNA^{Cys}$ and the resulting Sep-$tRNA^{Cys}$ is used as the substrate for a tRNA dependent modification of phosphoserine into cysteine (O'Donoghue et al. 2005; Sauerwald et al. 2005). It is worth mentioning that there is yet a 23rd aaRS, which catalyzes the pyrrolysylation (Pyl) of an unusual UAG-decoding tRNA in certain bacteria and archaea: the pyrrolysyl-tRNA synthetase (PylRS) (Polycarpo et al. 2004; Herring et al. 2007; Yuan et al. 2010). The 23 aaRSs are split almost equally

Table 12.1 Class division of aminoacyl-tRNA synthetases and their features

Features			Classes		
			Class I		Class II
Active site			Rossman fold		Antiparallel β-sheet
Motifs			HIGH and KMSKS		Motifs 1, 2 and 3
Approach onto tRNA acceptor stem			Minor groove side		Major groove side
Subclasses/quaternary structure					
Ia	IleRS	α	**IIa**	SerRS	α_2
	MetRS	α, α_2		ProRS	α_2
	LeuRS	α		ThrRS	α_2
	ValRS	α		GlyRS	α_2
				HisRS	α_2
Ib	CysRS	α, α_2	**IIb**	LysRS	α_2
	GlnRS	α_2		AspRS	α_2
	GluRS	α		AsnRS	α_2
Ic	TrpRS[a]	α_2	**IIc**	PheRS	$(\alpha\beta)2$, α
	TyrRS	α_2		GlyRS	$(\alpha\beta)2$
				SepRS	α_4
				PylRS	α2
				AlaRS	α_4, α2, α
Id	ArgRS	α			
Ie	LysRS[b]	α			

[a]TrpRS approaches the tRNA from the major groove side. The quaternary structures (second column) of these enzymes are monomeric (α), homodimeric (α_2), homotetrameric (α_4), or heterotetrameric ($(\alpha\beta)2$). There are two hydroxyl (OH) groups on the terminal ribose of the tRNA, 2′ and 3′; Class I enzymes esterify mostly the 2′OH, with the exception of CysRS and TyrRS, that can esterify on both hydroxyl groups. Class II enzymes esterify the 3′OH with the exception of PheRS, that prefers the 2′OH (Arnez and Moras, 2009; Perona and Hadd, 2012). AlaRS (alanyl-tRNA synthetase), ArgRS (arginyl-tRNA synthetase), AsnRS (asparaginyl-tRNA synthetase), AspRS (aspartyl-tRNA synthetase), CysRS (cysteinyl-tRNA synthetase), GlnRS (glutaminyl-tRNA synthetase), GluRS (glutamyl-tRNA synthetase), GlyRS (glycyl-tRNA synthetase), HisRS (histidyl-tRNA synthetase), IleRS (isoleucyl-tRNA synthetase), LeuRS (leucyl-tRNA synthetase), LysRS (lysyl-tRNA synthetase), MetRS (methionyl-tRNA synthetase), PheRS (phenylalanyl-tRNA synthetase), PylRS (pyrrolysyl-tRNA synthetase), SepRS (Phosphoseryl-tRNA synthetase), SerRS (seryl-tRNA synthetase), TrpRS (tryptophanyl-tRNA synthetase), TyrRS (tyrosyl-tRNA synthetase)

[b]LysRS is found in both aaRSs classes

into two classes (class I and class II) based mainly on the structure of their active site domains and location of the active site where adenosine triphosphate (ATP) is bound. The partition is further supported by functional and evolutionary relatedness within each class (Arnez and Moras 1997; Woese et al. 2000; Carter and Duax 2002; Klipcan and Safro 2004) as well as by their mechanistic behaviors (Table 12.1) (Arnez and Moras 2009). Additional common features are found among members of each class, resulting in further subdivisions (Table 12.1).

Class I enzymes are essentially monomeric enzymes with the exception of tyrosyl-tRNA synthetase (TyrRS), tryptophanyl-tRNA synthetase (TrpRS), and methionyl-tRNA synthetase (MetRS) in some species. They approach the tRNA 3′-end

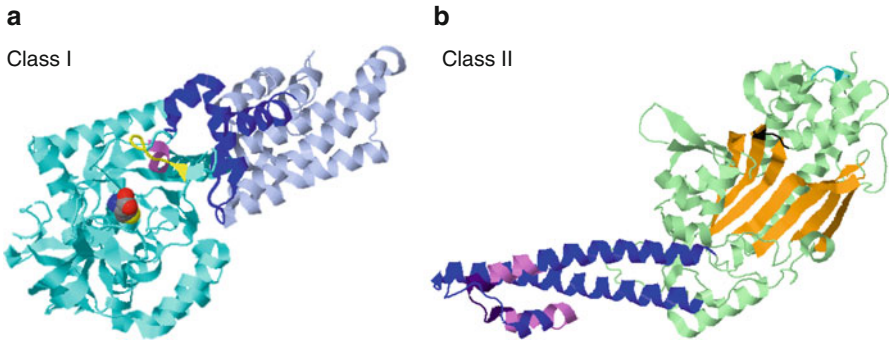

Fig. 12.1 Overall structures of Class I and Class II aminoacyl-tRNA synthetases represented by *T. brucei* MetRS (PDB ID:4EG1) (a) and SerRS (PDB ID:3LSQ) (b), respectively. (**a**) Tertiary structure of one subunit of the *T. brucei* MetRS homodimer complexed with methionine showing approximate positions of the Rossmann fold (*cyan*), the stem contact fold (*blue*) containing the KISKS signature (*purple*), and the anticodon binding helix bundle (*gray*). The HIGH motif is shown in yellow. Met bound in the active site is shown in CPK model. (**b**) Tertiary structure of the *T. brucei* SerRS homodimer showing the anti-parallel β sheet architecture (*orange*) and the coiled coil domain (*blue*) containing motif 1 (*pink*) and the metazoan-trypanosomatid specific insertion (*purple* + *pink*). Motif 2 is in cyan and motif 3 in purple. There is no publicized description of the *T. brucei* SerRS crystal structure

from the minor groove (with the exception of TrpRS) and prefer to charge the 2′-OH group of the terminal ribose of the tRNA, and their rate-limiting step is the release of the aminoacyl-tRNA. Their active site is composed of a Rossmann nucleotide-binding fold defined by the two conserved motifs, HIGH and KMSKS. The two motifs in the Rossmann fold interact with each other and ATP and bind amino acid, and are therefore responsible for catalysis (Fig. 12.1a) (Rould et al. 1989; Perona et al. 1993; Sekine et al. 2006).

Class II enzymes are usually dimeric or tetrameric. They approach the tRNA 3’ -end from the major groove and prefer to aminoacylate the tRNA at the 3′-OH, with the exception of phenylalanyl-tRNA synthetase (PheRS), which utilizes the 2′-OH (Sprinzl and Cramer 1973). The rate limiting step for class II aaRSs is the amino acid activation (Arnez and Moras 2009). Their active site possesses an anti-parallel β-sheet architecture composed by the signature motifs 1, 2, and 3. Motif 1 is important for dimerization and motifs 2 and 3 assist the binding of ATP and amino acid (Fig. 12.1b) (Eriani et al. 1995). The ATP exhibits a compact conformation which is unique for this class: ATP is surrounded by the β sheet formed partly by motifs 2 and 3 (Belrhali et al. 1994; Cavarelli et al. 1994; Cusack 1997).

3 Aminoacyl-tRNA Synthesis

The process of tRNA aminoacylation proceeds via a two-step reaction [steps (1) and (2)] (Arnez and Moras 1997, 2009). The first step is the activation of the amino acid by nucleophilic attack on the α-phosphate of the ATP, yielding an aminoacyl adenylate-aaRS intermediate complex (aa-AMP.aaRS) and a pyrophosphate (PP_i). In the second step, the amino acid is esterified to the 3′-end of the tRNA (A76) by the nucleophilic attack of the 2′- or 3′- ribose hydroxyl group to the carboxyl group of the aa-AMP, releasing aa-tRNA, AMP (adenosyl monophosphate) and aaRS. The reaction is driven by the hydrolysis of pyrophosphate. Therefore, the equivalent of two ATP molecules is consumed in the synthesis of each aminoacyl-tRNA. The tRNA binding, for many aaRSs, occurs in the presence of a divalent cation, usually Mg^{2+} ions (Mechulam et al. 1995).

(1) aaRS+ aa + ATP ⇌ aa-AMP.aaRS + PP_i
(2) aa-AMP .aaRS + tRNA ⇌ aa-tRNA + AMP + aaRS

4 The Diversity of the Aminoacylation System in Trypanosomatids

An *in silico* survey for aaRSs in the genome of *Leishmania major* identified 26 aminoacyl-tRNA synthetases. The aaRSs were present in all trypanosomatids checked (*L. major*, *Leishmania tarentolae*, *Leishmania mexicana*, *Leishmania infantum*, *Trypanosoma brucei brucei* 427, *T. brucei brucei* (TREU927), *Trypanosoma brucei gambiense*, *Trypanosoma cruzi* Esmeraldo, *Trypanosoma cruzi* Non-Esmeraldo, *Trypanosma congolense* and *Trypanosoma vivax*) but they differ in some aspects. There are two AspRSs in all *Leishmania* spp. and *T. brucei* subspecies; on the other hand *T. congolense* and *T. vivax* have only a single copy of AspRS. Moreover, with the exception of *T. congolense*, all the *Leishmania* spp. and *Trypanosoma* spp. carry a single HisRS. All trypanosomatids have two TrpRSs, but *T. cruzi* Non-Esmeraldo strain and *T. congolense* carry three. MetRS and GlnRS were not found in the *T. cruzi* Non-Esmeraldo strain, and only the alpha chain of the PheRS was found. In the *T. cruzi* Esmeraldo strain, AsnRS and ArgRS have not been found (Gowri et al. 2012). The lack of GlnRS and AsnRS in *T. cruzi* strains could be explained if an amidotransferase (GatCAB, GatFAB or GatDE - see below) is present in the genome of this parasite. In fact, *L. major* (and it might be the case for these *T. cruzi* strains) has genes for subunits of the amidotransferase - a GatA and a very distant homolog of GatC closely related to the GatF of yeast. However, a GatB gene was not found. The absence of ArgRS and one subunit of PheRS is unprecedented in the literature, and suggests that either these organisms are very unique in their aminoacylation pathways or that their genomes need better examination.

Only ten trypanosomatid aaRSs have been studied experimentally: AspRS, GlnRS, GluRS, HisRS, IleRS, LeuRS, LysRS, MetRS, PheRS, TrpRS and TyrRS. Crystal structures for *T. cruzi* and *T. brucei* HisRSs, *L. major* MetRS and TyrRS and cytosolic *T. brucei* TrpRS have been solved. Structures for the *T. brucei* SerRS alone and in complex with ATP exist in the Protein Data Bank, but a thorough description is not available. Six aaRSs have shown interesting characteristics regarding their use in the mitochondria and cytoplasm; these are AspRS, GluRS, GlnRS, IleRS, LysRS and TrpRS. Clearly, the number of studies on these aaRSs is below optimal and more research is needed in the area. Despite the overall conservation of the structural architecture and functional features, individual aaRSs of different organisms have evolved special unique structural elements with the addition of particular domains, and the understanding of these differences is essential for drug discovery efforts. These already published examples are examined in more detail below.

4.1 Direct tRNA Aminoacylation Pathways in Trypanosomatids

4.1.1 Aspartyl-tRNA Synthetase

AspRSs are homodimeric class II aaRSs belonging to the subclass IIb together with asparaginyl (AsnRS) and lysyl (LysRS) tRNA synthetases, with which they share strong structural similarities (Gampel and Tzagoloff 1989; Gatti and Tzagoloff 1991; Cusack 1995). A number of AspRS structures have been published, which have shown that there are important differences between enzymes of the three Domains of life at both structural and catalytic levels (Dietrich et al. 1980; Lorber et al. 1983; Ruff et al. 1991; Eiler et al. 1992; Cavarelli et al. 1993, 1994; Delarue et al. 1994; Schmitt et al. 1998).

Although there are no AspRS structures from trypanosomatids available, in 2009, Charrière and collaborators published a study revealing an interesting detail about the aspartylation system in these organisms that should be exploited for the design of new drugs. They showed that in *T. brucei*, one of the two essential AspRSs genes present in the genome codes for an AspRS with expanded substrate specificity. This enzyme is also present in the genomes of *T. cruzi*, *L. major*, *Leishmania infantum* and *Leishmania braziliensis*, in addition to the other AspRS genes. Despite the fact that both genes code for eukaryotic AspRS, while the AspRS1 is only able to recognize cytosolic $tRNA^{Asp}$ and is only present in the cytosol, the AspRS2, a mitochondrial targeted enzyme, can recognize both cytosolic and mitochondrial $tRNA^{Asp}$ (Charrière et al. 2009).

These results suggest that the $tRNAs^{Asp}$ must be different in the two cellular compartments. However, the two $tRNAs^{Asp}$ derive from the same single nuclear gene and therefore, their difference most likely comes from a post-transcriptional nucleotide modification specific for the mitochondrial $tRNA^{Asp}$ that would act as an antideterminant for AspRS1. The authors reason that this should be the scenario because

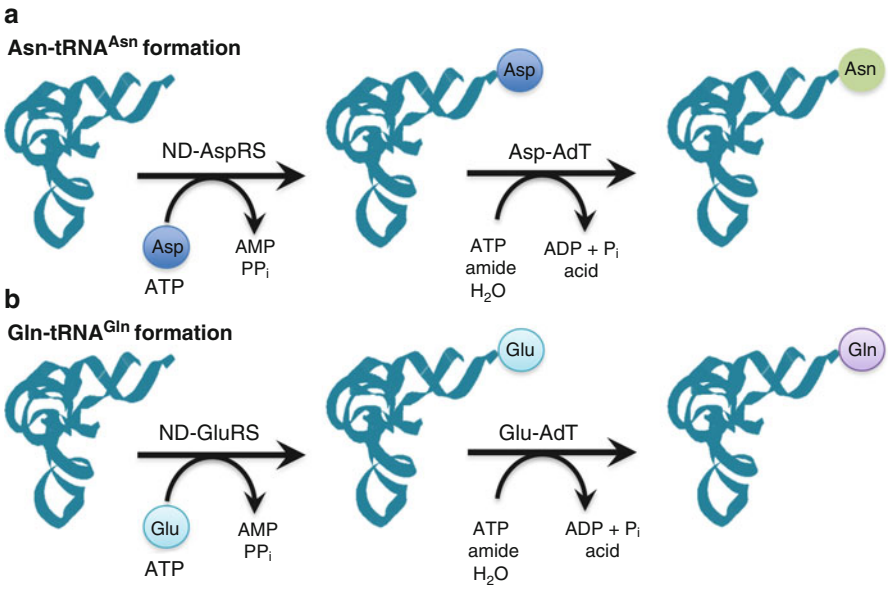

Fig. 12.2 Indirect pathways of (a) Gln-tRNA^{Gln} and (b) Asn-tRNA^{Asn} synthesis. (**a**) tRNA^{Gln} is glutamylated by an ND-GluRS to form Glu-tRNA^{Gln}. Glu-tRNA^{Gln} is then amidated by a Glu-AdT to form Gln-tRNA^{Gln}. (**b**) tRNA^{Asn} is aspartylated by an ND-AspRS to form Asp-tRNA^{Asn}. Asp-tRNA^{Asn} is then amidated by an Asp-AdT for form Asn-tRNA^{Asn}

the eukaryotic AspRSs use the anticodon and the discriminator base as identity elements and they have observed the absence of modifications in the anticodon of the cytosolic trypanosomatid tRNA^{Asp}. The nature and position of the postulated modification is unknown (Charrière et al. 2009).

The study of the mechanistic differences of the two trypanosomatid AspRSs in comparison to the human enzymes can lead to the rational design of drugs against one of the trypanosomal enzymes.

4.1.2 Glutaminyl-tRNA^{Gln} and Glutamyl-tRNA^{Glu} Synthesis

Most organelles synthesize Gln-tRNA^{Gln} through the transamidation pathway, where tRNA^{Gln} is first charged with Glu by a dual specificity GluRS. The resulting Glu charged onto tRNA^{Gln} is then amidated to form Gln-tRNA^{Gln} by a Glu-tRNA^{Gln} amidotransferase (Sheppard et al. 2008) (Fig. 12.2a). However, trypanosomatids are a special case, in that direct charging of the mitochondrial tRNA^{Gln} is possible. For *L. tarentolae*, a GlnRS activity was shown in the mitochondrial cellular fraction. This activity was only present when the mitochondrial tRNA$^{Gln}_{UUG}$ was given as a substrate. The cytosolic extract was able to charge both cytosolic and mitochondrial tRNA^{Gln}. These data indicated the existence of two different GlnRSs, one for each cellular

compartment. *L. tarentolae* has two $tRNAs^{Gln}$, of which only $tRNA^{Gln}_{UUG}$ is imported to the mitochondria. In contrast, in the cytosol both $tRNA^{Gln}_{UUG}$ and $tRNA^{Gln}_{CUG}$ can be found. These two tRNAs have 11 nucleotides that differ in their sequences and that contribute to the aminoacylation specificities of the two GlnRSs. More specifically, the nucleotides which differ in the two tRNA species are those flanking the anticodon stem, the 5′ nucleotide of the anticodon, and the acceptor stem. The nucleotide substitutions in the D loop or the T loop are not identity elements (Nabholz et al. 1997). These differences in the two tRNAs further support the notion that there are two different GlnRSs (one for the cytosol and one for the mitochondria).

A similar situation was found for *T. brucei*, in which a $tRNA^{Gln}$ is directly acylated by GlnRS, but the same enzyme is responsible for the charging of both mitochondrial and cytosolic $tRNAs^{Gln}$. The same is true for the *T. brucei* GluRS, that is able to charge mitochondrial and cytosolic $tRNA^{Glu}$ (Rinehart et al. 2004).

Sequences of other eukaryotic type GlnRSs and GluRSs were compared to those of *T. brucei* and, while the *T. brucei* GlnRSs and GluRS are eukaryotic like, they lack an N-terminal extension that in human GlnRS is needed for aminoacylation of $tRNA^{Gln}$. This extension aids in the formation of the multi-aminoacyl-tRNA synthetase complexes seen in higher eukaryotes. Also, the *T. brucei* GluRS is not presented as a bifunctional enzyme as in higher eukaryotes, in which the GluRS is linked to a ProRS by a connective domain made of repeated units of 50 amino acid residues. The *L. major* GlnRS also seems to have a short N-terminal extension and it is likely that trypansomatids don't form the multi-synthetase complex as other eukaryotes (Rinehart et al. 2004).

Last but not least, the trypanosomatids GlnRS and GluRS, also different from higher eukaryotes, possess short N-terminal regions predicted to be mitochondrial targeting sequences (Rinehart et al. 2004).

In conclusion, trypanosomatids and higher eukaryotes show plentiful of differences in their ways to achieve $tRNA^{Gln}$ and $tRNA^{Glu}$ aminoacylation, be it in the cytosol or in the mitochondria. These differences can aid in the design of new drugs against trypanosomatids.

4.1.3 Histidyl-tRNA Synthetase

HisRS is a dimeric, Mg^{+2} independent Class IIa aaRS for which structural characteristics include: (1) a catalytic domain, whose core fold is shared by all Class II aaRS; (2) a C-terminal domain, whose α/β fold is conserved among three Class IIa enzymes: HisRS, ThrRS, and ProRS, and by the eukaryotic/archaeal GlyRS and; (3) a unique conserved motif that consists of a 50- to 75-long α-helix insertion between motifs 2 and 3 of the catalytic domain. The prokaryotic sequences of this domain contain conserved residues that have been proposed to interact with the 3′strand backbone of the tRNA acceptor stem and to be important for histidine adenylation in the absence of the tRNA. The C-terminal domain holds the binding site for the tRNA anticodon loop, although cognate tRNA recognition is believed to be mediated not by the anticodon but by residues of the Class II motif 2 loop in the

catalytic domain. Furthermore, in many eukaryotes, the HisRS contains one or more copies of a short, N-terminal domain named WHEP. The WHEP domains are present in several non-homologous aaRSs and mediate different biological activities not related to aminoacyl-tRNA synthesis (Freist et al. 1999; Merritt et al. 2010).

The *T. brucei* and *T. cruzi* single HisRSs are the first eukaryotic HisRSs for which crystal structures have been obtained. Although they are very similar to each other (84 % identity), they present very little conservation when compared to bacterial and human enzymes. The sequence identity between both trypanosomal HisRS and previously studied bacterial homologs is on the order of 25 % in the catalytic domain and lower elsewhere. When compared to both human HisRS sequences (HARS1 and HARS2), trypanosomal enzymes present an identity of less than 30 % (Merritt et al. 2010).

The catalytic and anticodon binding domains of the trypanosomal HisRS show overall folds similar to those seen in bacterial homologs although there are differences in the residues forming the active site. The insertion domain is very different between human and bacterial counterparts, indicating that the interaction with the tRNA is different among these enzymes. The human N-terminal WHEP domain has been shown to be essential for its activity and the trypanosomal enzymes studied were truncations of this domain, that although were tested for their ability to activate histidine, were not tried in an aminoacylation reaction. In order to see if there was an equivalent of the WHEP domain in these kinetoplastida enzymes (*L. major* included), the authors used two different secondary-structure predictors that show they have a strong propensity for helix formation around residues 5–28, although it does not predict a second helical region that would pair with the first to form a WHEP-like 2-helix bundle (Merritt et al. 2010).

The differences seen in the active site are a result of the absence of a homologous residue to the Arg314 in *Escherichia coli*, which is important for the positioning of the ATP γ-phosphate prior to catalysis and seems to be played by a residue at a non-conserved region in trypansomatids. Also, the binding pocket occupied by the adenine ring in the *T. cruzi* complex with histidyl-adenosinemonophosphate (HAM), differently from *E. coli*, is formed by a different set of residues. The Arg311 needed for HAM sandwiching with Phe125 has no sequence or structural equivalent in the *T. cruzi* enzyme. Alternatively, trypanosomal enzymes have three residues that play that role: Cys365 and Val366, which provide a hydrophobic surface adjacent to the adenine and Arg164, which extends from the other side of the binding pocket, stacking against the other face of the adenine ring. The same group of amino acids were found in the *L. major* and apicoplast-specific *Plasmodium* spp. and *Toxoplasma* spp. HisRSs (Freist et al. 1999; Merritt et al. 2010).

What is interesting about this system is that the structural differences seen in the ATP binding mode and insertion domains cannot be generalized for the eukaryotic system. A corresponding set of bacterial key residues can be found in other eukaryotic HisRS sequences, including the two human homologs. Therefore, trypanosomas diverge radically from that. The results described above, together with the fact that a HisRS partial knockdown had a deep effect on the *T. brucei* bloodstream

forms, suggest HisRS as a possible drug target against trypanosomatids and maybe apicomplexans (Merritt et al. 2010).

4.1.4 Isoleucyl-tRNA Synthetase

Isoleucyl-tRNA synthetase, similar to GluRS and GlnRS, has only one copy in the genome of trypanosomatids and in order to allow mitochondrial protein synthesis to proceed normally, it must be targeted to both the cytoplasm and the mitochondria.

Although dually targeted proteins are known to exist in trypansomatids, the mechanism of dual localization was not well understood until recently. Previously, it was suggested that a mechanism of alternative splicing might be involved in this dual targeting as it was shown that a large number of spliced mRNA isoforms resulted from trans-splicing and changed abundance during the parasite life cycle (Kolev et al. 2010; Nilsson et al. 2010; Siegel et al. 2010; Gowri et al. 2012). As a result, it was shown that a long splice variant of the *T. brucei* IleRS gene encodes the mitochondrial isoform. This data was obtained by RNAi of the 5′UTR of the long splice variant, in which an accumulation of uncharged mitochondrial $tRNA^{Ile}$ was observed against a non-affected charging of cytosolic $tRNA^{Ile}$ (Rettig et al. 2012).

Different transcriptomic analyses suggest a situation similar to IleRS for four other aaRSs (AsnRS, ProRS, GluRS and GlnRS) (Kolev et al. 2010; Nilsson et al. 2010; Siegel et al. 2010; Rettig et al. 2012). Furthermore, a second set of two aaRSs were found, whose mRNAs are also differentially trans-spliced and the major splice site is upstream of the first AUG. In these cases, the shorter versions probably use the second AUG for translation initiation, uncovering a mitochondrial targeting sequence which is masked in the long form (Rettig et al. 2012).

4.1.5 Lysyl-tRNA Synthetase

LysRSs are unique in the fact that they can be found in the two classes of aaRSs. Most bacterial and some archaeal genomes contain a class I LysRS, while eukaryotes have a class II LysRS (Ibba et al. 1997; Ambrogelly et al. 2002). In trypanosomes there are two genes coding for LysRS, both class II enzymes. Besides their different sizes (584 and 634 amino acids long), a computer-based approach has identified an N-terminal mitochondrial signal peptide in the longer sequence (LysRS2), which is predicted to be mitochondrial. This enzyme also has a 90 residue extension in its C-terminus that is specific to the order Kinetoplastida. Both *T. brucei*'s LysRSs are essential for parasite growth and an experiment using N-terminal and C-terminal deletants of the LysRS2 has shown that only the N-terminal is needed for mitochondrial targeting. It has also been shown, by another assay, that both extensions are processed during mitochondrial transport. The

C-terminal extension is cleaved after the protein has been translocated and it is dispensable for mitochondrial localization (Español et al. 2009).

In order to understand better the role of the C-terminus extension in the LysRS2, adenylation assays using purified proteins obtained from the overexpression of different constructs of the LysRS2 genes [full-length. LysRS2, ΔN.LysRS2 (LysRS2 lacking residues 1–26), ΔC.LysRS2 (LysRS2 lacking residues 547–634), and ΔNΔC.LysRS2 (LysRS2 lacking residues 1–26 and 547–634)] were performed. This experiment showed that full-length LysRS2 had no detectable amino acid adenylation activity, while the ΔC.LysRS2 showed activity levels comparable to those of its cytosolic counterpart. The ΔNΔC.LysRS2, had the same level of activity as ΔC.LysRS2. These results suggested that the C-terminal extension is used to prevent LysRS2 from aminoacylating cytosolic $tRNA^{Lys}$. Only when it reaches the mitochondria, where the C-terminal is processed, it becomes active (Español et al. 2009).

Although it is not clear why there is a need for prevention of LysRS2 activity in the cytoplasm, it has been proposed that the C-terminal extension might have another role in the mitochondria (as has been shown for TrpRS and TyrRS domains in other organisms (Kise et al. 2004; Otani et al. 2011) and/or it is needed for $tRNA^{Lys}$ import into the organelle (Español et al. 2009).

4.1.6 Methionyl-tRNA Synthetase

MetRSs are class I enzymes, either monomeric or dimeric, that belong to the subclass Ia. They are interesting because they can recognize an initiator $tRNA^{Met}$ (recognizes AUG at the start of an mRNA) as well as an elongator $tRNA^{Met}$ (recognizes AUG other than the start codons in the mRNAs). Moreover, MetRSs from different species show clear structural divergence (Deniziak and Barciszewski 2001) but organisms belonging to all Kingdoms as well as organelle bear a minimal core enzyme composed of at least the active site.

The sequence of the catalytic core of *L. major* MetRS is 97 % identical to the *L. brasiliensis* and 89 % identical to the *L. infantum*, and is closely related to *Leishmania donovani*. When compared to *T. brucei* and *T. cruzi*, the leishmanial enzymes show an identity of 68 %, but if we consider only the active sites, they are 100 % identical. MetRSs from both *L. major* and *T. brucei* are characterized by several key structural features: (1) a Rossmann fold catalytic core with an inserted connective peptide (CP) domain; (2) a stem-contact fold (SCF) domain containing the conserved KMSKS signature (KISKS in trypanosomatids); and (3) an anticodon binding α helix bundle (Larson et al. 2011b; Koh et al. 2012).

The crystal structures of these enzymes in complex with their intermediates have been published and are very similar (Larson et al. 2011b; Koh et al. 2012). They differ only in the degree of closing of the active site in relation to the flexible CP knuckle and KISKS loop. While the *L. major* MetRS complexed with methionyl-adenylate (MAMP), PPi and Mg^{+2} distant of the CP kuckle and the KISKS loop for about 3.0 Å, being more "closed", the active site of *T. brucei* MetRS complexed

with methionine (Met), with a distance of 9.0 Å, is more "open". The active site of the *T. brucei* MetRS complexed with MAMP, with 7.0 Å, is in between these two states, not as "closed" as the MetRS complexed with MAMP and not as "open" as the one complexed with Met., The Pyrophosphate and Mg^{+2}, in the *L. major* MetRS structure complexed with MAMP, PPi and Mg^{+2} show multiple interactions with the KISKS motif and the CP domain, stabilizing a closed active site conformation. On the other hand, none of these interactions were observed for *T. brucei* MetRS complexed with MAMP, which shows an active site not as closed as the ones observed in the other MetRS structures.

Humans have a mitochondrial and a cytosolic MetRS. The mitochondrial enzyme has a higher sequence identity with the trypanosomal MetRS; although it is only 30 % identical to the trypanosomatid catalytic core overall, the active sites are much more highly conserved. It is shown that of the 27 residues closest to the products bound in the structure of *L. major*, only four differ from the human enzyme: *L. major* MetRS Tyr218 is a Phe in human mitochondrial MetRS, Trp515 is a His, Ile523 is a Met and Gly514 is a Ser. The carbonyl oxygen of the Gly514 points towards the ligand while the Ser side chain in the human mitochondrial enzyme points away from the ligand in the direction of a hole between the side chains of Phe238, Lys310, and Asp383, as deduced from the *L. major* structure for which the corresponding residues are respectively, Arg401, Lys485, and Asp558. Among the residues surrounding the latter, five amino acids differ: Val482 and Val483 in *L. major* MetRS, which are both an Ile in human mitochondrial MetRS, and the tripeptide Ser553-Asn554- Phe555, which is Gly-Val-Pro in the human mitochondrial enzyme (Larson et al. 2011b).

Depending on the type of ligand bound, MetRSs can adopt dramatically different conformations (Ghosh and Vishveshwara 2008; Koh et al. 2012). Five inhibitor-bound complexes (I-state) were determined for the *T. brucei* MetRS, showing that upon their binding the methionine pocket is enlarged, the CP domain is open, the KISKS motif is flexible and an auxiliary pocket is formed. There are drastic differences between the I-state and the methionine bound state (M-state), involving backbone shifts of domain and secondary structure elements, combined with small and large side-chain movements. A small Met pocket, a closed CP domain, an open/flexible KISKS motif, and a nonexistent auxiliary pocket characterize the M-state. The methionyl-adenylate bound state (P-state) differs from the M-state in only the KISKS motif, which shows a closed form (Koh et al. 2012) Kohs work has been able to show that even though the amino acid sequences of human and trypanosomatid MetRSs active sites seem very similar, when one looks at a 3D structure, these differences are magnified, showing important aspects of these two enzymes that make them divergent. This idea is supported by the fact that substitutions of only two residues in human and *Staphylococcus aureus* (type II) IleRSs have increased its K_i value for mupirocin by one order of magnitude (Nakama et al. 2001). Considering the description above, it doesn't come as a surprise that five potent inhibitors (all containing a substituted benzyl group connected by a linker to an aminoquinolone or to a benzimidazole) of *T. brucei* and *T. cruzi* MetRS have already been described, constituting a platform for the further development of at

least anti-Human African trypanosomiasis and anti-Chagas disease compounds (Shibata et al. 2011; Koh et al. 2012). The tertiary structure of the *T. brucei* MetRS is shown in Fig. 12.1a.

4.1.7 Seryl-tRNA Synthetase

The first crystal structure of a SerRS to be published (Cusack et al. 1990) provided the evidence of the existence of two classes of aaRSs as it was observed that they did not contain a dinucleotide binding fold as part of their catalytic domain as described for other aaRSs up to that time (Leberman et al. 1991). SerRSs are homodimeric enzymes of the class II aaRS belonging to subclass IIa. In addition to its characteristic class II active site domain, the structures of most SerRSs include a coiled-coil domain at the N-terminal that is essential for the recognition of the elbow and the long variable loop of $tRNA^{Ser}$ and, in the case of eukaryotic enzymes, a C-terminal extension important for protein stability and amino acid recognition (Geslain et al. 2006).

There are three types of recognition of $tRNA^{Ser}$ described (Geslain et al. 2006): (1) recognition of some base pairs of the acceptor stem but not of the discriminator base 73 (Normanly et al. 1992; Biou et al. 1994; Cusack et al. 1996; Himeno et al. 1997). This is seen in bacteria and *S. cerevisiae*; (2) recognition of the discriminator base and the orientation of the variable loop. This has been seen in human and archaeal SerRSs (Achsel and Goss 1993; Wu and Goss 1993; Metzger et al. 1997; Heckl et al. 1998); (3) recognition of the acceptor stem, the variable loop and the discriminator base of the $tRNA^{Ser}$. This mode of recognition is seen in methanogenic archaeal (Bilokapic et al. 2004; Bilokapic et al. 2006). SerRSs also charge $tRNA^{Sec}$ with serine in the first step of Sec-$tRNA^{Sec}$ formation (see Sect. 1.3.2.3).

The trypanosomatids' genomes only code for one SerRS gene each and their products are probably used in both cytoplasm and mitochondria, although a mitochondrial targeting sequence has not been identified (Geslain et al. 2006). There are two structures for the *T. brucei* SerRS available in PDB (ID 3LSQ and 3LSS), but a thorough description has not been published yet. Nevertheless, the protein, which is composed of 477 amino acids, behaves as the expected homodimer and contains the coiled coil domain seen in homologous enzymes. The trypanosomatid coiled coil domain contains 26 residues insertion only observed in metazoans. As seen for the *T. cruzi* enzyme, the mode of recognition of $tRNA^{Ser}$ observed for the trypanosome SerRS shows that the discriminator base G73 and the G1 base are essential as well as the existence (not the sequence) of the long variable loop. The entire stem and anticodon loop are not recognized by the *Trypanosoma* SerRS. Thus the mode of $tRNA^{Ser}$ recognition in trypanosomatids fits well with that described for human and archaeal enzymes, and separate them from other eukaryotic enzymes such the one from *S. cerevisiae*, which is in agreement with the observed clustering of the trypanosomal SerRSs with metazoans in a phylogenetic analysis (Geslain et al. 2006). Although the $tRNA^{Ser}$ aminoacylation system of the trypanosomatid SerRS is not especially different in comparison to metazoans, the serylation of $tRNA^{Sec}$ has some

properties that should be checked (see Sect. 1.3.2.3). The tertiary structure of the *T. brucei* SerRS is shown in Fig. 12.1b.

4.1.8 Tryptophanyl-tRNA Synthetase

TrpRSs are Zn^{+2} dependent homodimeric enzymes of the class I aaRSs belonging to the subclass Ic (Kisselev et al. 1981; Cusack 1995). They have an additional AIDQ (Eukarya and Archaea)/GXDQ (Bacteria) motif positioned between the canonical HIGH and KMSKS class I motifs; this motif is involved in ATP binding in the human enzyme (Xu et al. 2001; Shen et al. 2006). All trypanosomatids have two TrpRSs, but *T. cruzi* Non-Esmeraldo strain and *T. congolense* carry three. For *T. brucei*, it is already known that both TrpRSs (TrpRS1 and TrpRS2) are essential for growth and needed to form cytosolic and mitochondrial Trp-$tRNA^{Trp}$, respectively (Charrière et al. 2006; García et al. 2007). These two enzymes are 44 % identical. All of the mitochondrial $tRNA^{Trp}_{CCA}$ in *L. tarentolae* and a percentage of the same tRNA in *T. brucei* undergoes C34U editing to be able to decode the mitochondrial non-standard Trp UGA codon (Crain et al. 2002; Charrière et al. 2006). Also, these tRNAs present a 2-thio modification in the U33 and U34. In *T. brucei* it was shown that the edited U34 and the thiolated U33 are independent antideterminants for the TrpRS1, which is then specific for cytosolic unmodified and unedited $tRNA^{Trp}$ (Crain et al. 2002; Charrière et al. 2006; Bruske et al. 2009).

What we see here is that in a single aminoacylation system we find at least two aspects that can be studied further in a drug design project: (1) The enzymes responsible for the editing and modification of $tRNA^{Trp}$ (not a subject of this chapter) and (2) The essential TrpRSs.

The crystal structure of the *T. brucei* cytosolic TrpRS has been partially obtained, sharing a 56 % identity with the human enzyme (Merritt et al. 2011). Although the portion of the enzyme expected to interact with the tRNA anticodon arm was not solved, a good superposition with the helical regions that contain residues involved in binding the anticodon in the human (Lys 431, Ser 378, and Thr 427) and yeast TrpRS-$tRNA^{Trp}$ complexes was possible. The amino acids Lys 431 and Ser 378, responsible for the recognition of the anticodon base C35 in humans, are conserved in both trypanosomal TrpRSs. The Thr 427 in *T. brucei* TrpRS2 is a serine. As Thr 427 is responsible for the anticodon C34 recognition, this amino acid change could account for the different specificities of the two trypanosomal TrpRSs. However, the presence of serine in this position in other TrpRS sequences that have the same substrate as the trypanosomal TrpRS1 ($tRNA^{Trp}_{CCA}$) suggests that this difference is not the one accountable for *T. brucei*'s TrpRSs recognition specificities. Can the differential trypanosomal TrpRSs specificities be related to the modification of the U33? Since the human U33 in the TrpRS:$tRNA^{Trp}$ complex extends away from protein, it is possible that the thiolation present in *T. brucei* might induce a change in the tRNA structure that alters its interaction with the TrpRS1. Although not strong, this explanation is possible, but since we don't have the structures of the different trypanosomal TrpRSs with their tRNAs, this question cannot yet be answered.

The available trypanosomatid TrpRS1 crystal structure is mostly disordered and the only other region where it is possible to gain insight regarding differences between the human cytosolic TrpRS is near the human active site residue Trp 203. The structural equivalent in *T. brucei* is the residue Phe 120. Most probably, the phenylalanine sidechain in this protozoan homolog plays the role of the human homolog's tryptophan, in stacking against the central W of the V*XX*W*XX*V (*X*- any amino acid) motif upon ATP binding. The V*XX*W*XX*V motif, present in a region called the eukaryote-specific extension (ESE) that corresponds to amino acids 82–154 in humans, forms a hairpin upon ATP binding that mediates the activation of tryptophan to form tryptophanyl-AMP in the first half reaction of $tRNA^{Trp}$ aminoacylation. The hairpin is stabilized by the interaction of the residues Val85, Tpr 88 and Val 90 with the active site residues Trp 203 and Phe 339 in humans.

This discussion suggests that there is an urgent need for the TrpRS2 structure, as well as a better TrpRS1 structure. The ideal crystal structures are those that will yield information about the interaction between the two TrpRSs and their substrates. These would lead to a better understanding of the differences between the trypanosomal TrpRSs themselves and the human enzyme.

4.1.9 Tyrosyl-tRNA Synthetase

Tyrosyl-tRNA synthetases are class I aaRSs of the subclass Ic together with TrypRSs, both of which have an AIDQ ATP binding motif in addition to the classical HIGH and KMSKS motifs, although TryRSs have a class II mode of tRNA recognition, which is approached by the variable arm and the major groove side, instead of the minor groove as in other class I aaRSs (Yaremchuk et al. 2002; Tsunoda et al. 2007). Although the recognition mode of Tyr-AMP is the same across the three Domains of life, the aminoacylation of its cognate $tRNA^{Tyr}$ exhibits some differences, and the main reason for the lack of cross-reactivity between archaeal/eukaryotic and bacterial TyrRS-$tRNA^{Tyr}$ pairs most probably lies in the last base pair of the acceptor stem (C1-G72 vs G1-C72, respectively) (Tsunoda et al. 2007). Although the crystal structure of most of the TyrRSs are homodimers, these enzymes in solution show one tRNA bound per dimer and show half-of-the-sites reactivity regarding the binding of tyrosine or Tyr-AMP; the anticodon loop binds to one subunit of the dimeric TyrRS molecule and the acceptor stem to the other.

TyrRSs were previously thought to exist only as homodimers (Cusack 1995), a belief that changed as more genome sequences were unveiled and showed that a small group of organisms have TyrRS sequences in which the N-terminal and the C-terminal halves form a single double-length polypeptide chain. This group forms a clade in the TyrRS phylogenetic tree where we find the trypanosomatids, plants, other basal eukaryotic organisms and mimivirus, which have their origin on the euryarchaeota branch (Bonnefond et al. 2005; Larson et al. 2011b).

Trypanosomatids have only one TyrRS, which presents a double length sequence that might have originated from gene duplication or tandem insertion of an

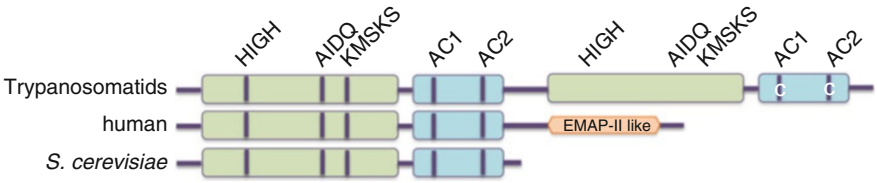

Fig. 12.3 Primary structure and motifs of trypanosomatids' TyrRS in comparison to human and *S. cerevisiae*. The catalytic domains are shown in green and the anticodon-binding domains are in blue. The HIGH (HIAQ in most trypanosomatids) and KMSKS active site motifs, common to all class I catalytic domains, are assigned, as well as the AIDQ motif (GLDQ in most trypanosomatids), characteristic of the ATP binding site in TyrRS and TrpRS. The AC1 motif corresponds to the residues of the eukaryotic/archaeal TyrRS anticodon-binding domain that interact with the anticodon stem of $tRNA^{Tyr}$. The AC2 motif contains the residues that specifically recognize the anticodon bases G34 and U/ψ35 in other organisms. The endothleial monocyte-activating polypeptide II-like domain (EMAP-II like domain) of human TyrRS has leukocyte and monocyte chemotaxis activity, stimulating the production of myeloperoxidase, tumor necrosis factor-α and tissue factor (see: Wakasugi and Schimmel, 1999) (Adapted from Larson et al. 2011b)

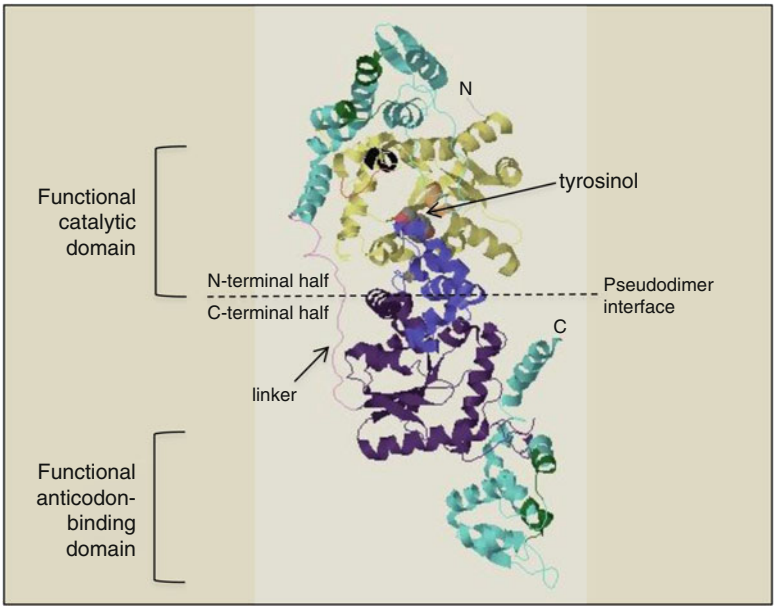

Fig. 12.4 *L. major* TyrRS. The asymmetric *L. major* pseudo-dimer shown is that of the tyrosinol:TyrRS complex (PDB ID: 3p0i). The catalytic domain is shown in light yellow for the N-terminal (functional) copy, in purple for the C-terminal (presumed nonfunctional) copy in *L. major* TyrRS. The anticodon-binding domain is shown in cyan in both halves of the enzyme; only the C-terminal is functional. The linker residues connecting the N-terminal and C-terminal halves are shown in pink. The crystallographically observed bound tyrosinol molecule is shown in CPK model (gray and magenta). AC1 (residues 244–250 and 578–584, in the N-terminal half and C-terminal half, respectively) and AC2 (residues 313–319 and 636–642, in the N-terminal half and C-terminal half, respectively) in both halves are shown in green. CP1 is shown in blue (residues 120–166 in the N-terminal half and 485–503 in the C-terminal half). The active site motifs in the N-terminal half are also shown: HIAQ (residues 46–49, *black*), GLDQ (residues 182–185, *orange*), KMSKS (residues 22–226, *red*) (Adapted from Larson et al. 2011b)

exogenous TyrRS. The C-terminal extension (second half) is a full TyrRS like domain that lacks the canonical HIGH (HIAQ in the first half of the *L. major* sequence), AIDQ (GLDQ in the first half of the *L. major* sequence) and KMSKS motifs (Fig. 12.3). Taking the two *L. major* TyrRS halves separated, they show only 17 % identity. The full-length TyrRSs of *L. major*, *T. brucei* and *T. cruzi* present 60–70 % identity between them.

The crystal structures of the *L. major* TyrRS show that it is an intrinsically asymmetric pseudo-dimer that contains a functional active site only in the N-terminal "monomer" (Larson et al. 2011a) (Fig. 12.4). The pseudo-dimer simulates the TyrRS homodimers observed for some homologs. Residues 355–373 connect the two halves of the pseudodimer. The N-terminal half has two α helices (one and two) split by a strand β1. The latter structural elements are not present in the C-terminal half and the region comprehending residues 355–373 have no structural equivalent in canonical TyrRS dimers. Most of the interdomain interface in the pseudo-dimer consists of residues from the connective peptide one region (residues 120–166 in the N-terminal half and residues 458–503 in the C-terminal half) as in other class Ic aaRSs. Interactions involving helices α4 (residues 74–84) and α4′ (residues 416–426) also contribute to the dimer interface. The Rossman fold and the anticodon binding domains are in agreement with the expected class Ic core secondary structure and fold, showing only a slight difference in the relative orientation of the two halves.

Two distinct conformational states of the active site have been observed in the *L. major* TyrRS structures published (Larson et al. 2011a). All of them show conformational differences in the KMSKS loop and the α4 (residues 73–78) –α5 (84–103) helices that distinguish their active site from other TyrRS:tyrosine analog complexes previously reported. Nevertheless, the position of tyrosinol within the *L. major* TyrRS active site is indistinguishable from the previous position observed for tyrosine itself and for the tyrosyl moiety of tyrosyladenylate in structures of human, yeast and archaeal TyrRSs complexes (Larson et al. 2011b).

One of the conformational states observed in some of the tyrosinol complexes shows the loop containing residues 146–154 positioned so that the residues at the tip of the loop interact with the α and β phosphates of ATP or with the single phosphate of tyrosyladenylate in a more closed conformation than that observed for bacterial TyrRS and the related human mitochondrial enzyme, although it is very similar to archaeal and other eukaryotic enzymes (Larson et al. 2011b).

The second conformational state is observed in the fisetin complexes and in the triclinic tyrosinol complexes, where helix α4 (residues 73–78) and α5 (84–103) run parallel to each other in an extended conformation. The residues between the two helices form a cap across them. This state results in a more extensive interaction across the pseudo-dimer interface in place of the interaction with the residues preceding the HIGH motif. In this case, the active site is more open in spite of the closed conformation presented by the KMSKS loop (Larson et al. 2011b).

The *L. major* TyrRS has only one active site present in the N-terminal half of the enzyme, which undoubtedly points to the C-terminal half being responsible for anticodon binding. The sequence motifs AC1 and AC2 are usually associated with the

recognition of the tRNA anticodon stem, and while both halves of the trypanosomatids' TyrRS possess these motifs, they are structurally different. The loop connecting strands β7 (residues 291–293) and β8 (residues 296–298) is much shorter in the N-terminal copy of the anticodon-binding domain, which probably makes it unable to bind $tRNA^{Tyr}$. Additionally, residues 256–271 that follow the AC1 motif in the N-terminal anticodon-binding domain and form a hairpin structure are not present in the C-terminal copy of the anticodon-binding domain, nor in TyrRSs structures from yeast or Mimivirus of plants, being characteristic of TyrRS sequences from the plant/plastid clade (Larson et al. 2011b).

The yeast: tyrosyladenylate complex revealed 26 residues within 4 Å of the intermediate reaction product tyrosyladenylate that are most probably associated with the active site of the TyrRS. Models superimposing the human and the *L. major* TyrRS structures to the yeast complex showed that the active site of the *L. major* TyrRS has 16 amino acids that differ from the human cytosolic TyrRs and 14 amino acids that differ from the mitochondrial human enzyme. An essential difference between the human and the *L. major* active sites that can be extended to the other trypanosomatids is the absence of a metal ion in the latter. The trypanosomatid enzyme doesn't have any of the residues that coordinate K^+ in the human TyrRS: tyrosinol complex and two of these residues (Phe39 and Leu96) result in an additional area of hydrophobic surface compared to the human cytosolic enzyme (Larson et al. 2011b). The particularities observed in the primary sequences of the trypanosomal TyrRSs that were complemented by the tertiary structure obtained with the *L. major* crystal structures, culminating in very important active site sequence and conformational differences between human and trypanosomatid TyrRSs, open a number of possibilities for the design of selective anti-trypanosomal drugs.

4.2 tRNA Dependent Aminoacyl-tRNA Synthesis

The direct aminoacylation of tRNAs by aaRSs is the way by which most aa-tRNAs are formed, yet there are a small number of aa-tRNAs that are produced by the modification of a non-cognate amino acid on the tRNA by a tRNA dependent amidotransferase. This is the case for Asn-$tRNA^{Asp}$ and Gln-$tRNA^{Gln}$ formation in most prokaryotes and eukaryotic organelles (Fig. 12.2), archaeal and eukaryotic Sec-tRNASec synthesis, Cys-$tRNA^{Cys}$ biosynthesis in some methanogenic organisms and fmet-$tRNA^{fmet/met\text{-}i}$ formation in prokaryotes and organelles of eukaryotic organisms. In Kinetoplastida the only described tRNA dependent amino acid synthesis pathways are those for Sec-$tRNA^{Sec}$ and fmet-$tRNA^{Met}$ (Tan et al. 2002; Cassago et al. 2006; Lobanov et al. 2006).

4.2.1 Indirect Routes to Gln-tRNA^{Gln} and Asn-tRNA^{Asn} Formation

In the last decade it has been accepted that only eukaryotes and a handful of bacteria have a full set of 20 aaRSs. By performing detailed genomic analysis and genetic experiments researchers have shown that most prokaryote genomes don't encode an AsnRS and that the majority of bacterial and all known archaeal genomes do not encode a GlnRS (Sheppard et al. 2008).

In these organisms, in order to get Gln-tRNA^{Gln} and Asn-tRNA^{Asn}, there must be a non-discriminating (ND) GluRSs (Lapointe et al. 1986) and AspRSs (Becker et al. 1997; Feng et al. 2005, Bernard et al. 2006) in their genomes. These enzymes have a dual tRNA specificity, being able to glutamylate tRNA^{Glu} and tRNA^{Gln} in the case of the GluRS and to aspartylate tRNA^{Asp} and tRNA^{Asn} in the case of the AspRS. The mischarged tRNAs are then used as substrates of a tRNA dependent amidotransferase to form the correct acylated substrate for protein synthesis (Fig. 12.2). There have been three different tRNA-dependent amidotransferases described up to now: GatDE (Tumbula et al. 2000), GatCAB (Curnow et al. 1997) and GatFAB (Sheppard et al. 2008; Frechin et al. 2009).

GatDE is an archaeal specific enzyme and is responsible for the transamidation of Glu-tRNA^{Gln}. GatCAB is found in Archaea, Bacteria and eukaryotic organelles (Pujol et al. 2008). In archaea, GatCAB (Curnow et al. 1997; Tumbula et al. 2000) is only present when AsnRS is not (Tumbula et al. 2000; Roy et al. 2003). Therefore gatCAB can generate Asn-tRNA^{Asn} as well as Gln-tRNA^{Gln}, depending on the genomic context (Sheppard et al. 2008). GatFAB was reported in yeast as a Glu-tRNAGln amidotransferase, and has homologs in *Kluyveromyces lactis*, *Candida glabrata* and *L. major* genomes (Frechin et al. 2009; Gowri et al. 2012).

In general, the indirect routes for Gln-tRNA^{Gln} and Asn-tRNA^{Asn} formation are not present in trypanosomatids. Nonetheless, it is not possible to find the gene coding for AsnRS in *T. cruzi* Esmeraldo strain (Gowri et al. 2012), which could indicate the presence, in the Esmeraldo strain, of an indirect pathway for the synthesis of these translation substrates. Although it seems strange that only one strain would use a different pathway for Asn-tRNA^{Asn} formation, peculiarities are not scarce among trypanosomatids.

4.2.2 Formylmethionyl-tRNA Synthesis

As in bacteria, mitochondria of eukaryotic organisms depend upon formylmethionyl-tRNA to initiate protein synthesis. The tRNA^{fMet} is charged with methionine by the methionyl-tRNA synthetase. The methionyl-tRNA^{fMet} is then modified by methionyl-tRNA formyltransferase (MTF) yielding a formylmethionyl-tRNA^{fMet}. The formyl donor is the N10-formyltetrahydrofolate (FTHF), a common donor of formyl groups in biosynthesis reactions. The formylation reaction involves a highly specific protein-tRNA recognition process. In trypanosomatids, which have to import all the tRNAs to the mitochondria to allow organellar protein synthesis, a special situation is encountered, where all the tRNAs are of the eukaryotic-type only. This case has

been studied in detail in *T. brucei* and, although we can find two distinct $tRNAs^{Met}$ in the cytosol of these parasites as in all eukaryotes, an elongator $tRNA^{Met}$ (tRNAMet-e) and an initiator $tRNA^{Met}$ ($tRNA^{Met\text{-}i}$), the $tRNA^{Met\text{-}e}$ is the only imported and formylated $tRNA^{Met}$ in the mitochondria of *T. brucei*. Therefore, the $tRNA^{Met\text{-}e}$ is used as an elongator and as an initiator $tRNA^{Met}$ in this compartment, but only as an elongator in the cytoplasm. Interestingly, the trypanosomatid $tRNA^{Met\text{-}e}$ lacks all of the known architectural features recognized by the mitochondrial MTF in the bovine system and consequently its mitochondrial MTF recognizes different identity determinants of those known to be important for mitochondrial or bacterial MTFs. In fact, *T. brucei* MTF has an unusual sequence, in which the linker region, also present in other MTFs, is longer. Also, the trypanosomatid MTF has twice the size of any other MTFs (Tan et al. 2002). The knockdown of the *T. brucei* MTF was not shown to be deleterious for its growth, but the MTF activity in this experiment was not totally abolished and the small fraction of fmet-$tRNA^{Met\text{-}e}$ present in the mitochondria could be enough for the parasite's survival. *In vivo* experiments to test the survival of the knocked down parasites have not been tried in mice nor in insects. Also, as stated by Martin (Martin 2002), further knowledge about the compensatory changes that may happen with the mitochondrial translational system upon genetic manipulation of parasites are needed to help us have a final answer about the essentiality of this enzyme.

4.2.3 The Selenocysteinyl-tRNA Formation Pathway

The belief of the existence of 61 tRNAs for the 20 standard amino acids and three codons used as terminators of protein synthesis has been settled in our minds since the unraveling of the genetic code (Crick 1968). Nevertheless, since 1979 it is known that the human and yeast mitochondrial genes of cytochrome oxidase subunit II have UGA coding for tryptophan and AUA for methionine (Chance et al. 1979). This discovery was followed by similar findings in many others organisms (Lozupone et al. 2001) and several code variants are known (e.g. *Tetrahymena* and *Paramecium* translate UAA and UAG as glutamine) (using only UGA as stop) (Lozupone et al. 2001; Sánchez-Silva et al. 2003). Euplotes instead translates UGA as cysteine, using UAA and UAG for termination (Lozupone et al. 2001; Sánchez-Silva et al. 2003). The conviction of a universal code was accompanied by another concept: there were only 20 amino acids cotranslationally inserted into a polypeptide. The observations that the 21st amino acid, selenocysteine, was coded by UGA and was cotranslationally inserted during protein synthesis by its own specific tRNA again challenged the understanding of the genetic code (Böck et al. 1991). As mentioned above, today we know of another amino acid cotranslationally inserted into proteins, pyrrolysine (the 22nd amino acid), that shows a very narrow distribution among all the organisms whose genomes have been sequenced until now (Hao et al. 2002). Pyrrolysine has not been observed in eukaryotic genomes yet. Conversely, selenocysteine is widely distributed among the three Domains of life and it is a special amino acid for the fact that it is the carrier of selenium, which plays an

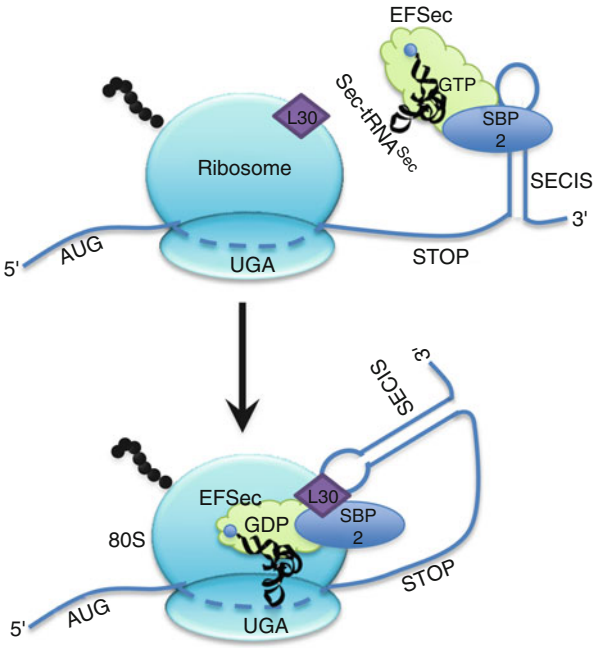

Fig. 12.5 Model for Selenocysteine insertion in eukaryotes. The SECIS binds SBP2, which recruits EFsec and Sec-tRNA^{Sec}. Upon association with the ribosome, SBP2 exchanges for L30. A conformational change in the SECIS results in the release of the Sec-tRNA^{Sec} and GTP hydrolysis (Adapted from Chavatte et al. 2005)

essential catalytic role as a component of selenoproteins. Most selenoproteins are oxidoreductases, preventing damage to cellular components by regulating the redox state of proteins or having other redox functions (Lobanov et al. 2009). To achieve selenocysteine synthesis, eukaryotes first serylate tRNA^{Sec} by seryl-tRNA synthetase (SerRS), giving Ser-tRNA^{Sec}. Ser-tRNA^{Sec} will be the substrate for O-phosphoseryl-tRNA kinase (PSTK), resulting in O-phosphoseryl-tRNA^{Sec} (Sep-tRNASec), which is converted by O-phospho-L-seryl-tRNASec: L-selenocysteinyl-tRNA synthase (SepSecS) into Sec-tRNA^{Sec} (Carlson et al. 2004; Kaiser et al. 2005; Yuan et al. 2006). SepSecS uses selenophosphate, produced by selenophosphate synthase (SPS2) (Xu et al. 2007) as the selenium donor. In order to get selenocysteine inserted in response to a UGA codon, a specific elongation factor that recognizes Sec-tRNA^{Sec} (eEFSec) (Fagegaltier et al. 2000), and a stem loop present far downstream of the UGA codon in the selenoprotein's mRNA must be present. This stem loop structure is called a Sec-insertion sequence element or SECIS-element (Berry et al. 1993) and it interacts with eEFSec by the means of the SECIS binding protein (SBP2) (Copeland et al. 2000) and the ribosomal protein L30 (Chavatte et al. 2005). Previously it was thought that SBP2 worked as a static anchor recruiting eEFSec to the SECIS element (Kinzy et al. 2005). However, Caban and Copeland (Caban and Copeland 2012) have shown that the SBP2-ribosome interaction leads to the modification of a specific set of rRNA residues, H89 and ES31, attributing a more dynamic role for SBP2 during Sec incorporation. Nonetheless, these are very recent findings and more detailed studies of the precise

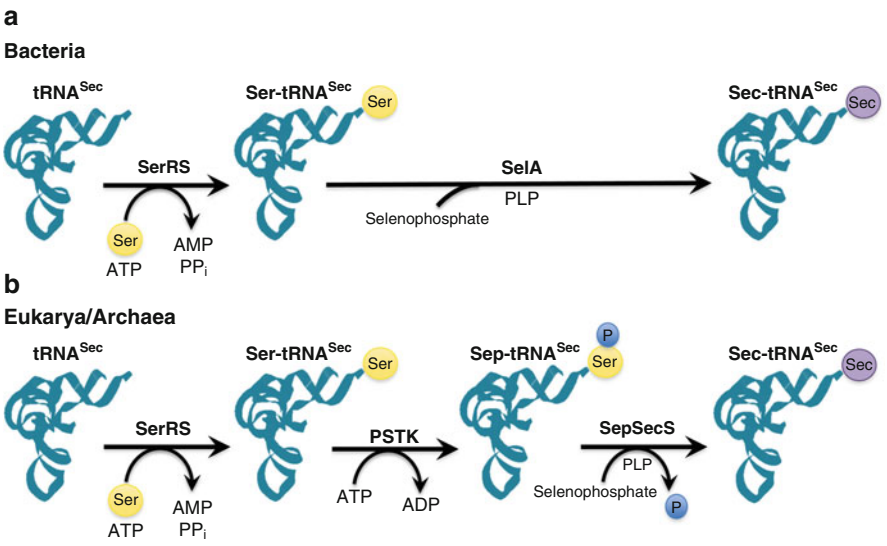

Fig. 12.6 Sec-tRNA^{Sec} formation pathway in the different Domains of life. In all organisms of the three Domains of life in which there is selenocysteine (Sec), first SerRS aminoacylates tRNA-^{Sec}, forming Ser-tRNA^{Sec}. (**a**) In Bacteria, the serine (Ser) bound to the tRNA is directly converted to Sec by SelA (Selenocysteine synthase) in the presence of selenophosphate, yielding Sec-tRNA^{Sec}. (**b**) In Eukarya and Archaea, the Ser on tRNA^{Sec} is phosphorylated by PSTK (O-phosphoseryl-tRNA kinase), forming Sep-tRNA^{Sec}. The phosphoserine (Sep) bound to the tRNA is then converted to Sec by SepSecS (Sep-tRNA:Sec-tRNA synthase), in the presence of selenophosphate, which yields Sec-tRNA^{Sec}. PLP (pyridoxal phosphate) is a cofactor of SelA and SepSecS (Sheppard et al. 2008). For a review on bacterial Sec-tRNA^{Sec} synthesis see Bock et al. (2005)

functional role of SBP2 are necessary (Caban and Copeland 2012). Regardless of the fact that Sec incorporation in all Domains of life requires an in-frame UGA codon that specifies Sec, a unique Sec-tRNASec, a unique elongation factor to deliver the tRNA and a cis-acting structural element in the selenoprotein mRNA, the mechanisms of Sec cotranslational insertion vary greatly among them. A eukaryotic model for Sec insertion into selenoproteins is shown in Fig. 12.5 and the differences in the selenocysteinylation of tRNA^{Sec} in Bacteria and Eukarya/Archaea are given in Fig. 12.6 (for review see refs: Sheppard et al. 2008; Squires and Berry 2008; Yoshizawa and Böck 2009; Cobucci-Ponzano et al. 2012).

Two independent articles report the search for selenoprotein genes in Kinetoplastida; the results of these studies were complementary to each other (Cassago et al. 2006; Lobanov et al. 2006). Almost all the components of the eukaryotic Sec insertion system were identified, including the tRNAs^{Sec}. As for selenoproteins and their SECIS elements, three were found by Lobanov and coworkers (2006) and one by Cassago (2006). Among these proteins there were homologs of mammalian SelK and SelT and two novel Kinetoplastida-specific selenoproteins designated SelTryp (Lobanov et al. 2006), and Sel1 (Cassago et al. 2006). Different

from other organisms, the SPS2 coding sequence doesn't have an in-frame UAG codon in any of the trypanosomatids (Jayakumar et al. 2004; Cassago et al. 2006; Sculaccio et al. 2008).

Along with the fact that trypanosomatids have two new uncharacterized selenoproteins, an interesting observation that warrants further investigation, the only seryl-tRNA synthetase present in the genome of *T. cruzi* shows a K_{cat}/K_m for serylation of $tRNA^{Sec}$ that is seven times higher than that for $tRNA^{Ser}$, a very different value from what has been shown in other organisms. The human seryl-tRNA synthetase charges $tRNA^{Ser}$ tenfold more efficiently than $tRNA^{Sec}$ and the *E. coli* enzyme shows a 100-fold difference in favor of $tRNA^{Ser}$ (Geslain et al. 2006). Given the necessity of a more "specialized" serylation reaction for $tRNA^{Sec}$, together with the observation that auranofin, a highly specific inhibitor of eukaryotic selenoenzymes, is very toxic for bloodstream and procyclic stages of *T. brucei* (Lobanov et al. 2006), and given the importance of SPS2 for protection against H_2O_2 generated reactive oxygen species in bloodstream and procyclic *T. brucei* 29–13 cells (Costa et al. 2011), one can deduce that the Kinetoplastida selenoprotein system is permeated with interesting facts that need to be better understood.

5 Trypanosomal Aminoacyl-tRNA Synthesis as a Target for Antibiotics

One of the main targets of antibiotics is the protein synthesis machinery where one can find inhibitors of nearly all the stages of translation. In recent years there has been a growing interest in compounds that inhibit aaRSs and some success has been achieved in finding molecules that are specific for different classes of pathogenic organisms (Vondenhoff and Van Aerschot 2011). As described, for a molecule to be considered a good drug target, the inhibitor should be able to induce a loss of function that triggers the pathogen's death, it should be suitable for the design of a specific inhibitor and it should be difficult for the pathogen to develop resistance by mutation (Hurdle et al. 2005; Vondenhoff and Van Aerschot 2011). Also, more recently it has become common thinking that new drugs should be able to simultaneously hinder the function of more than one target, as it is less probable that the pathogen will be able to mutate all of them (Morphy and Rankovic 2005).

Aminoacyl-tRNA synthetases serve as all-in-one targets for drug design as they fit most of these principles. For example, they present enough evolutionary divergence between the Domains of life and at the same time, they can be greatly conserved across families of pathogens. There is also the possibility of inhibiting more than one phylogenetically related synthetase. Also, regardless of the vast conservation of core structural features, aaRSs from the different Domains of life have evolved distinctive structural features as additional inserted domains that give them special identities and even functions; these individual characteristics may enable specific drug design (Hurdle et al. 2005).

Of all the antibiotics in use nowadays that target aminoacyl-tRNA synthesis, only mupirocin is validated for clinical use. Mupirocin was originally isolated from *Pseudomonas fluorescens* and is an IleRS inhibitor that shows a 8,000-fold higher K_i for pathogenic over human aaRSs, being more effective against Gram-positive bacteria (Fuller et al. 1971; Hughes and Mellows 1980). It has also been recently tested against *Plasmodium falciparum* IleRSs and it kills the parasite at nanomolar concentrations, with a specificity towards the apicoplast enzyme, a more distant homolog of the human enzyme than the cytosolic one (Istvan et al. 2011). Mupirocin has not been tested against trypanosomatids yet.

Besides mupirocin, there are several inhibitors of aaRSs, some of which have been isolated from organisms, while others have been found by high throughput screening of compounds (Vondenhoff and Van Aerschot 2011). For diverse reasons these compounds are not in clinical use. However, their existence suggests that specific aaRS inhibitors should not be difficult to find.

For trypanosomatids there have been screenings for inhibitors of only LysRS and LeuRS (Farrera-Sinfreu et al. 2008; Ding et al. 2011; Koh et al. 2012; Zhao et al. 2012). Benzoxaborole derived *T. brucei* LeuRS inhibitors with IC50 as low as 1.6 μM were discovered. The most potent enzyme inhibitors in this study also showed excellent *T. brucei* parasite growth inhibition activity (Ding et al. 2011). In a separate investigation, a number of 2-pyrrolinones were discovered to be good LeuRS inhibitors and they have potential as lead compounds for the design of other drugs (Zhao et al. 2012).

As far as LysRS is concerned, a library of proline derivatives specifically designed to mimic the lysyl adenylate complex was prepared on solid-phase support which led to the identification of three compounds that *in vitro* inhibit the *T. brucei* LysRS. Inhibition constants (*K*i) for the compounds that showed strong inhibitory effect were between 34 and 10 μM. Their K_i values were comparable to the *K*m for lysine and $tRNA^{Lys}$, which suggests that they are good competitors against the natural LysRS substrates (Farrera-Sinfreu et al. 2008).

Another recent study involved solving the structures of *T. brucei* MetRS in complex with five inhibitors, and a low molecular weight compound. The sequence identity of the catalytic core of *T. brucei* MetRS with both *L. major* and *T. cruzi*'s enzymes is 68 %, but is 100 % when only considering the active sites. The sequence of the catalytic core of *L. major* MetRS is 89 % and 97 % identical to *L. infantum* and *L. brasiliensis* enzymes, respectively. Furthermore, these complexes are valuable models for further development of anti-trypanosomal MetRS compounds (Koh et al. 2012; Larson et al. 2011b).

Clearly, the repertoire of distinguished features seen in trypanosomatid aaRSs is specially suited for research on rational drug design. Only a few compounds have been tested against a very small group of aaRSs. The diseases caused by trypanosomatids have a detrimental impact on public health, and, together with the fact that the existing drugs are very toxic and vaccines are unavailable, resistant strains are starting to appear. There is an urgent need for new drugs against these diseases; aaRSs present a promising target for drugs that will ultimately reduce the suffering from trypanosomatid-related illness.

6 Conclusions

The investigation of trypanosomal aaRSs has only begun recently and considerably more research is necessary in order to better understand their characteristics. Nevertheless, based on what has been published until now, it is evident that these enzymes have unique features. Furthermore, the trypanosomal tRNA aminoacylation-dependent pathways have interesting attributes that warrant further exploration. While the aaRSs were traditionally considered to be a highly conserved group of enzymes that play a universal role in tRNA aminoacylation, studies performed in the last few decades have demonstrated how diverse they can be, even if looking exclusively at their aminoacylation modes, which have been better elucidated by the numerous crystal structures available and experimental studies performed. On close examination, important differences between aaRSs or better yet, tRNA aminoacylation pathways of different organisms are evident, which should be exploited for the rational design of drugs. Lastly, the existence of accessory domains appended to the aaRSs (some known to confer upon aaRSs non-aminoacylation related roles in the cell, see reference Paul and Schimmel 2013) can make aaRS specific for the same amino acid in different organisms, structurally unique. These distinct characteristics of the trypanosomatid tRNA aminoacylation systems should be explored in more depth in order to expand the possibilities for the rational design of drugs against these parasites.

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Chapter 13
The Expected Outcome of the *Trypanosoma cruzi* Proteomic Map: A Review of Its Potential Biological Applications for Drug Target Discovery

Rubem F.S. Menna-Barreto and Jonas Perales

Abstract Chagas disease is a neglected tropical illness endemic to Latin America, and its treatment remains unsatisfactory. This disease is caused by the hemoflagellate protozoan *Trypanosoma cruzi*, which has a complex life cycle involving three evolutive forms in both vertebrate and invertebrate hosts. Targeting metabolic pathways in the parasite for rational drug design represents a promising research field. This research area requires high performance techniques and proteomics become a powerful tool in this context. Here, we review advances in the construction of proteomic maps of the different forms of *T. cruzi*, emphasizing their biological applications towards the identification of alternative candidates for drug intervention.

Abbreviations

2-DE	Two-dimensional electrophoresis
GPI	Glycosilphosphatydilinositol
LC-MS/MS	Liquid chromatography and tandem mass spectrometry
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
PAF	Platelet-activating factor

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1 State of the Art

The hemoflagellate protozoan *Trypanosoma cruzi* is the etiological agent of Chagas disease, a neglected illness endemic to Latin America that emerges in non-endemic countries due to the globalization of immigration (Schmunis 2007). The evolution of the disease involves an acute phase, where a patent parasitemia can be detected, followed by a progressive chronic phase (Marin-Neto et al. 2009; Rassi et al. 2009). In the later phase, the disease pathogenesis involves cardiac and/or digestive alterations that are likely derived from prolonged inflammation induced by the persistent parasitemia (Rocha et al. 2007; Sosa-Estani et al. 2009).

The current treatment for Chagas disease is based on two nitroheterocycles (nifurtimox and benznidazole) that were introduced four decades ago. These compounds are highly efficacious against the acute infection, but they also lead to substantial side effects and have limited activity against different parasite isolates and strains. The poor efficacy of these drugs during the chronic phase reinforces the need to search for novel active compounds against this disease (Urbina and Docampo 2003; Jannin and Villa 2007; Soeiro and DeCastro 2011). For this reason, the investigation of parasite-specific molecules as alternative chemotherapy targets has been extensively performed, with the aim of reducing the toxicity to mammalian cells (reviewed in De Souza 2002). It is well known that the *T. cruzi* life cycle involves different hosts and life forms, and the mammalian stage trypomastigotes and amastigotes are the relevant forms for clinical drug intervention. However, the insect stage epimastigotes are still extensively employed as experimental models due to their proliferative characteristics in axenic cultures.

Trypanosomatids have many special molecular and cell biology features, making the development of the *T. cruzi* genome project a crucial step for the advancement of chemotherapy (El-Sayed et al. 2005). Among these uncommon characteristics are the organization of the open reading frames into long polycistronic regions and post-transcriptional processing that makes the regulation of gene expression in these protozoa peculiar (Clayton and Shapira 2007). The regulation depends on the stability and translation of each mRNA, and the protein content is directly related to this post-translational control (Parodi-Talice et al. 2004; Andrade et al. 2008). More recently, the presence of non-translated mRNA was detected in the *T. cruzi* cytosol (Holetz et al. 2010), reinforcing the notion that RNA-based techniques may be limited for the evaluation of parasite gene expression. Therefore, the proteomic approach has become increasingly attractive.

The application of high-throughput proteomics is crucial for the functional and structural analysis of diverse experimental conditions and can have implications for the discovery of the physiological role of proteins and accurate genome annotations (Ferella et al. 2008). The *T. cruzi* proteomic map has been assessed for the identification of evolutive stage-specific proteins, and certain molecules involved in drug resistance have also been described (Parodi-Talice et al. 2004; Atwood et al. 2005; Andrade et al. 2008; Menna-Barreto et al. 2010).

2 *T. cruzi* Proteomic Map: An Overview

The first *T. cruzi* proteomic analysis was performed by Paba and colleagues (2004) on epimastigotes, culture-derived trypomastigotes and amastigotes (Berenice strain) using two-dimensional electrophoresis (2-DE) and mass spectrometry based on matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF). Differences between epimastigote and trypomastigote protein contents were additionally established during the *in vitro* differentiation of trypomastigotes into amastigotes in axenic acidic conditions. Nineteen proteins, including heat shock proteins, elongation factors, glycolytic enzymes and cytoskeletal components, were identified. Interestingly, paraflagellar rod proteins were more highly expressed in trypomastigotes than in epimastigotes and amastigotes (2.4 and 15-fold, respectively), suggesting that this structure is more prominent in the infective forms. The data also revealed an 8.6-fold increase in enolase 2 expression in trypomastigotes in relation to epimastigotes. Tielens and Van Hellemond (1998) described that *T. brucei* bloodstream forms are more dependent on glycolysis in comparison to the insect forms. Recently, Gonçalves and colleagues (2011) demonstrated similar features in *T. cruzi*, indicating that the mitochondria of epimastigotes are more efficient than this organelle of trypomastigotes. Consequently, the insect forms are not as glycolytic as the bloodstream trypomastigotes.

Several months after the first *T. cruzi* proteome publication, a description of the protein content of epimastigotes (Dm28c clone) was performed by a similar approach (Parodi-Talice et al. 2004). From 70 colloidal Coomassie-stained spots, 22 different proteins were identified, most of which belonged to the metabolic pathways of the parasite. Other identified proteins included structural proteins related to cytoskeleton assembly, chaperonins and proteins involved in polypeptide biosynthesis. Several spots corresponding to prostaglandin F2a synthase, an important enzyme for the antioxidant defenses of the parasite, were also identified, implicating a crucial role for the detoxification process (Kubata et al. 2002).

In 2005, a large-scale descriptive proteomic study was performed by comparing the protein content between *T. cruzi* (Brazil strain) epimastigotes, metacyclic trypomastigotes and culture-derived amastigotes and trypomastigotes (Atwood et al. 2005). Employing a shotgun liquid chromatography and tandem mass spectrometry (LC-MS/MS) approach, Atwood and colleagues described 2,784 proteins, 838 of which were detected in all the life-stages. The number of identified proteins was dependent on the evolutive form of the parasite; the metacyclic form had the highest number of identified proteins (2,339), followed by amastigotes (1,871), epimastigotes (1,861) and trypomastigotes (1,486). In this work, it was also observed that 36 % of the proteins identified (1,008/2,784) were hypothetical proteins. Among the main protein groups identified were surface proteins, such as *trans*-sialidase, GP63 and mucins, and molecules associated with polypeptide biosynthesis and degradation (ribosomal, proteasomal and chaperonins). From these data, several hypotheses were created to explain the differences in protein expression between the different life stages. For example, proteins involved in vesicular trafficking were

preferentially found in amastigotes, suggesting that trafficking processes are active in this stage. Another interesting observation was that the antioxidant defenses of the parasite increased dramatically during the differentiation of epimastigotes into metacyclic trypomastigotes. Key antioxidant defense enzymes, such as ascorbate peroxidase, mitochondrial tryparedoxin peroxidase, trypanothione synthase and iron superoxide dismutase, were highly expressed in the metacyclic forms, suggesting a preadaptation of the parasite to the oxidative burst inside the vertebrate host, particularly in phagocytes. It will be necessary to validate these hypotheses by different techniques.

Although Atwood and coworkers (2005) identified proteins from metacyclic trypomastigotes and epimastigotes, the comparisons were performed on completely differentiated parasites, and the changes in protein levels of parasites undergoing differentiation needed to be clarified. A proteomic study examining metacyclogenesis (Dm28c) was then performed, where 43 differentially expressed proteins were identified by MALDI-TOF (Parodi-Talice et al. 2007). As previously observed by Parodi-Talice and colleagues (2004) in epimastigotes, the proteins related to metabolism were the most identified, as well as electron transport and antioxidant defenses enzymes, proteases, structural organization and hypothetical proteins were also identified**.** Parodi-Talice and coworkers (2007) hypothesized that the increase in glutamate dehydrogenase expression could be due to increased amino acid uptake by the parasite during nutritional stress. They also suggested that post-translational modifications were an essential strategy of the parasite for regulating gene expression during differentiation.

In order to specifically investigate the alkaline protein content, a 6–11 pH gradient 2-DE was standardized for epimastigote and trypomastigote (Berenice strain) samples (Magalhães et al. 2008). As expected, using the separation range of pH 6–11, the most frequently identified proteins were related to amino acid metabolism and dehydrogenases in epimastigotes and *trans*-sialidases and paraflagellar rod proteins in trypomastigotes. These authors also suggested the use of this approach to further evaluate the alkaline proteome of *T. cruzi* amastigotes during the differentiation process.

In 2009, the detergent-solubilized membrane proteins were investigated in *T. cruzi* insect forms, stationary epimastigotes and metacyclic trypomastigotes (G strain) by LC-MS/MS (Cordero et al. 2009). Employing the neutral detergent Triton X-114, 280 and 98 proteins were identified in metacyclic forms and epimastigotes, respectively. Most of them displayed lipid post-translational modifications, such as GPI-anchor, myristoylation or prenylation. Conversely, the majority of identified polypeptides expressed by proliferative epimastigotes were related to metabolic pathways, likely due to the high energy requirements of parasite mitosis. Interestingly, the huge repertoire of surface glycoproteins observed in the infective forms has been associated with the participation of glycoconjugates in adhesion and host cell invasion processes. In the same year, the proteome of CL Brener strain epimastigotes was assessed for the first time by a 2-DE and MALDI-TOF approach (Sodré et al. 2009). This analysis revealed multiple tubulin and heat shock protein isoforms and prostaglandin F2a synthase, as previously

observed (Kubata et al. 2002; Parodi-Talice et al. 2004), and arginine kinase, an important enzyme of the polyamine pathway, for the first time.

T. cruzi is exposed to different environmental conditions during its life cycle, and the success of the infection is dependent on the capacity of the parasite to adapt. The temperature changes between the triatomine midgut (28°C) and the mammalian bloodstream (37°C) strongly indicate the adaptation potential of *T. cruzi*. Recently, epimastigotes (Ninoa strain) were submitted to heat shock stress (37 and 42°C), and the proteomic profile was evaluated by 2-DE and MALDI-TOF analysis (Pérez-Morales et al. 2012). Twenty-four spots were modulated by the temperature increase, including heat shock protein 70, ATPases, ATP synthase, thiol transferase, ubiquitin hydrolase, surface glycoproteins, retrotransposon hot-spot protein, tryparedoxin peroxidase and several hypothetical proteins. The overexpression of surface molecules related to infectivity and virulence during the temperature increase suggested a possible connection between the stress response and the infection. This was the first report of the involvement of proteins other than chaperonins in the *T. cruzi* heat shock stress response.

More recently, an in-depth quantitative MS-based proteomic approach was employed to evaluate the metacyclogenesis process in *T. cruzi* (de Godoy et al. 2012). By analyzing the different intermediary stages of the parasites undergoing differentiation and identifying the peptides by LC-MS/MS, de Godoy and colleagues performed the most comprehensive quantitative proteomic study in *T. cruzi* to date, quantifying approximately 3,000 proteins involved in metacyclogenesis. Among the proteins differentially expressed in the metacyclic forms, *trans*-sialidase was one of the most abundant, presenting 18 isoforms that were upregulated in trypomastigotes. These data were in accordance with previous studies that demonstrated the role of cyclic-AMP in the induction of metacyclogenesis (Gonzales-Perdomo et al. 1988). Cytoskeletal proteins (tubulin, actin, dyneins and paraflagellar rod components) were also remarkably upregulated upon differentiation to trypomastigotes, most likely due to the important morphological changes that occur during metacyclogenesis. Changes in the kinetoplast and flagellar pocket positions, as well as in the flagellar attachment zone, could explain such increases in cytoskeletal protein levels. Surprisingly, four nuclear ribonucleoproteins involved in the splicing process and five histones were upregulated in metacyclic trypomastigotes, suggesting a possible increase in gene regulation in the infective forms of the parasite, although this hypothesis must be tested experimentally. The most important proteins downregulated in the metacyclic forms were ribosomal proteins, tRNA synthetase and elongation factors, as previously described by Marchini and colleagues (2011). However, the reason for this downregulation remains unclear.

2.1 Sylvatic Isolates

Polymorphic DNA analysis was employed to separate the taxa into phylogenetic lineages called discrete typing units, referred to as *T. cruzi* I to VI, considering

eco-epidemiological features, pathogenicity and the parasite biology (Zingales et al. 2009). A preliminary comparative assessment of the proteomic profile was performed between epimastigotes of isolates 3663 (*T. cruzi* III) and 4167 (*T. cruzi* IV) and reference strains CL Brener and Dm28c by 2-DE and MALDI-TOF/TOF (Kikuchi et al. 2010). Fifty-seven proteins identified from these isolates were involved in different cellular functions, such as parasite metabolism, protein assembly, stress response and cell-to-cell communication.

Surprisingly, the 4167 strain was more infectious *in vitro* than the 3663 isolate. The proteomic analysis confirmed that this difference is related to the increased expression of proteins associated with virulence, including glutathione and tryparedoxin peroxidases, S-adenosylmethionine synthetase, arginine kinase and cruzipain. Flow cytometric assays confirmed the evidence that cruzipain is more highly expressed in 4167, CL Brener and Dm28c rather than in the 3663 strain, suggesting a correlation between virulence and the heterogeneity of the parasite surface composition. This is the only report on the *T. cruzi* IV proteomic profile, and further studies are needed to fully comprehend the crucial characteristics of these important parasite stocks in nature.

2.2 Sub-Cellular Fractions

The first large-scale study of *T. cruzi* protein localization was performed on the insect form (CL Brener strain) using LC-MS/MS, and demonstrated the importance of enriching organellar fractions for the identification of novel proteins (Ferella et al. 2008). In this work, 38 novel proteins that had not been detected in the whole epimastigote proteome were identified. Immunofluorescence assays were performed with five hypothetical proteins that were localized to organelles, such as the endoplasmic reticulum, acidocalcisomes, mitochondria and cytosolic vesicles. Ferella and colleagues (2008) also described the expression of almost all the enzymes from the tricarboxylic acid cycle and the iron-sulfur subunit of succinate dehydrogenase, which were not identified by the whole cell approach (Atwood et al. 2005). Therefore, this type of analysis is fundamental because it provides the subcellular localization and identification of many proteins, including those that might not have previously been identified using different approaches.

Subsequently, the reservosome sub-proteome was the first specific *T. cruzi* subcellular profile to be published (Sant'Anna et al. 2009). This organelle represents an endpoint of the endocytic pathway and is only found in the epimastigote forms. Reservosomes are acidic compartments where proteins and lipids are stored. They are not considered to be typical lysosomes due to the absence of lysosomal molecular markers, such as acid phosphatases and differences in pH (lysosomal pH is 5 and reservosomal pH is 6). Reservosome fractions were obtained from epimastigotes (Dm28c), the purification process was monitored by transmission electron microscopy, and the protein identifications were performed by LC-MS/MS. Of the 709 parasite proteins identified, cruzipain, serine carboxypeptidase, ABC transporters

and protein tyrosine phosphatase had previously been described in reservosomes, and a novel P-type H^+ -ATPase was detected for the first time. Many hydrolases, such as cysteine peptidases, α-mannosidases, acid phosphatases, calpains, lipases and serine carboxypeptidase were also identified, indicating that the reservosomes are a main site of lysosomal hydrolases, despite the lack of some well-known lysosomal markers of other organisms.

Among the adverse environmental conditions that *T. cruzi* is exposed to during its life cycle, the osmotic variation between mammalian blood and the insect midgut is a strong barrier. Previous osmotic stress studies have shown that the contractile vacuole complex participates in the parasite regulatory volume process (Rohloff and Docampo 2008). Despite its fundamental importance for parasite homeostasis, knowledge of this structure and its physiological implications for trypanosomatids is very poor. In 2011, Ulrich and coworkers examined the sub-proteome of the contractile vacuole complexes of CL Brener epimastigotes using one-dimensional gel electrophoresis and LC-MS/MS. A total of 220 proteins were identified in the contractile vacuole-enriched fraction, including 74 hypothetical proteins and 39 members of the dispersed gene family 1, whose function in the parasite is unclear. Amastins, calpains, transmembrane glycoproteins, transporters, channels and vacuolar-H^+-pyrophosphatases were also detected in these structures. The identifications were validated by ultrastructural immunolocalization; Rab11, Rab32, AP180, ATPase subunit B, VAMP1, and phosphate transporters predominantly localized to the vacuole bladder, and two isoforms of TcSNARE and calmodulin localized to the spongiome. These data provided insights into the biogenesis of these organelles and helped to formulate hypotheses regarding the possible physiological roles of the *T. cruzi* contractile vacuole. Furthermore, the data showed that subcellular proteomics can be essential in the determination of protein localization and function. However, the possibility of contaminants from other cellular compartments represents an important limitation of this approach. Therefore, further morphological and biochemical assays must be performed to confirm the data.

2.3 Post-Translational Modifications

The first *T. cruzi* glycoproteomic analysis was performed in culture trypomastigotes (Brazil strain) by LC-MS/MS in 2006 (Atwood et al. 2006). In order to increase the number of identified proteins, the parasites were subcellularly fractioned, and each fraction was isotopically labeled. Twenty-nine glycoproteins were identified, 11 of which were *T. cruzi*-specific glycoproteins that were detected at the protein level for the first time. Among those identified proteins, the mucin-associated surface proteins and dispersed gene family members displayed N-linked glycan post-translational modifications.

Another essential post-translational modification that has already been studied in *T. cruzi* by proteomic approaches is phosphorylation. Phosphorylation events are directly implicated in cell signaling transduction networks, which regulate numerous

biological processes. The involvement of kinases in the parasite biology could represent an interesting possibility for drug intervention. The *T. cruzi* phosphoproteome was assessed in Y strain and Dm28c epimastigotes by LC-MS/MS (Nakayasu et al. 2009; Marchini et al. 2011). The first published analysis revealed 119 distinct proteins associated with important biological processes, such as cell motility, metabolism, ion transport and differentiation (Nakayasu et al. 2009). In 2011, Marchini and colleagues identified 753 phosphoproteins and 2,572 phosphorylation sites using a highly accurate LC-MS/MS approach. These data will be reviewed in more detail in Chap. 15.

Recently, additional insights into the post-translational modifications of Y strain epimastigotes were obtained by employing 2-D gel specific-staining protocols with the fluorescent dyes Pro-Q-Emerald and Pro-Q-Diamond for glycoproteins and phosphoproteins, respectively (Beghini et al. 2012). Fifteen glycoproteins were identified, including ATPase subunits, tubulin, heat shock proteins, peroxiredoxin, prostaglandin F2a synthase, sterol 24-c-methyltransferase, tryparedoxin peroxidase and tyrosine aminotransferase. Additionally, 22 phosphoproteins were identified, including 14-3-3 protein, prolyl oligopeptidase, actin, tubulin, aminoacylase, chaperonins, enolase, initiation factor, glutamine synthetase, several hypothetical proteins, paraflagellar rod protein, peroxiredoxin, proteasome subunit, pyrroline-5-carboxylate synthetase and spermidine synthase.

3 Susceptibility and Drug Resistance

The development of drug resistance is an important challenge for chemotherapy approaches and poses a major clinical problem for bacterial, fungal and parasitic infections. In Chagas disease, the increase of benznidazole resistance has led to a rise in therapeutic failure, enhancing a genotypic variety of the parasite. The molecular mechanisms involved in *T. cruzi* resistance and susceptibility to benznidazole were investigated in 2008 by 2-DE and MALDI-TOF/TOF using Y strain epimastigotes as a model (Andrade et al. 2008). For these analyses, resistant parasites were selected *in vitro* and *in vivo*. Thirty-six distinct proteins, including calpain, several other peptidases (metallocarboxipeptidase, peptidase M20, and mitochondrial peptidases), tyrosine amino transferase, cyclophilin A, glutamate dehydrogenase, iron superoxide dismutase, nucleoside diphosphate kinase, peroxiredoxin and a hypothetical protein, were described in the resistant parasites, suggesting an adaptation to drug stress conditions. Interestingly, the overexpression of prostaglandin F2α synthase in the susceptible epimastigotes may indicate the participation of benznidazole in the detoxification processes of anion radical production, which could kill the parasite (Murta et al. 2006).

In the search for alternative treatments for Chagas disease, our group has been working on the trypanocidal effect of naphthoquinones and their derivatives for the last 15 years, and three naphthoimidazoles are the most promising compounds (Menna-Barreto et al. 2005, 2007). The proteomic analysis showed that the

mitochondrial proteins were the most frequent differentially expressed proteins in the treated parasites, reinforcing the morphological data. Additionally, glutamamyl carboxypeptidase, cystathionine beta-synthase, tyrosine aminotransferase, sterol 24-c-methyltransferase, elongation factors, proteasomal subunits, chaperonins and tubulin were also involved in the epimastigote response to drug treatment (Menna-Barreto et al. 2010). One of the proteins that were overexpressed upon treatment with the three naphthoimidazoles was trypanothione synthetase, a key enzyme in the antioxidant pathway of trypanosomatids. Because naphthoimidazoles do not possess redox properties that could lead to the generation of reactive oxygen species, these mechanisms are still under investigation in our laboratory.

4 Molecular Candidates for Drug Interventions and Vaccine Development

In the search for the ideal drug alternative to benznidazole in the clinical treatment of Chagas disease, the promising compound should have high trypanocidal activity, strong efficacy against acute and chronic infections, low costs and reduced side effects (Nwaka and Hudson 2006). It is crucial that this novel drug interferes with a vital pathway that is exclusive to *T. cruzi*. Many proteomic studies have been conducted to discover new and interesting molecular targets in the parasite.

The parasite surface molecules involved in recognition and entry into the host cells could be good targets for drug intervention. Platelet-activating factor (PAF) plays a crucial role during infection by triggering *T. cruzi* differentiation, and the impairment of this process by incubation with a PAF antagonist suggests the presence of a specific receptor in the parasite. Bioinformatic analysis of 23,000 protein sequences led to the identification of 29 hypothetical proteins that share G protein-coupled receptor features with the PAF receptor. However, further evaluation must be performed to test those receptors as chemotherapy targets (Kawano et al. 2011). Kikuchi and colleagues (2010) proposed that specific cruzipain inhibitors could be valid drug alternatives when the cysteine protease is highly expressed on the parasite surface and is associated with the internalization process. In the same work, these authors suggested arginine kinase as a drug target. This enzyme is involved in the conversion of L-arginine to phosphoarginine. It also participates in the oxidative response and infectivity, as it was upregulated in two highly virulent strains (Kikuchi et al. 2010). The absence of arginine kinase in mammalian cells, together with its importance in parasite ATP synthesis, credits this enzyme as a potential target for new chemotherapies (Silber et al. 2005; Sodré et al. 2009). Glutamate dehydrogenase participates in the reversible deamination of L-glutamate to alpha-ketoglutarate because amino acids are the main carbon source for epimastigotes (Sodré et al. 2009). The involvement of amino acid biosynthesis and protein metabolism in the trypanocidal activity of trypanocidal compounds was previously reported, providing further evidence for the modulation of tyrosine aminotransferase expression as part of the mechanism of action of naphthoimidazoles and benznidazole (Andrade

et al. 2008; Menna-Barreto et al. 2010). Tyrosine aminotransferase is a crucial enzyme in the turnover of methionine, tyrosine, tryptophan and phenylalanine, suggesting that an increase in the misbalance of amino acids could represent an unexplored target for therapeutic drugs.

Our group has made many efforts to detect the expression of parasite proteins that do not share significant sequence similarities with the human proteome. This has been a rational approach that allows us to search for ideal targets while reducing the potential side effects to host cells and tissues. Some promising molecular targets are mentioned below based on our results with Y strain epimastigotes (Beghini et al. 2012). Among these targets, prostaglandin F2 alpha synthase is present in different *T. cruzi* strains (Parodi-Talice et al. 2004; Murta et al. 2006; Andrade et al. 2008; Sodré et al. 2009; Menna-Barreto et al. 2010). In *T. brucei,* prostaglandin F2 alpha synthase is involved in NADPH-catalyzed prostaglandin production, but the precise mechanism is still not clear. Its function has been associated with drug detoxification, especially for quinones in trypanosomatids (Kubata et al. 2000), suggesting that the inhibition of this enzyme may be a good target for Chagas disease chemotherapy.

Another crucial biosynthetic pathway in trypanosomatids is the ergosterol biosynthesis pathway, which diverges from mammals that only produce cholesterol. Sterols are essential for membrane fluidity, and consequently, the impairment of ergosterol production represents a promising alternative for specific anti-*T. cruzi* therapeutics (Urbina 2009). Menna-Barreto and coworkers (2010) described 24-C-methyltransferase, which is responsible for catalyzing the transfer of a methyl group from S-adenosyl-methionine to zymosterol and other Δ^{24} sterols, as a good option for drug interventions.

The *T. cruzi* redox system is regulated by reactive oxygen species levels, which are important for the signaling of a variety of cellular processes (Gonçalves et al. 2011). Interestingly, previous work has shown that the antioxidant machinery of the parasite is related to ROS resistance and increased virulence and infectivity, indicating that redox balance affects the success of infection (Piacenza et al. 2009). Trypanothione synthetase, peroxiredoxins and superoxide dismutases are fundamental enzymes for the anti-oxidant defenses in trypanosomatids (Andrade et al. 2008; Irigoín et al. 2008; Menna-Barreto et al. 2010). Therefore, the inhibition of a molecular target in this pathway could lead to a misbalance of ROS and parasite death.

Calpains are calcium-regulated cytosolic cysteine peptidases that participate in different cellular processes, such as cytoskeleton rearrangement, signal transduction and apoptosis, but the detailed role of these proteases is still unclear (Goll et al. 2003). In trypanosomatids, genomic analysis has demonstrated the presence of diverse calpains (Ersfeld et al. 2005), whose function is associated with differentiation and infectivity in *T. cruzi* (Ennes-Vidal et al. 2010, 2011). Evidence of the presence of a calpain-like cysteine peptidase in different parasite forms, together with the absence of a similar sequence in humans, suggests its potential as a drug target (Atwood et al. 2005; Andrade et al. 2008; Sant'Anna et al. 2009; Sodré et al. 2009; Kikuchi et al. 2010).

Phosphoenolpyruvate carboxykinase is a key enzyme involved in gluconeogenesis, and the three-dimensional structure of the *T. cruzi* enzyme has already been

solved (Trapani et al. 2001). This molecule, which exists in both cytosolic and mitochondrial isoforms, converts oxaloacetate into phosphoenolpyruvate (Cazzulo 1994). Due to significant differences in sequence and substrate specificity between the *T. cruzi* and human enzymes, it could be a promising target for the development of new anti-*T. cruzi* drugs (Beghini et al. 2012).

Nucleoside phosphorylase is a crucial enzyme in purine metabolism and is involved in the nucleotide salvage pathways, which have been previously described in trypanosomatids (Miller et al. 1987; Silva et al. 2011). Because the purine and pyrimidine salvage pathways are fundamental for nucleoside recovery after nucleotide degradation, this enzyme may also be used for future drug interventions.

The I/6 autoantigen was also a predicted protein in *T. cruzi* (El-Sayed et al. 2005) but was described at the transcriptional level in *T. brucei* (Detmer et al. 1997). Beghini and coworkers (2012) described this molecule for the first time at the protein level. It is possible that the I/6 autoantigen is a calcium-binding microtubule-associated protein that is involved in cross-linking microtubule filaments. The impairment of this structural protein could compromise important cytoskeleton-dependent processes, such as mitosis or vesicle trafficking, leading to parasite death.

T. cruzi sub-fractional proteomic studies have strongly increased the number of identified specific organelle proteins (Ferella et al. 2008; Sant'Anna et al. 2009; Ulrich et al. 2011). The characterization of different proteins in uncommon structures that are present in clinically relevant forms of the parasite, such as acidocalcisomes, glycosomes, the paraflagellar rod, the contractile vacuole and many others, endorses these proteins as promising candidates for the development of new drugs. However, despite the suggestion by Sant'Anna and coworkers (2009) of the reservosomal membrane pumps as a promising chemotherapeutic target, the absence of reservosomes in trypomastigotes and amastigotes strongly indicates that these molecules would not be good candidates for drug design.

While the *T. cruzi* plasma membrane provides many interesting potential novel drug targets, the parasite surface molecules may also lead to the discovery of new antigens for the development of Chagas diagnostic tests or vaccines (Cordero et al. 2009). Atwood and coworkers (2006) demonstrated a complex pool of N- and O-linked glycan residues on the trypomastigote surface, and 11 parasite-specific glycoproteins were described (i.e., mucin-associated surface proteins, dispersed gene family proteins, among others). These data provided a huge repertoire of potential cell vaccine targets to be validated, as well as insights for the design of novel specific inhibitors. Recently, Nakayasu and coworkers (2012) published a Y strain trypomastigote study to increase the proteomic identification of potential vaccine target candidates in *T. cruzi* by LC-MS/MS. Fourteen percent of the non-redundant sequences were glycosylphosphatidylinositol-anchored surface proteins related to pathogenesis. An immunobioinformatic analysis revealed the prediction of the binding of many proteins to major histocompatibility complex classes I and II, and 45 highly immunogenic epitopes were identified after a stringent analysis. These epitopes must be further validated for the development of an efficient Chagas disease vaccine. Additionally, peculiarities in the parasite signaling pathways could become interesting possibilities for drug interventions. Phosphorylation represents an essential step

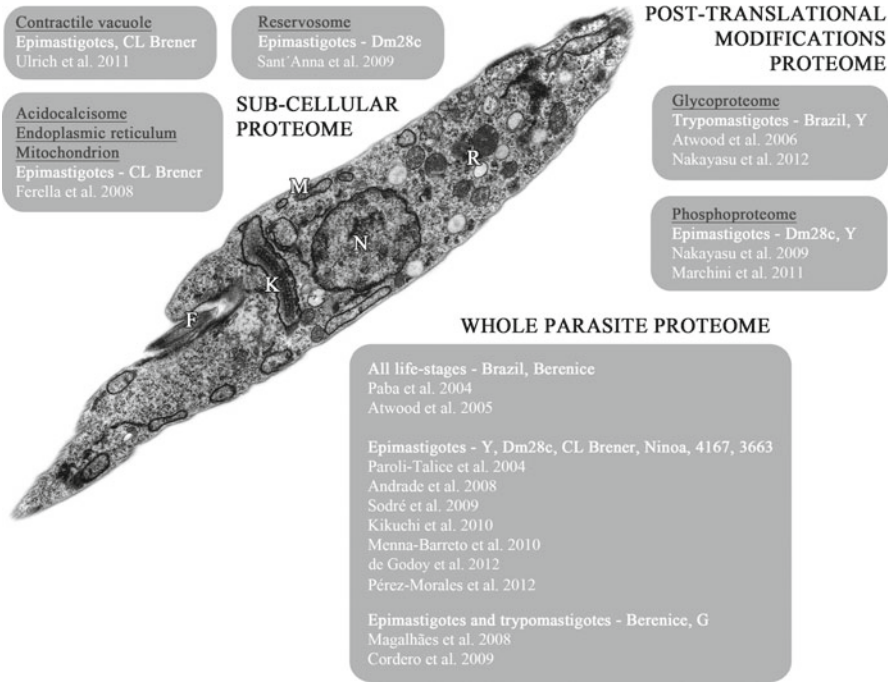

Fig. 13.1 Typical ultrastructural aspects of *T. cruzi* epimastigote and a summary of all the proteomic studies published on this parasite. All the references listed in this figure are cited in the text. *N* nucleus, *M* mitochondrion, *F* flagellum, *R* reservosomes, *K* kinetoplast

in cellular signaling and consequently to parasite survival, kinases, such as GSK3, may make excellent drug targets, as suggested by Nakayasu and colleagues (2009). New insights into the *T. cruzi* glycoproteome and phosphoproteome were investigated by 2D gel staining protocols with fluorescent dyes (Beghini et al. 2012). Seventy-five percent of the phosphoproteins identified after 2-DE and Pro-Q-Diamond labeling were also present in the phosphoproteomic study published by Marchini and colleagues (2011), validating this quick approach for the preliminary description of protozoan phosphoproteins. However, the true biological outcomes of these post-translational modifications have to be further characterized by other techniques. A summary of all the proteomic studies in *T. cruzi* is displayed in Fig. 13.1.

5 Chagas Disease Chemotherapy: Challenges and Perspectives

In 1909, a Brazilian researcher named Carlos Chagas discovered the protozoan *T. cruzi* and the illness that was subsequently referred to as Chagas disease. More than one century later, this neglected disease is still a serious public health problem,

particularly in endemic countries, such as Bolivia. One of the greatest challenges has been to find an effective chemotherapy alternative to benznidazole, and many medicinal chemistry research groups are working on the development of compounds that are being tested against the parasite. Intense interdisciplinary efforts have been made to increase the trypanocidal activity of these drugs and to reduce host toxicity (Romanha et al. 2010). Recent improvements in high-throughput screening facilities for the search for new trypanocidal compounds have allowed for the evaluation of several new potential drugs against intracellular amastigotes, a crucial step for the development of active compounds that will be evaluated in clinical trials (Buckner and Navabi 2010). Likewise, the use of synergic combinations and more practical formulations (e.g., pediatric) of currently available commercial drugs could also represent a rapid strategy and should not be neglected (Abad-Franch et al. 2010).

Indeed, the molecular mechanisms of action of the trypanocidal compounds are very poorly studied. Several previous biochemical and morphological studies indicated that the parasite organelles, structures and metabolic pathways, including the mitochondria, reservosomes, ergosterol and phospholipid pathways, respectively, were differentially affected by drugs in different forms of the parasite (Rodrigues et al. 2001; De Souza and Rodrigues 2009; Menna-Barreto et al. 2009c; Vannier-Santos and DeCastro 2009). Interfering with lipid biosynthesis led to important impairments of the plasma membrane, such as macromolecule uptake, ion exchange and many other essential cellular processes (Soeiro et al. 2010). It had previously been reported that several compounds deregulated lipid content, leading to alterations in the parasite plasma membrane (Santa-Rita et al. 2000, 2006; De Souza and Rodrigues 2009). Along with other promising targets of anti-*T. cruzi* drugs, the microtubule structures are consistently altered by drug treatment. All the trypanosomatids contain a set of stable microtubules, known as subpellicular microtubules, which are responsible for the morphological shape of the protozoa (Teixeira et al. 2012). Moreover, *T. cruzi* flagella contain a paraflagellar rod together with the typical axonemal conformation (9+2) (Rocha et al. 2010; Teixeira et al. 2012). Treatment with aromatic diamidines and naphthoquinones led to disorganization of the subpellicular microtubules, resulting in the partial loss of this structure and the appearance of unusual parasites containing multiple flagella (Silva et al. 2007; Batista et al. 2010; Fernandes et al. 2012).

Structures enriched for DNA (nucleus and kinetoplast) are commonly affected by different compounds. The *T. cruzi* genome is 60.3 Mb and is composed of 41 small chromosomes, presenting differences in the regulation of the transcription machinery in comparison to other eukaryotes offering another attractive target for drug interventions (Weatherly et al. 2009; Soeiro et al. 2010). Previous studies have demonstrated that drugs that interact with *T. cruzi* DNA, such as aromatic diamidines and naphthoquinone derivatives, induce severe chromatin condensation and fragmentation (Menna-Barreto et al. 2005, 2007, 2009a, c; De Souza et al. 2006). One of the most particular cellular characteristics of trypanosomatids is the presence of a single mitochondrion. Interestingly, all protozoa mitochondrial DNA is contained in a specialized mitochondrial region called the kinetoplast, which represents nearly 20 % of the total parasite genome (Shapiro and Englund 1995). Other features, such as the presence of alternative oxidases and the lack of an

Table 13.1 The main ultrastructural targets of drugs against *T. cruzi*

Drug	Parasite target	Putative action	Reference
Naphthoimidazoles	Mitochondrion; kinetoplast; reservosomes; plasma membrane; Golgi; endoplasmic reticulum; autophagosome-like structures	Interaction with DNA	Menna-Barreto et al. 2005, 2007, 2009a, b
Naphthoquinones	Mitochondrion; plasma membrane; nucleus; myelin-like figures	ROS generation	Menna-Barreto et al. 2009a, c
Lysophospholipids analogues	Mitochondrion; reservosomes; plasma membrane	Interference with lipid synthesis	Santa-Rita et al. 2000, 2005, 2006; Menna-Barreto et al. 2009a
Geranyl geraniol	Mitochondrion; kinetoplast; myelin-like figures; endoplasmic reticulum	Interference with lipid synthesis	Menna-Barreto et al. 2008, 2009a
Anti-cytoskeleton agents	Mitochondrion; plasma membrane; reservosomes; flagellar pocket	Mitosis blockage	Dantas et al. 2003; Menna-Barreto et al. 2009a
Propolis extracts	Mitochondrion; kinetoplast; reservosomes; myelin-like figures; vacuolization; plasma membrane; Golgi	Not determined	Dantas et al. 2006; Salomão et al. 2011; Menna-Barreto et al. 2009a
Aromatic diamines	Mitochondrion; kinetoplast; microtubules	Interaction with DNA	De Souza et al. 2004, 2006; Silva et al. 2007; Batista et al. 2010

antioxidant pool, make the mitochondrion of these parasites an interesting drug target (Stoppani 1999; Fang and Beattie 2003). Several compounds have been shown to induce major mitochondrial damage, including swelling of the organelle, loss of membrane potential and impairment in respiratory rates (Rodrigues et al. 2001; De Souza et al. 2004; Menna-Barreto et al. 2005, 2007, 2009c; Silva et al. 2007; De Souza and Rodrigues 2009; Vannier-Santos et al. 2009; Batista et al. 2010). Table 13.1 summarizes the potential ultrastructural targets of several trypanocidal drugs and their suggested mechanisms of action.

6 Concluding Remarks

Despite this review regarding the potential cellular targets of trypanocidal compounds, the great majority of drugs that are active against *T. cruzi* utilize modes of action that are not well understood. Previous proteomic efforts have attempted to

identify the proteins involved in anti-*T. cruzi* drug activity; however, these studies had all been performed with epimastigotes until recently. The effects of drugs on the clinically relevant trypomastigotes and amastigotes must be studied further by proteomics and other high throughput approaches. These studies could provide additional information regarding the parasite cell biology and could introduce novel strategies for drug design against this neglected disease.

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Chapter 14
Proteomics Advances in the Study of *Leishmania* Parasites and Leishmaniasis

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Abstract *Leishmania* spp. are digenetic parasites which cause a broad spectrum of fatal diseases in humans. These parasites, as well as the other trypanosomatid, regulate gene expression at the post-transcriptional and post-translational levels, so that a poor correlation is observed between mRNA content and translated proteins. The completion of the genomic sequencing of several *Leishmania* species has enormous relevance to the study of the leishmaniasis pathogenesis. The combination of the available genomic resources of these parasites with powerful high-throughput proteomic analysis has shed light on various aspects of *Leishmania* biology as well as on the mechanisms underlying the disease. Diverse proteomic approaches have been used to describe and catalogue global protein profiles of *Leishmania* spp., reveal changes in protein expression during development, determine the subcellular localization of gene products, evaluate host-parasite interactions and elucidate drug resistance mechanisms. The characterization of these proteins has advanced, although many fundamental questions remain unanswered. Here, we present a historic review summarizing the different proteomic technologies applied to the study

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of *Leishmania* parasites during the last decades and we discuss the proteomic discoveries that have contributed to the understanding of *Leishmania* parasites biology and leishmaniasis.

Abbreviations

1DE	One dimensional electrophoresis
2D-DIGE	Two dimensional differential gel electrophoresis
2DE	Two dimensional electrophoresis
2D-LC-MS/MS	Two dimensional liquid chromatography coupled with tandem mass spectrometry
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamine tetra acetic acid
ESI	Electrospray ionization
ESI-MS	Electrospray ionization mass spectrometry
GFP	Green fluorescent protein
GO	Gene ontology
GP46	Glycoprotein 46
GP63	Glycoprotein 63 also called leishmanolysin and major surface peptidase
GTP	Guanosine triphosphate
HSP60	Heat shock protein 60
HSP70	Heat shock protein 70
HSP90	Heat shock protein 90
HSP100	Heat shock protein 100
IEF	Isoelectric focalization
IgG	Immunoglobulin g
IgM	Immunoglobulin m
iMAC	Metal affinity chromatography
iTRAQ	Isobaric tags for relative and absolute quantification
KMP-11	Kinetoplastid membrane protein 11
LC-MS/MS	Liquid chromatography coupled with tandem mass spectrometers
MALDI	Matrix assisted laser desorption ionization
MALDI-MS	Matrix assisted laser desorption ionization mass spectrometry
MALDI-MS/MS	Matrix assisted laser desorption ionization tandem mass spectrometry
mRNA	Messenger ribonucleic acid
MUDPIT	Multidimensional protein identification
PTMs	Post translational modifications
Rab7	Ras-related protein 7

RNA	Ribonucleic acid
SbIII	Trivalent antimonials
SbV	Pentavalent antimonials
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SHERP	Small hydrophilic endoplasmic reticulum-associated protein
SILAC	Stable isotope labeling by amino acids in cell culture
SKRP	Small-kinetoplastid-related-protein
TCA	Trichloroacetic acid
Th1	T helper 1
WHO	World health organization

1 Proteomics Technologies Applied to the Study of *Leishmania* Parasites

Protozoan parasites of the genus *Leishmania* are the etiological agents of leishmaniasis, a complex of diseases that presents a wide spectrum of clinical manifestations ranging from self healing cutaneous lesions to disseminated, mucocutaneous and visceral infections (Murray et al. 2005). Although the establishment of the primary *Leishmania* infection and the development of the clinical symptoms of leishmaniasis depend on the host immune response, the genetic content of each different species and isolate can also influence the course of the disease (Murray et al. 2005; Reithinger et al. 2007). To date, the genomes of four different *Leishmania* species have already been sequenced and annotated (Ivens et al. 2005; Peacock et al. 2007; Rogers et al. 2011). Comparative genomic analyzes revealed that most of the genes (7392) are conserved between the four species while 14, 19, 67 and 2 genes are specific to *L. (L.) major*, *L. (L.) infantum*, *L. (L.) braziliensis* and *L. (L.) mexicana,* respectively (Rogers et al. 2011). In trypanosomatids, the absence of RNA polymerase II promoters leads to the formation of constitutively expressed polycistronic precursor mRNAs. Thus, the expression of individual genes is controlled mainly at the post-transcriptional level, depending on mechanisms that (i) stabilize the RNA, (ii) initiate translation and (iii) stabilize the protein translated (Clayton 2002; Haile and Papadopoulou 2007). For this reason, most of the genes are conserved among the *Leishmania* species and a poor correlation is observed between the transcripts and the proteins expressed by the parasite. In this sense, proteomics is the most suitable tool for the identification of virulence markers, control targets and mechanisms of drug resistance (Cuervo et al. 2010; Paape and Aebischer 2011).

One-dimensional electrophoresis and the Edman sequencing were seminal biochemical techniques for the structural characterization of proteins and for the determination of its molecular weight (Laemli 1970; Matsuidara 1987). Since 1975, the two-dimensional electrophoresis (2DE) has been improved allowing the simultaneous determination of the molecular weight and the isoelectric point of proteins (O'Farrel 1975). By introducing this methodology, it was possible to separate a large amount of proteins and generate descriptive maps for various biological

systems. In the early eighties, the first protein maps of *Leishmania* spp. were published using the 2DE technique. In these studies, the authors searched for virulence markers (Handman et al. 1981) and proteins capable of differentiating between species (Saravia et al. 1984). However, during this period, the lack of reproducibility of 2DE plus the absence of high-throughput protein identification methods made the maps essentially descriptive. The identification of proteins and peptides by mass spectrometry only became possible in the late 80s, when two soft ionization techniques allowed the conversion of these molecules into intact ions. Until the current days, matrix assisted laser ionization/desorption (MALDI) and electrospray ionization (ESI) are the most commonly used techniques to ionize proteins and peptides prior to the analysis by mass spectrometry (Karas and Hilenkamp 1988; Tanaka et al. 1988; Fenn et al. 1989). While the MALDI-MS is used to analyze simple mixtures of peptides (for example, those previously fractionated by 2DE), the ESI-MS, integrated with systems of liquid chromatography, is more suitable for the identification of proteins in complex samples (Aebersold and Mann 2003). The improvement of techniques for the ionization of proteins and the development of methods for appropriate lysis of *Leishmania* led to the production of several protein maps of this parasite (Acestor et al. 2002). At first, most of these maps used 2DE to report proteins expressed by different species of *Leishmania* spp. (Góngorra et al. 2003; Brobey et al. 2006; Cuervo et al. 2007) or proteins differentially expressed according to the developmental stage of the parasite (El Fakhry et al. 2002; Bente et al. 2003; Nugent et al. 2004; Walker et al. 2006). In this period, protein spots were detected by staining the gels with silver or colloidal Coomassie G-250. However, in order to analyze quantitative differences among the spots detected, various studies started using fluorescent dyes, mainly the Cy Dyes (Morales et al. 2010; Pescher et al. 2011) and Sypro Ruby (Morales et al. 2008). Although the use of fluorescent dyes has turned 2DE into a quantitative tool of high sensitivity, linearity, and dynamic range, the separation of proteins by gel limits the applicability of the technique to the resolution of soluble and abundant proteins (Bantscheff et al. 2007; Görg et al. 2009). These limitations have been surpassed by the development of *shotgun proteomics* techniques such as Multidimensional Protein Identification (MudPIT) that uses liquid chromatography coupled to tandem mass spectrometers (LC-MS/MS) for the identification and characterization of proteins within complex biological systems (Domon and Aebersold 2006). The LC-MS/MS is not a quantitative technique by itself and can be used as a descriptive tool. However, in general, most of the proteomic studies aim to compare two or more physiological conditions within a biological system. For this purpose, the labeling of peptides with stable isotopes is the most used approach for the quantification of proteins analyzed by LC-MS/MS. Labeling based on Isobaric Tags for Relative and Absolute Quantification (iTRAQ), Stable Isotope Labeling by Amino Acids in Cell Culture (SILAC) and Metal Affinity Chromatography (iMAC) have been used in several proteomic analysis of *Leishmania* parasites, enabling not only the quantification of proteins but also the characterization of post-translational modifications (Rosenzweig et al. 2008a; Chawla et al. 2011; Hem et al. 2010; Biyani and Madhubala 2012).

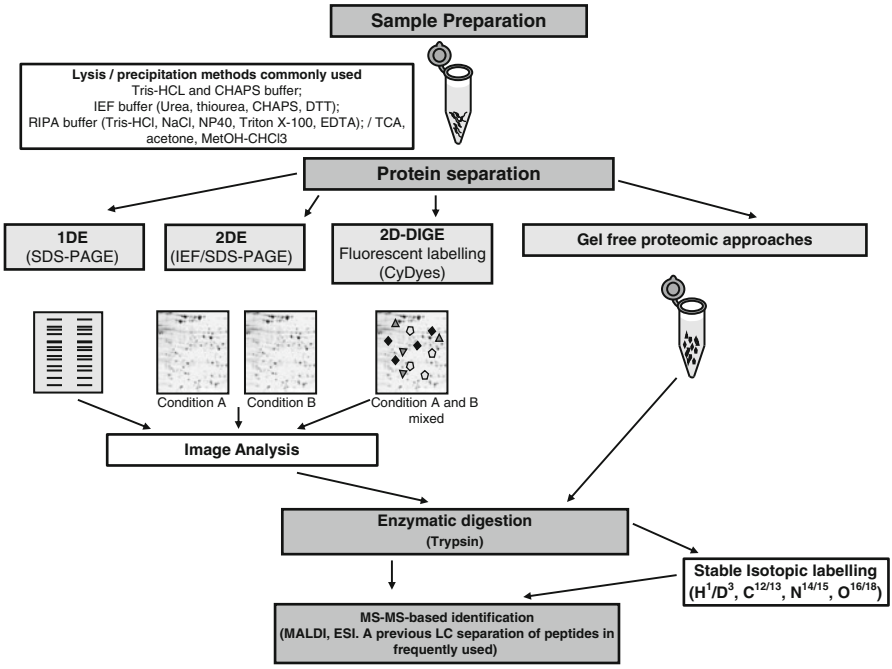

Fig. 14.1 Common gel-based and gel-free approaches used in proteomic studies of *Leishmania* parasites. Before protein separation, *Leishmania* parasites are lysed and precipitated using different methods, some mentioned above. Gel-based approaches include separation of proteins in one dimension according to their molecular weights by SDS-PAGE or in two dimensions, using isoelectric focalization (IEF) and SDS-PAGE. Then, different conditions can be analyzed and compared. In 2D-DIGE, various fluorescent CyDyes are used to label proteins in different samples which are mixed before separation by 2DE. Differences in the levels of individual protein spots are determined after image analysis. Finally, the selected gel bands/spots are enzymatically digested, which is done mainly by trypsin. In quantitative gel free proteomic approaches, proteins undergo direct enzymatic digestion, which can be followed by stable isotopic labelling of peptides. Here, it is important to note that both peptides and proteins can be chemically labelled by isotopes. Metabolic labelling of live cells in culture by SILAC is another common quantification methods. After digestion, proteins are identified by mass spectrometry

The proteomics technologies enable the mapping and the identification of proteins found on the cell surface, within the cytosol or secreted into the extracellular medium. In turn, these proteins may be involved in diverse mechanisms such as cytoadhesion, cell invasion, differentiation and cell cycle progression. As protein expression in protozoa of the genus *Leishmania* is regulated at the post-transcriptional level, the different proteomic approaches can assist in the discovery of novel metabolic pathways and potential therapeutic targets. In this chapter, it is intended to discuss how different proteomic methodologies have contributed to the understanding of the biology of *Leishmania*, giving emphasis to the advancements made until the current day and perspectives (Fig. 14.1).

2 Comparative Proteomic Analysis Between Promastigote and Amastigote Stages of *Leishmania* spp

In *Leishmania* species, the differentiation from the promastigote to amastigote form is triggered by several environmental changes such as (i) elevation of temperature, (ii) decrease of pH within the phagolysosome, (iii) increased exposure to oxygen and nitrogen reactive species, (iv) intense extracellular proteolytic activity and (v) nutritional starvation (Besteiro et al. 2007). Promastigotes and amastigotes of *Leishmania* show different mRNA levels for genes associated with protein translation, signal transduction and carbohydrate metabolism (Leifso et al. 2007). However, most of the *Leishmania* genome is reported to be constitutively expressed, with limited stage specific mRNA expression (Leifso et al. 2007; Rochette et al. 2008; Deplege et al. 2009; Adaui et al. 2011). Thus, the proteome has been widely used for the comparative study between the different stages of *Leishmania*. In the studies quoted below, the authors attempted to detect proteins as well as post-translational modifications and isoforms of proteins involved in the cellular differentiation of the parasite during its life cycle. A summary of the major proteins highlighted by the authors in such studies is presented in Table 14.1.

The first comparative proteomic analyzes were carried out by 2DE and detected 2,000 protein spots in both promastigotes and axenic amastigotes of *L. (L.) infantum*, *L. (L.) donovani* and *L. (L.) mexicana* (El Fakhry et al. 2002; Bente et al. 2003; Nugent et al. 2004). For the three species, about 3-5 % of the spots were considered to be differentially expressed. The comparative proteomic analysis of *L. (L.) infantum* detected 62 spots exclusive to the amastigote form of the parasite. However, after digestion and analysis of the peptides by mass spectrometry, only two proteins were identified: isocitrate dehydrogenase and triosephosphate isomerase involved in the Krebs cycle and in the glycolytic pathway respectively (El Fakhry et al. 2002). At that time, the genome of *L. (L.) major* was not available and the obtained amino acid sequences were identified by homology-based search using the *Basic Local Alignment Search Tool* (BLAST) algorithm (Altschul et al. 1990). After the genome of *L. (L.) major* was sequenced and made available in a public database, the number of identified proteins increased considerably. Of 104 spots regulated during *L. (L.) donovani* differentiation, 67 proteins were identified (Bente et al. 2003). In a similar way, 47 proteins were identified from 147 spots differentially expressed by promastigotes and amastigotes of *L. (L.) mexicana* (Nugent et al. 2004). In general, these studies agreed that the glycolytic pathway is more active in promastigotes while amastigotes use the fatty acid oxidation and gluconeogenesis as their main energy sources (Bente et al. 2003; Nugent et al. 2004). Also, differentially regulated proteins in promastigotes and amastigotes were gathered in several functional categories such as: (i) cytoskeleton components, (ii) response to stress, (iii) amino acid metabolism, (iv) carbohydrate metabolism, (v) detoxification and (vi) proteolysis, indicating that the parasites of the genus *Leishmania* undergo a variety of metabolic changes during their differentiation (Bente et al. 2003; Nugent et al. 2004).

Table 14.1 Metabolic pathways and proteins highlighted by the authors which are up (+) or down-regulated (−) during *Leishmania* spp. differentiation from (i) procyclic to metacyclic promastigote and (ii) from promastigote to amastigote form

(i) From procyclic to metacyclic promastigote		
Metabolic pathways and proteins	***Leishmania* species**	**References**
Paraflagellar rod protein 1D (+) α and β-tubulin (+)	*L. (L.) major*	Mojtahedi et al. 2008
Cysteine proteinase b (+) Trypanoredoxin (+)	*L. (L.) mexicana*	Nugent et al. 2004
RNA helicase (−)	*L. (L.) major*	Mojtahedi et al. 2008
GP63 and GP46 (+)	*L.(L.) infantum*	Yao et al. 2010
Metabolic enzymes (−)	*L.(L.) infantum*	Yao et al. 2010
Transporters nutrient and protons (−)	*L.(L.) infantum*	Yao et al. 2010
(ii) From promastigote to amastigote		
Glycolytic pathway (−) Fatty acid oxidation (+)	*L. (L.) donovani, L. (L.) mexicana, L. (L.) infantum*	Bente et al. 2003, Nugent et al. 2004, Rosenzweig et al. 2008a, Brotherton et al. 2012, Paape et al. 2010
Gluconeogenesis (+)	*L. (L.) donovani, L. (L.) mexicana, L. (L.) infantum*	Bente et al. 2003, Nugent et al. 2004, Rosenzweig et al. 2008a, Brotherton et al. 2012, Paape et al. 2010, Biyani and Madhubala 2012
Mitochondrial respiration (+)	*L. (L.) donovani*	Bente et al. 2003, Rosenzweig et al. 2008a, Biyani and Madhubala 2012
Stress response/heat shock proteins (+)	*L. (L.) donovani, L. (L.) mexicana, L. (V.) panamensis*	Bente et al. 2003, Nugent et al. 2004, Walker et al. 2006, Rosenzweig et al. 2008a, Morales et al. 2008, Morales et al. 2010, Hem et al. 2010, Biyani and Madhubala 2012
Protein synthesis (−)	*L. (L.) donovani*	Rosenzweig et al. 2008a, Biyani and Madhubala 2012
Proteins with basic pH (+)	*L. (L.) mexicana*	Paape et al. 2008, 2010
Rab 7 and GTP-binding protein (+)	*L. (L) donovani*	Biyani and Madhubala 2012
Tryparedoxin peroxidase (+)	*L. (L.) donovani*	Pescher et al. 2011
Methylthioadenosine phosphatase (+)	*L. (L.) donovani*	Pescher et al. 2011

Proteins expressed by promastigotes and amastigotes belonging to the *Leishmania (Viannia)* subgenus were also mapped by 2DE. In the single comparative study carried out, 11 spots expressed uniquely by *L. (V.) panamensis* amastigotes were analyzed by mass spectrometry (Walker et al. 2006). Six spots were identified as 7 distinct proteins belonging to diversified functional classes such as: (i) carbohydrate metabolism (fructose 1,6-bisphosphate aldolase, glucose 6-phosphate dehydrogenase

and pyruvate dehydrogenase), (ii) stress response (heat shock protein HSP83), (iii) cytoskeleton components (tubulin), (iv) amino acid metabolism (cysteine synthase) and (v) cell cycle regulation (*ran*-binding protein) (Walker et al. 2006). Subsequently, the first reference map for the promastigote form of *L. (V.) braziliensis* was generated, in which 101 spots were detected and 75 proteins were identified by MALDI-MS/MS. Among the proteins identified in this study, the authors highlighted the importance of "elongation factor 1-beta" that has been associated with the metastatic spread of *L. (V.) braziliensis* and may have influence on the biological behavior of the species, which is responsible for distinct clinical manifestations of cutaneous leishmaniasis (Cuervo et al. 2007).

The differentiation from non-infective procyclic promastigotes into metacyclic infective parasites is essential for the infection of the vertebrate host, being a prerequisite for resistance to complement-mediated lysis and intracellular survival (Franke et al. 1985). Promastigotes cultured *in vitro* undergo metacyclogenesis during the stationary phase of growth, which is characterized by inhibition of cell division and down-regulation of protein synthesis (Sacks and Perkins 1984). Nevertheless, some proteins such as "cysteine proteinase b" and "small hydrophilic endoplasmic reticulum (ER)-associated protein" (SHERP) are up-regulated in metacyclic promastigotes (Mottram et al. 1997; Knuepfer et al. 2001). Separation of proteins by 2DE revealed 1,200 spots belonging to both procyclic and metacyclic promastigotes of *L. (L.) major* (Mojtahedi et al. 2008). Of this total, 19 isoforms of proteins were considered to be differentially expressed. Proteins with synthetic activity, such as RNA helicase, were down-regulated in metacyclic promastigotes, what is in agreement with the general down-regulation of protein synthesis reported previously (Sacks and Perkins 1984). On the other hand, proteins involved with the parasite motility, such as "paraflagellar rod protein 1D", "α-tubulin" and "β-tubulin" were up-regulated in metacyclic promastigotes (Mojtahedi et al. 2008). Another comparative analysis, this time between procyclic and metacyclic promastigotes of *L. (L.) infantum*, revealed 52 spots which were differentially expressed during the metacyclogenesis (Nugent et al. 2004). The most interesting findings of this study were (i) the identification of "cysteine proteinase b" exclusively in the metacyclic promastigotes and (ii) the up-regulation of "trypanoredoxin" in these parasites (Nugent et al. 2004). These enzymes have been detected in metacyclic forms of *Leishmania* by other biochemical methods and their up-regulation in such infective stage, identified by 2DE, confirm their importance as virulence factors.

All the studies discussed above detected proteins of high abundance (such as α and β tubulin) on their maps. These proteins undergo extensive modifications and have multiple isoforms that hide the low abundance proteome. In order to increase the coverage of proteins detected, two pre-fractionation methods have been developed by independent groups enabling the identification of new protein isoforms differentially expressed in promastigotes and amastigotes of *L. (L.) infantum*. The first methodology used digitonin to permeabilize the cell membrane and allowed the detection of 3,186 spots, of which 42 were differentially expressed (Foucher et al. 2006). Another pre-fractionation strategy, using ammonium sulfate, allowed the detection of 2,200 spots and the identification of 168 proteins of *L. (L.) infantum*

of which 76 were differentially expressed by promastigotes and axenic amastigotes (McNicoll et al. 2006). Although the number of identifications increased after the use of pre-fractionation methods, there was not a corresponding increase in the differentially expressed proteins of *L. (L.) infantum*. Also, McNicoll et al. (2006) observed a poor correlation between mRNA transcribed and differentially expressed proteins derived from the same gene. The same study detected multiple protein isoforms related to the same gene, indicating that these proteins undergo post-translational modifications (McNicoll et al. 2006). Further analysis complemented this result demonstrating that promastigotes and amastigotes of *L. (L.) major* express 94 % of its mRNA constitutively (Leifso et al. 2007).

The comparative proteomic studies previously described in this review have focused on two or three stages of *Leishmania* differentiation and used 2DE for the mapping of differentially expressed proteins. Rosenzweig and colleagues (2008a) used an axenic differentiation model and a quantitative approach based on iTRAQ to analyze in detail the changes in protein expression during the differentiation of *L. (L.) donovani*. The analysis by LC-MS/MS identified and quantified 21 % of the proteome of the parasite during 7 stages of the differentiation. Also, iTRAQ analysis detected many enzymes and allowed the authors to create a dynamic metabolome of *L. (L.) donovani* differentiation (Rosenzweig et al. 2008a). Briefly, during differentiation, fatty acid oxidation became the main source of metabolic energy of these parasites and enzymes in the glycolytic pathway became down-regulated. In turn, differentiating parasites up-regulated gluconeogenesis and started producing sugars from glycerol and amino acids. In line with the increased oxidation capacity, Krebs cycle enzymes, respiratory chain and oxidative phosphorylation proteins were up-regulated. Down-regulation of proteins from the translational machinery and an increase in the condensation of the DNA were also observed. As expected, most changes in proteins of physiological significance occurred during the third and fourth differentiation stages, when promastigotes initiated their morphological transition into amastigotes (Rosenzweig et al. 2008a).

Most of the comparative proteomic analyzes among the different *Leishmania* stages used axenic amastigotes, maintained in culture medium with pH and temperature similar to those within the intracellular environment. Although axenic amastigotes can display some biochemical markers of the intracellular form, they cannot be grown from all species of *Leishmania* (Holzer et al. 2006). Furthermore, microarray analysis has demonstrated that axenic amastigotes are more closely related to promastigotes than the intracellular amastigotes (Holzer et al. 2006). In order to obtain intracellular amastigotes free from contaminants of the host cell, Paape et al. (2008) used fluorescent transgenic promastigotes of *L. (L.) mexicana* to infect mice *in vivo* and macrophages *in vitro*. The amastigotes obtained were further purified by lysis, centrifugation and sorting by flow cytometry. For comparative analysis, the soluble proteins from both promastigotes and amastigotes were processed for analysis by 2DE and MALDI-MS/MS, while the insoluble material was separated by 1DE and analyzed by LC-MS/MS. In total, 509 proteins were identified, of which 301 were specific of promastigotes, 51 were specific of amastigotes and 187 were common to both stages (Paape et al. 2008). Although results from samples analyzed by

MALDI-MS/MS and LC-MS/MS were complementary (having only 10 % of the proteins identified in both sets) comparison with the genome sequence-predicted proteome revealed only 6 % of coverage (Paape et al. 2008). To increase the total number of identifications and detect putative proteins secreted by the intracellular form of *L. (L.) mexicana*, the purification of amastigotes by fluorescence activated sorting was combined with a high resolution gel free proteomic analysis (Paape et al. 2010). In total, 1,764 proteins were identified, of which 741 had not been reported in previous studies. Also, the analysis of the supernatant allowed the identification of 143 proteins possibly secreted by *L. (L.) mexicana* amastigotes (Paape et al. 2010). Despite the use of different techniques, both studies agreed on some major points. Firstly, several proteins with enzymatic function in intracellular amastigotes were detected, indicating that adaptation to the intracellular environment requires high metabolic activity of these parasites, just as with the axenic amastigotes. Secondly, the two studies demonstrated that amastigotes of *L. (L.) mexicana* express a greater amount of basic protein than the promastigotes, which can function as an attempt to buffer the protons released into the phagolysosome and neutralize the total charge of this environment (Paape et al. 2008, 2010).

Recently, a study used intracellular amastigotes of three different *L. (L.) donovani* clones and the isobaric tagging method iTRAQ to analyze changes in the protein expression during the differentiation of the parasite (Biyani and Madhubala 2012). Most of the results described by this study agreed with those presented by Rosenzweig et al. (2008a) that used axenic amastigotes of *L. (L.) donovani*. For example, enzymes involved in gluconeogenesis, mitochondrial respiration and stress response were up-regulated in both intracellular and axenic amastigotes. However, the up-regulation of proteins associated with the traffic of vesicles in amastigotes such as "putative Rab7" and "GTP binding protein" is a new finding of this study. The increase in the vesicular transport may be important for the survival of the parasite inside the phagolysosomes being necessary for nutrient acquisition and release of metabolites and virulence factors (Biyani and Madhubala 2012).

The first simultaneous comparison between axenic and intracellular amastigotes of *L. (L.) donovani* demonstrated that both share more than 93 % of the protein profile (Pescher et al. 2011). Of 2,152 proteins detected through 2D-DIGE analysis, 152 were differentially expressed in axenic and intracellular amastigotes (Pescher et al. 2011). Nevertheless, the same study reported that axenic amastigotes showed a significant increase in both cell size and nuclear diameter when compared with intracellular amastigotes. Also, axenic amastigotes demonstrated a drastic reduction in the capacity of infecting the host and surviving in the intracellular environment (Pescher et al. 2011). Thus, the differences in cell size and infectivity observed between axenic and intracellular amastigotes are linked to a minor set of differentially expressed proteins with increased abundance in axenic amastigotes (113 spots) or splenic amastigotes (39 spots). In fact, proteins such as "tryparedoxin peroxidase" and "methylthioadenosine phosphatase", required for anti-oxidant resistance and growth under nutritionally deprived conditions, were up-regulated in intracellular amastigotes (Pescher et al. 2011).

Many studies have used *Leishmania* procyclic promastigotes as a model due to the ease of cultivation *in vitro*. Nevertheless, the metacyclic promastigotes are the infective form to the vertebrate host, while the amastigotes are responsible for the pathogenesis of the disease. As shown above, many metabolic pathways are regulated differentially in these developmental stages, and different proteins could be targeted for therapeutic development. However, there are still many gaps in the knowledge on this topic, especially with regard to parasites belonging to the *L. (Viannia)* subgenus. In this context, quantitative proteomic approaches applied to the analysis of the protein profile from metacyclic promastigotes and intracellular amastigotes is a promising field for the discovery of new virulence markers.

3 The Importance of Post-Translational Modifications (PTMs) for *Leishmania* Differentiation

Leishmania spp. undergoes drastic biological changes during its life cycle, but only a small fraction of its genes is differentially regulated between promastigotes and amastigotes (Bente et al. 2003; Nugent et al. 2004; Foucher et al. 2006; Leifso et al. 2007; Rosenzweig et al. 2008a). Post-transcriptional and post-translational controls, including the mRNA stability, translation initiation and protein folding are the major mechanisms of gene regulation in *Leishmania*, contributing for the increase in the complexity from genome to proteome level (Fasel et al. 2008). The complexity of proteome is further increased by PTMs, chemical modifications that can determine protein structure, cellular localization, activity state and interactions with other proteins, cells or organisms (Fasel et al. 2008; Wastling et al. 2012). Although many studies have reported qualitative and quantitative changes in the protein profiles of promastigotes and amastigotes, little is known about the role played by PTMs during *Leishmania* differentiation. Rosenzweig et al. (2008b) used a quantitative approach based on iTRAQ and LC-MS/MS to elaborate the first high-throughput analysis of PTMs during *L. (L.) donovani* differentiation. A total of 16 phosphorylated, 20 methylated, 26 acetylated, 9 hexosylated and 4 fucosylated distinct peptide modifications were detected. In the same way that alterations in the abundance of proteins (Rosenzweig et al. 2008a), most significant changes in the PTMs mentioned above occurred during later differentiation times, after promastigotes had initiated their morphological transition into amastigotes (Rosenzweig et al. 2008b).

A further analysis of *L. (L.) donovani* promastigote and amastigote protein extracts used iMAC, 2DE, Pro-Q-Diamond staining and MALDI-MS/MS to detect phosphorylated peptides. In total, 221 spots were identified, of which 48 % had not yet been reported in studies that analyzed the protein profile of total extracts (Morales et al. 2008). Treatment of the phosphoprotein enriched fractions with λ-PPase (an enzyme that cleaves phosphorylated serine, threonine and histidine) drastically reduced the number of spots revealed by Pro-Q-Diamond staining, validating the specificity of iMAC pre-fractionation. This treatment also confirmed

that the protein isoforms with distinct experimental pI and molecular weight detected in the 2D gels resulted from multiple phosphorylations. Although some proteins such as cyclophilin 40 and MAP kinase homolog LmaMPK10 were differentially regulated by promastigotes and amastigotes, most of the *L. (L.) donovani* phosphoproteome was considered to be constitutively expressed. However, the authors noted that high molecular weight phosphoproteins or phosphoproteins associated with membrane or nucleus were possibly underrepresented by 2DE analysis (Morales et al. 2008). Indeed, in a further study by the same group, quantitative analysis by 2D-DIGE revealed 318 spots differentially expressed by promastigotes and amastigotes, which represented 38 % of the phosphoproteins detected. Interestingly, amastigote phosphoproteins with increased abundance were almost exclusively protein chaperones, including several isoforms of HSP90 and HSP70, stress induced protein STI1 and cyclophilin 40. According to Pathway Architect™ software analysis, these chaperones play an important role on signal transduction as they assemble in multimeric complexes and get phosphorylated during interaction with ribosomal client proteins in an amastigote specific manner (Morales et al. 2010).

To extend the coverage of *Leishmania* amastigote phosphoproteome, total protein extract was enriched with iMAC and analyzed by LC-MS/MS. In two biological repeats, 445 phosphorylated proteins were identified, of which 48 % were annotated as hypothetical proteins (Hem et al. 2010). Based on *Saccharomyces cerevisiae* orthologs, proteins responsible for biological functions such as (i) stress response, (ii) translation initiation, (iii) catabolic processes and (iv) protein transport were enriched in Gene Ontology analysis. Physical proof of phosphorylations was provided by the enrichment of phosphopeptides on titanium dioxide columns and subsequent analysis by 2D-LC-MS/MS. A total of 181 phosphorylation sites were detected in 126 proteins. Phosphorylation occurred mostly on serine residues (86 %), but threonine (12 %) and tyrosine (2 %) residues were also modified (Hem et al. 2010). According to gene database annotation, the identified phosphoproteins were gathered into four different categories including: (i) signal transduction (covering protein phosphatases and kinases), (ii) protein folding (including protein chaperones), (iii) transcription/translation (covering translation initiation factors and RNA helicases), and (iv) RNA/protein turnover (including proteins with peptidase and nuclease activity). Also, the identification of amastigote specific phosphorylated chaperones support the results previously obtained by 2D-DIGE analysis, indicating the importance of chaperone phosphorylation for *Leishmania* infectivity and showing that stress response is an important target for drug development (Hem et al. 2010).

4 Proteome of *Leishmania* Cell Fractions

Due to its high hydrophobicity and low solubility, proteins from the plasma membrane are generally underrepresented in proteomic screens. Recently, a new protocol combined sonication, serial centrifugation and free-flow zone electrophoresis (a liquid-based method that separate charged particles according to their isoelectric points) for the

enrichment of *L. (L.) infantum* plasma membrane proteins prior to the separation by 1DE and the analysis by mass spectrometry (Brotherton et al. 2012). Interestingly, 52 % of the membrane enriched proteins were identified for the first time when compared with a previous *L. (L.) donovani* large scale proteomic screen (Rosenzweig et al. 2008a). For promastigotes and amastigotes, this protocol allowed the detection of 260 proteins containing at least one transmembrane domain according to the TMHMM v2.0 software (http://www.cbs.dtu.dk/services/TMHMM/) (Brotherton et al. 2012). These proteins were assigned into several functional categories such as transport, metabolism, transcription, translation, surface, protein folding and proteolysis. The enrichment of membrane fractions was confirmed by fluorescence microscopy of the promastigotes expressing the folate biopterin transporter fused with GFP. Total proteins and membrane fractions from GFP-tagged parasites were also blotted against the proteins elongation factor 1α and α-tubulin to exclude cytosolic and cytoskeletal localization (Brotherton et al. 2012). Another efficient protocol for the enrichment of plasma membrane proteins, based on high speed centrifugation and extraction by octyl glucoside, enabled the identification of proteins differentially expressed by *L. (L.) chagasi* (syn *L. (L.) infantum*) during the metacyclogenesis (Yao et al. 2010). After digestion of the proteins and LC-MS/MS analysis, 93 proteins were detected of which 36 and 58 were specific to the membrane fractions of metacyclic and logarithmic promastigotes, respectively. Metabolic enzymes and transporters of both nutrient and protons were uniquely present in the membrane fractions of the logarithmic promastigotes while known virulence factors such as GP63 and GP46 were detected exclusively in the membranes of the metacyclic parasites (Yao et al. 2010). As the metacyclic promastigotes do not expend energy to undergo cell division, these findings are consistent with the general decrease of the protein synthesis previously reported for this infective stage. In these studies, detergent extraction was a successful tool for the enrichment of membrane fractions, as it allowed the identification of proteins that had not yet been detected in the whole promastigote extracts (Brotherton et al. 2012; Yao et al. 2010; Nugent et al. 2004; Mojtahedi et al. 2008).

Leishmania intracellular material has also been enriched by the use of digitonin, a non-ionic detergent that permeabilizes membranes in a selective way, according to their different concentrations of sterol. While the sterol-rich plasma membrane is affected by 100 μM of digitonin, the sterol-poor membranes of the organelles are permeabilized by higher concentrations of the detergent, ranging from 0.5 mM to 5 mM. Serial digitonin extractions resulted in five different fractions of proteins, which were separated by 2DE and analyzed by mass spectrometry. Almost no overlap in spot pattern was observed between the gels, demonstrating the efficacy of the fractionation method (Foucher et al. 2006). Also, 153 protein spots were identified, of which 70 % had not yet been detected in previous analyzes. The subcellular localization of the protein spots was accessed by blast against the Human Protein Reference Database (http://www.hprd.org/). Proteins were also investigated for their putative mitochondrial localization by using the MitoProt II software (Claros and Vincens 1996). Most of the identified proteins were found in cytosol and in mitochondria, but the pre-fractionation also allowed the detection of proteins from endoplasmic reticulum, nucleus and glycosome. In total, 47 hypothetical

Table 14.2 Proteins most commonly associated with plasma membrane, microsomal fraction and secretion of *Leishmania* spp according to proteomic approaches

Proteins from plasma membrane	***Leishmania* species**	**References**
GP63	*L.(L.) infantum*	Yao et al. 2010
GP46	*L.(L.) infantum*	Yao et al. 2010
Proteins from microsomal fraction	***Leishmania* species**	**References**
GP63	*L. (L.) amazonensis*	De Oliveira et al. 2006
GP46	*L. (L.) amazonensis*	De Oliveira et al. 2006
Tryparedoxin peroxidase	*L.(L.) major*	De Oliveira et al. 2006
GTP-binding rab protein	*L. (L.) amazonensis*	De Oliveira et al. 2006
Secreted proteins	***Leishmania* species**	**References**
GP63	*L. (L.) donovani*	Silverman et al. 2010b
Elongation-Factor 1β	*L. (L.) donovani, L. (V.) braziliensis*	Cuervo et al. 2009; Silverman et al. 2010b
KMP-11	*L. (L.) donovani*	Silverman et al. 2010b
Iron superoxide dismutase homolog	*L. (L.) donovani*	Silverman et al. 2010b
HSP70	*L. (V.) braziliensis*	Cuervo et al. 2009
Tryparedoxin peroxidase	*L. (V.) braziliensis*	Cuervo et al. 2009
Activated protein kinase receptor C LACK	*L. (V.) braziliensis*	Cuervo et al. 2009
Acid phosphatase	*L. (V.) braziliensis*	Cuervo et al. 2009

proteins were identified in this study and their location was confirmed by fluorescence microscopy of GFP-fused proteins detected in both cytosolic and mitochondrial fractions (Foucher et al. 2006).

The microsomal fraction of *Leishmania* was also purified for proteomic analysis. De Oliveira et al. (2006) developed a protocol that combined serial centrifugations and sonication for the detection of proteins associated with transport vesicles. The identification of proteins potentially secreted by the parasite, such as GP63, GP46, tryparedoxin peroxidase and cytosolic nucleoside diphosphate kinase constituted the main findings of this study, indicating that the microsomal fraction is rich in vesicles addressed to the cell membrane for secretion. The GTP binding Rab-protein, involved in the intracellular traffic and localized mainly in the Golgi vesicles was also identified, confirming the efficacy of the protocol. With regard to the detection of abundant proteins from the cytosol (such as HSP60), it was suggested that they may be temporarily associated with the organelles or structures under examination, not necessarily constituting a contamination (De Oliveira et al. 2006). A summary of the major proteins highlighted by the authors in the studies mentioned above is showed in Table 14.2.

5 Secretome

Metabolites, enzymes and virulence factors released by pathogens can interfere in cell signaling and modulate the host immune response. For this reason, proteins secreted by distinct protozoan parasites have been considered key factors for the

establishment of infection and the further development of pathogenesis. The first analysis of *L. (L.) donovani* secretome used a quantitative approach based on stable isotopic labeling of amino acids in culture (SILAC) to differentiate the proteins actively secreted from those associated with the cell body of the parasite (Silverman et al. 2008). Since presence of degradation products and contamination with proteins from the serum are two major problems related to the study of secreted proteins, assays were performed in serum-free conditioned medium containing a nontoxic peptidase inhibitor. The analysis by LC-MS/MS identified 358 proteins in the conditioned medium and, based on quantitative SILAC labeling, 151 were considered to be actively secreted by the parasites. In the same study, a screen against the *Leishmania* genome database revealed that 217 proteins were predicted to have a classical amino-terminal secretion signal peptide. However, only two proteins identified in the conditioned medium contained this signal peptide. On the other hand, 10 % of the proteins identified by LC-MS/MS were previously detected in exosome-like vesicles released from other eukaryotic cells, indicating that *Leishmania* could utilize nonclassical secretion pathways to regulate the liberation of the secreted proteins (Silverman et al. 2008). To investigate this possibility, the exosomes from promastigotes of *L. (L.) donovani* were isolated through high speed centrifugations in different conditions of pH and temperature (Silverman et al. 2010a). Subsequent analysis of the exossomal global proteome by LC-MS/MS detected 329 proteins, 52 % of them shared with the total secretome of *L. (L.) donovani* confirming that exossomes transport much of the material secreted by the parasite (Silverman et al. 2008; 2010a). It was also observed that *L. (L.) donovani* exosomes interact with human monocytes *in vitro*, modulating the release of cytokines. The anti-inflammatory nature of the modulation, characterized by increased IL-10 production, suggested that the exossomal cargo contributed to the establishment of infection (Silverman et al. 2010b). In this sense, the presence of immunogenic proteins in *Leishmania* secretion was investigated by MALDI and LC-MS/MS through the comparison between wild type and mutant HSP100$^{-/-}$ parasites. In previous studies, HSP100$^{-/-}$ *L. (L) donovani* showed reduced ability to infect macrophages *in vitro*. Similarly, exosomes from HSP100$^{-/-}$ parasites did not express proteins known as virulence factors. In turn, GP63, elongation factor 1β, kinetoplast membrane protein 11 and a putative homolog of iron superoxide dismutase were some of the immunogenic proteins detected in exosomes from wild type parasites (Silverman et al. 2010b).

In a previous study, Cuervo and colleagues (2009) used 2DE and MALDI-MS/MS to characterize the profile of proteins secreted by promastigotes of *L. (V.) braziliensis* in conditioned medium. In total, 270 spots were detected of which 42 were identified and organized into categories according to GO and KEEG Ontology (http://www.genome.jp/kegg/genes.html) annotation of biological processes. The SignalP 3.0 and Secretome P 2.0 servers were used to investigate whether identified proteins had the amino-terminal secretion signal peptide (Bendtsen et al. 2004a, b). According to these analyzes, 62 % of the proteins identified were predicted to be secreted, and among them 5 % were predicted to contain the secretion signal peptide. On other hand, 57 % of the secreted proteins were released from cells by

non-classical secretion mechanisms observed in both eukaryotic and prokaryotic organisms. The promastigote forms of *L. (V.) braziliensis* also secreted proteins involved with signal transduction, intracellular survival and modulation of the host immune response. In this sense, the authors highlighted the roles of HSP70, acid phosphatase, elongation factor 1β, tryparedoxin peroxidase and activated protein kinase C receptor LACK, proteins which take part on protein folding, cell cycle regulation and resistance to the oxidative burst, respectively (Cuervo et al. 2009). These results are in agreement with those proposed by Silverman et al. (2008, 2010a), suggesting that no-conventional secretion mechanisms must be preserved among the distinct species of this parasite. A summary of the major proteins identified and discussed by the authors mentioned above is showed in Table 14.2.

6 Proteomics as a Tool for Mapping Drug Resistance in *Leishmania*

For over 60 years, pentavalent antimonials (SbV) have been used as first line drugs in the treatment of leishmaniasis. However, about 15 years ago, patients unresponsive to the treatment and descriptions of resistant strains started to be reported (Faraut-Gambarelli et al. 1997; Lira et al. 1999; Palacios et al. 2001; Abdo et al. 2003; Rojas et al. 2006; Croft et al. 2006). Currently, drug resistance is a major concern in India and in Nepal, where over 60 % of the patients are unresponsive to treatment with SbV (WHO 2010). For antileishmanial activity, SbV has to be reduced to the trivalent form (SbIII) by either the parasite or the macrophage (Callahan et al. 1997; Ephros et al. 1999; Shaked-Mishan et al. 2001). SbV and the more toxic and active trivalent form can interact with several parasite targets including trypanothione reductase, glutathione synthetase, and enzymes from both fatty acid β-oxidation chain and glycolytic pathway (Berman et al. 1982; Cunningham et al. 1994; Wyllie et al. 2004). Antimonials can also lead *Leishmania* parasites to apoptotic cell death, which is mainly characterized by nuclear DNA fragmentation (Sereno et al. 2001; Sudhandiran and Shaha 2003). With the increasing incidence of resistance to antimonials, second-line drugs such as amphotericin B, pentamidine, paromomycin and the oral drug miltefosine have been used as alternatives for the treatment of Leishmaniasis in various regions (WHO 2010). However, high toxicity, questionable efficacy and cross-resistance are recurrent problems (WHO 2010). In this sense, proteomic analysis of susceptible and resistant parasite strains may contribute for the identification of novel targets, therapeutic development and reversing of the resistant phenotype (Cuervo and De Jesus 2012).

Comparative 2DE analysis of *L. (L.) donovani* isolated from SbIII resistant and susceptible kalazar patients in India showed that proteins associated with the induction of cell death were differentially expressed by each strain (Vergnes et al. 2007). Heat-shock proteins, which take part on *Leishmania* stress response, were up-regulated in the resistant strain. Indeed, growth inhibition assays showed that promastigotes of the susceptible strain transfected with HSP83 gene were more than

twofold resistant to SbIII. HSP83 transfectants were also cross-resistant to miltefosine, an oral drug used for the treatment of visceral and cutaneous leishmaniasis. In both cases, HSP83-overexpressing parasites were protected against decrease in mitochondrial membrane potential and nuclear DNA fragmentation, suggesting that HSP83 may interact with other proteins to negatively regulate *Leishmania* mitochondria-dependent apoptotic pathway (Vergnes et al. 2007). In the same study, 2DE analysis detected down-regulation of small-kinetoplastid-related-protein (SKRP) in the resistant parasites. This protein is often associated with calpain-like peptidases which are one of the key effectors of programmed cell death in *Leishmania*. As expected, overexpression of SKRP increased the sensitivity of resistant parasites to SbIII and SbV, enhancing DNA fragmentation. However, overexpression of SKRP had the opposite effect on miltefosine treated parasites, protecting them against decrease in mitochondrial membrane potential and nuclear DNA fragmentation (Vergnes et al. 2007). These findings reveal that proteins may have contrasting roles in the resistance mechanisms of *Leishmania*, highlighting the importance of further studies to detect resistance pathways in different species and strains.

Many studies use *Leishmania* promastigotes as a model to study the mechanisms of drug resistance. In order to check whether similar mechanisms are observed in amastigotes, El Fadili et al. (2009) selected SbIII resistant *L. (L.) infantum* by direct drug pressure under axenic conditions. Comparative analysis between resistant and susceptible amastigotes by 2DE revealed several down-regulated proteins in the resistant strain such as: LACK receptor, β-tubulin, proteasome 26 subunit, pyruvate kinase and the kinetoplastid membrane protein 11 (KMP-11). The authors gave special attention to KMP-11, which was associated with SbIII resistance for the first time (El Fadili et al. 2009). Previous studies have suggested that KMP-11 is an amphipatic membrane protein involved with the increase of the lipid bilayer pressure (Jardim et al. 1995; Fuertes et al. 1999). In this sense, a reduction in KMP-11 expression could change SbIII interaction with transporters such as aquaglyceroporin, which is known to facilitate the uptake of trivalent metalloids (Gourbal et al. 2004). Down-regulation of KMP-11 could also alter the interaction of SbIII with efflux systems increasing drug transport out of the parasites (El Fadili et al. 2009). Western-blot analysis using a monoclonal antibody specific for KMP-11 confirmed down-regulation of this protein in the SbIII resistant strain. However, northern-blot revealed that down-regulation was not correlated with a decrease in the mRNA levels (El Fadili et al. 2009). Indeed, N-terminal acetylation of KMP-11 indicates that the protein stability may be compromised in the SbIII resistant strain, which could increase KMP-11 degradation rate in this condition (Rosenzweig et al. 2008a, b).

Two studies published by the same group investigated proteins differentially expressed by *L. (L.) donovani* strains resistant to SbIII and paromomycin. In the first study, a comparative analysis between SbIII susceptible and resistant parasites was conducted through a quantitative approach based on iTRAQ and LC-MS/MS (Biyani et al. 2011). In the second study, proteins differentially expressed by paromomycin resistant and susceptible strains were detected by SILAC labeling and LC-MS/MS (Chawla et al. 2011). Despite the use of different labeling methods,

up or down regulation of proteins involved with (i) metabolism, (ii) stress response, (iii) processing of DNA and RNA, (iv) protein translation, (v) intracellular survival, and (vi) cytoskeleton were common to both SbIII and paromomycin resistant parasites. Within the biological function "metabolism", the increase in the expression of enzymes involved with glycolysis was the more noticeable. Interestingly, it has been demonstrated that pyruvate acts as a peroxide scavenger in mammalian cells (Wang et al. 2007; Babich et al. 2009). Thus, it is suggested that up-regulation of enzymes from the glycolytic pathway can provide resistant *Leishmania* strains an extra protection against oxidative stress (Biyani et al. 2011). Up-regulation of heat shock proteins also play an important role on the protection of resistant parasites against oxidative stress and apoptotic cell death (Vergnes et al. 2007; Biyani et al. 2011; Chawla et al. 2011). Regarding the mechanism of action of paromomycin, proteomic analysis by SILAC and LC-MS/MS showed that proteins related to vesicular trafficking and fusion had an increased expression in the resistant strain. Additionally, transmission electron microscopy of resistant parasites showed an increase in the number of vesicular vacuoles in the cytoplasm when compared with the wild-type strain (Chawla et al. 2011). Proteomic analysis also revealed that ribosomal proteins involved in the translational machinery had increased expression in the resistant strain. It was suggested that after entering the cell paromomycin acts by inhibiting protein synthesis (Chawla et al. 2011). Thus, the increased expression of proteins related to the translation machinery in the resistant strain could represent a compensatory mechanism for the action of the compound (Chawla et al. 2011). Indeed, this suggestion agrees with results previously observed with *Escherichia coli* which indicate that protein synthesis is inhibited when paromomycin interacts with the ribosomal subunits (Davis 1987).

Recently, Walker et al. (2012) used NP-40 and Triton X-114 detergents to enrich membrane proteins from both SbIII resistant and susceptible *L. (V.) panamensis*. After 2DE separation and LC-MS/MS analysis, several spots were detected but only nine proteins were differentially expressed between resistant and susceptible strains. It is suggested that rather than being involved with a specific resistance mechanism HSP70 related protein-1, HSP70 and stress-induced protein 1 may be associated with general response to stress. On the other hand, GTP-binding protein and ATPase β-subunit may be associated with metal-thiol efflux while elongation factor-2 and translation release factor take part on the modulation of protein synthesis, demanded for the maintenance of the resistant phenotype. Finally, up-regulation of S-adenosylmethionine synthetase and S-adenosylhomocysteine may be involved with the synthesis of glutathione and trypanothione precursors in the resistant strain, playing an essential role in the process of antimony detoxification (Walker et al. 2012). A summary of the major proteins highlighted by the authors in the studies mentioned above is showed in Table 14.3.

Proteomic analysis identified many proteins directly and indirectly related to the development of resistant phenotypes in *Leishmania*. Some of them, such as heat-shock proteins and enzymes from the glycolytic pathway, are up-regulated in strains resistant to antimonials, miltefosine and paromomycin and could be exploited for reversing resistance to all three compounds (Vergnes et al. 2007; Biyani et al. 2011;

Table 14.3 *Leishmania* spp. proteins most commonly associated to drug resistance and/or considered immunogenic antigens according to proteomic approaches. Ant: antimonials; Milt: Miltefosine; Par: paromomycin

Protein name	Associated with resistance to			Immunogenic	*Leishmania* species	References
	Ant	Milt	Par			
HSP83	X	X	X	X	*L. (L.) donovani, L. (L.) infantum*	Gupta et al. 2007, Vergnes et al. 2007, Biyani et al. 2011, Chawla et al. 2011, Costa et al. 2011
HSP70	X		X	X	*L. (L.) donovani, L. (L.) infantum, L. (V.) panamensis*	Dea-Ayuela et al. 2006, Gupta et al. 2007, Biyani et al. 2011, Chawla et al. 2011, Coelho et al. 2011, Walker et al. 2012
Elongation Factor 2			X	X	*L. (L.) donovani, L. (V.) panamensis*	Forgber et al. 2006, Gupta et al. 2007, Chawla et al. 2011, Walker et al. 2012
Triosephosphate isomerase	X		X	X	*L. (L.) donovani*	Gupta et al. 2007, Biyani et al. 2011, Chawla et al. 2011
Enolase			X	X	*L. (L.) donovani*	Forgber et al. 2006, Gupta et al. 2007, Chawla et al. 2011
Manose-1-phosphate guanysil-transferase	X			X	*L. (L.) donovani, L. (L.) infantum*	Biyani et al. 2011, Costa et al. 2011
Aldose-1-epimerase	X			X	*L. (L.) donovani, L. (L.) infantum*	Biyani et al. 2011, Costa et al. 2011
3,2-trans-enoyl-Co-A isomerase			X	X	*L. (L.) donovani, L. (L.) infantum*	Chawla et al. 2011, Costa et al. 2011
Calreticulin				X	*L. (L.) donovani, L. (L.) infantum*	Gupta et al. 2007, Coelho et al. 2011
ATP-dependent RNA-helicase			X	X	*L. (L.) donovani, L. (L.) infantum*	Chawla et al. 2011, Coelho et al. 2011
KMP-11	X			X	*L. (L.) donovani*	El Fadili et al. 2009, Costa et al. 2011

Chawla et al. 2011). However, drug resistance is a multifactorial problem that involves *Leishmania* genetic diversity, host immune response and host genetic background (Cuervo and De Jesus 2012). In this sense, genome sequencing of other *Leishmania* species and quantitative proteomics studies involving a larger number of them may reveal novel proteins associated with the development of resistance phenotypes.

7 Proteomic Analysis of *Leishmania* Antigens and Vaccine Candidates

Visceral leishmaniasis is a major parasitic disease in Asia, Africa and South America affecting about 0.2 to 0.4 million people every year (Murray et al. 2005; Alvar et al. 2012). As mentioned above, resistance to antimonials in India and Nepal hampers disease control. Furthermore, the available anti-leishmanial drugs are highly toxic leading patients to drop out the treatment, especially in cases of co-infection with HIV and association with malnutrition (Murray et al. 2005; WHO 2010). For these reasons, pioneering studies analyzed the proteomes of promastigotes of *L. (L.) donovani* and *L. (L.) infantum* in search of disease-specific biomarkers and new therapeutic targets. Using ammonium sulfate precipitation and SDS-PAGE separation Garg et al. (2006) obtained a fraction of proteins from *L. (L.) donovani* which showed significant Th1 responses in cured patients and hamsters. Characterization of this fraction by 2DE and MALDI-MS/MS revealed proteins, such as dissufide isomerase, enolase and triosephosphate isomerase, which were previously identified as immunogenic in other organisms and may also represent potential therapeutic targets against *Leishmania* (Gupta et al. 2007). Further characterization of that fraction, allowed the identification of calreticulin, a protein involved with glycoprotein folding and considered a therapeutic candidate as it may affect the targeting of proteins associated with the virulence of the parasite (Gupta et al. 2007).

In order to investigate the heterogeneity of the *L. (L.) donovani* responses, sera of four patients from Bihar (India) were collected for analysis by 2DE-Western blot (Forgber et al. 2006). In this assay, the parasites used as a source of antigens were isolated from two patients that lived in adjacent districts of Bihar. In total, 330 different antigens were detected, of which 68 were related to proteins in 2DE gels. Some antigens were shared between the two parasite isolates, but extensive differences were observed in the patterns of the spots and no immunodominant antigens could be detected (Forgber et al. 2006). The reactivities were found to be weaker when both parasites and sera were isolated from the same patient, indicating that the immune system may use selection processes that increase tolerance to some antigens (Forgber et al. 2006). It was also observed that antigenicity of the proteins did not correlate with their expression level in 2DE gels. Six protein spots which correlated with Western blot signals were analyzed by MALDI-MS/MS and five antigens were identified, among them, HSP70 and elongation factor 2, which were previously reported as being immunogenic proteins (Forgber et al. 2006; Gupta et al. 2007). A similar study using rabbit hyperimmune serum raised against promastigote

extracts also combined 2DE with Western blot analysis to investigate antigenic proteins of *L. (L.) infantum*. In this study, MALDI-MS/MS identified several antigenic proteins such as ATPase β subunit, transkelotase, proteasome subunit, succinyl-diaminopimelate desuccinylase, tubulin α chain, HSP70 and several hypothetical proteins with unknown function (Dea-Ayuela et al. 2006).

Dogs are important targets for the control of *Leishmania* transmission in places where visceral leishmaniasis is a zoonosis (WHO 2010). Currently, there are three vaccines licensed for prophylaxis of non-infected dogs against canine visceral leishmaniasis. Fucose-manose-ligand-saponin vaccine is commercialized under the name of Leishimune® in Brazil (da Silva et al. 2001; Santos et al. 2007). Leish-Tec®, a vaccine composed of recombinant A2 antigen of *Leishmania* amastigotes and adjuvanted by saponin, is also commercialized in Brazil (Fernandes et al. 2008). Finally, CaniLeish®, a vaccine composed of proteins secreted by *L. (L.) infantum*, was licensed for commercialization in Portugal, Spain, France, Greece and Italy (Lemesre et al. 2005, 2007; Bourdoiseau et al. 2009). Although these three vaccines are available for immunization of dogs against visceral leishmaniasis, proteomic studies still contribute for the identification of new immunogenic *Leishmania* antigens. Recently, Costa et al. (2011) used an immunoproteomic approach to identify *Leishmania* antigens that are immunogenic for dogs. In this study, proteomic analysis was performed with a highly virulent strain of *L. (L.) chagasi* (syn *L. (L.) infantum*) isolated from a dog in Brazil. Briefly, whole promastigote extracts were separated by 2DE, transferred by Western blot and incubated with a pool of sera from dogs infected with various stages of visceral leishmaniasis. MALDI-MS/MS analysis identified 41 proteins, of which 3 and 38 were reactive with IgM and total IgG respectively (Costa et al. 2011). The identification of antigens was followed by *in silico* analysis to predict B cell epitopes. Mannose-1-phosphate guanyltransferase, α-tubulin and HSP83 were recognized by IgM in sera from acutely infected dogs, but only HSP83 had a B cell epitope recognized in its structure. In turn, eight proteins (HSP83, 3,2-trans-enoyl Co-A isomerase, ribonucleoprotein p 18 mitochondrial precursor, aldose 1-epimerase and four hypothetical proteins) were recognized by IgG in sera from chronically infected animals and all of them were predicted to have B cell epitopes. Bioinformatic methods were also applied to identify immunogenic proteins with T cell epitopes. Interestingly, four proteins identified specifically in the amastigote form of *L. (L.) infantum* by 2D-DIGE presented high content of T cell epitopes and were selected as antigens with greater potential to be immunogenic for T cells and vaccine development (Costa et al. 2011). As *Leishmania* amastigotes are responsible for causing the clinical manifestations of leishmaniasis in mammals, the mapping of antigens specific from this stage has great importance. Coelho et al. (2012) used a pool of symptomatic and asymptomatic dogs' sera to perform immunoblots against promastigotes and axenic amastigotes of *L. (L.) infantum*. Separation of proteins by 2DE detected 350 protein spots in promastigote and 200 spots in amastigote total extracts. (Coelho et al. 2012). Spots which reacted with sera from symptomatic or asymptomatic dogs were analyzed by MALDI-MS/MS and several proteins were identified. Most of them were expressed by both *Leishmania* stages and reacted with sera from symptomatic dogs, including proteins involved

with stress response, carbohydrate metabolism, protein synthesis and some known virulence factors such as KMP-11 (Coelho et al. 2012). Three proteins (ATP-dependent RNA helicase, A2, and amastin) were detected exclusively in axenic amastigotes and may constitute important therapeutic targets. Finally, several proteins proved to be reactive against sera of asymptomatic dogs. Despite the fact of being potential diagnostic antigens, proteins expressed by dogs which can control parasite replication may be correlated with protective responses and thus represent potential vaccine candidates (Coelho et al. 2012).

8 Concluding Remarks

As reviewed above, proteomic approaches are particularly useful tools for the identification of proteins and mechanisms involved in several aspects of the biology of *Leishmania* parasites. A summary of the proteins highlighted by the authors in such studies was presented in Tables 14.1, 14.2, and 14.3. New roles for already well-characterized proteins as well as the assignment of roles for proteins of unknown function are being defined for several *Leishmania* species using those proteomics tools. However, the recurrent identification of a group of proteins that correspond to the most abundant ones (both in *Leishmania* and in several biological systems) precludes the identification of the less abundant proteins and obscures the studied phenomena (Gygi et al. 2000; Petrak et al. 2008). For this reason, new proteomics technologies together with specific bioinformatic tools and databases may be valuable for a better exploitation and interpretation of the *Leishmania* proteomics and for revealing intra and inter-specific particularities.

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Chapter 15
Towards the Phosphoproteome of Trypanosomatids

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Abstract The identification and localization of protein phosphorylation sites provide clues to what proteins or pathways might be activated in a given condition, helping to improve our understanding about signaling networks. Advances in strategies for enrichment of phosphorylated peptides/proteins, mass spectrometry (MS) instrumentation, and specific MS techniques for identification and quantification of post-translational modifications have allowed for large-scale mapping of phosphorylation sites, promoting the field of phosphoproteomics. The great promise of phosphoproteomics is to unravel the dynamics of signaling networks, a layer of the emerging field of systems biology. Until a few years ago only a small number of phosphorylation sites had been described. Following large-scale trends, recent phosphoproteomic studies have reported the mapping of thousands of phosphorylation sites in trypanosomatids. However, quantitative information about the regulation of such sites in different conditions is still lacking. In this chapter, we provide a historical overview of phosphoproteomic studies for trypanosomatids and discuss some challenges and perspectives in the field.

Abbreviations

2D-DIGE	Two dimensional differential gel electrophoresis
2DE	Two dimensional electrophoresis
AGC	Kinases containing PKA, PKG, and PKC
ALK	Anaplastic lymphoma kinase
aPK	Atypical protein kinase

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CAMK	Group of kinases containing calcium/calmodulin-dependent kinases
CaMKK	Calmodulin-dependent protein kinase kinase
CDK1	Cyclin-dependent kinase 1
CID	Collision-induced dissociation
CK	Casein-kinase
CMGC	Group of kinases containing cyclin-dependent kinases, mitogen-activated protein kinases, Glycogen synthase kinases, and CDK-like kinases.
CRK	CT10 regulator of kinase
DGF	Dispersed gene family
DHB	2,5-dihydroxybenzoic acid
DYRK	Dual-specificity tyrosine-regulated kinase
ECD	Electron capture dissociation
EGFR	Epidermal growth factor receptor
ePK	Eukaryotic protein kinase
ERK	Extracellular signal-regulated kinase
ERLIC	Electrostatic repulsion- hydrophilic interaction chromatography
ESI	Electron spray ionization
ETD	Electron transfer dissociation
FRAP	FKBP12-rapamycin-associated protein
GMD	Glycosomal malate dehydrogenase
GSK3	Glycogen synthase kinase 3
HCD	Higher-energy C-trap dissociation
HILIC	Hydrophilic interaction chromatography
HOP	HSP organizing protein
HSP	Heat shock protein
IDA	Iminodiacetic acid
IgE-HRF	Immunoglobulin E-dependent histamine-releasing factor
IMAC	Immobilized metal affinity chromatography
iTRAQ	Isobaric tag for relative and absolute quantification
LC	Liquid chromatography
m/z	Mass-to-charge-ratio
MALDI	Matrix-assisted laser desorption/ionization
MAP kinase	Mitogen-activated protein kinase
MOAC	Metal oxide affinity chromatography
mRNA	Messenger RNA
MS	Mass spectrometry
MSA	Multi-stage activation
NEK	NimA related kinase
NTA	Nitrilotriacetic acid
PEK	Pancreatic eukaryotic initiation factor-2alpha kinase
Pf2αS	Prostaglandin F2-alpha synthase
PK	Protein kinase
PKA	Protein kinase A
PP	Protein phosphatase

PTM	Post-translational modification
PTP	Tyrosine-specific phosphatase
RIO2	Right open reading frame 2
RP	Reverse phase
SAX	Strong anion exchange
SCX	Strong cation exchange
SH2	Src homology 2
SILAC	Stable isotope labeling by amino acids in cell culture
SIMAC	Sequential elution from IMAC
STE	Group of kinases homolog of Sterile
STI	Stress inducible protein
STP	Serine/threonine phosphatase
TCTP	Translationally controlled-tumor-protein
TED	Tris-(carbomethyl)-ethylendiamine
TFA	Trifluoroacetic acid
TGF-β	Transforming growth factor beta
TOF	Time of flight
TPR	Tetratricopeptide repeat
ULK	UNC-51-like kinases

1 Introduction

As discussed in the first chapter, trypanosomatids are unicellular eukaryotes with many peculiarities in both cellular and molecular aspects. These parasites possess a complex life cycle alternating between vertebrate and invertebrate hosts and in order to survive under these distinct conditions, must quickly recognize and adapt to their environment. It is still unclear which signaling pathways are involved in this adaptation, and the knowledge available for better characterized organisms is frequently not applicable to trypanosomatids. Assuming that protein phosphorylation, driven by protein kinases and phosphatases, effectively participates in the regulation of signaling pathways, the comprehension of this process is of paramount importance. This is even more evident in trypanosomatids, in which the control of gene expression occurs post-transcriptionally, giving a notable significance to post-translational modifications in these organisms, such as protein phosphorylation, ubiquitilation, glycosylation, among others. Therefore, the discovery and characterization of protein phosphorylation is one of the most important steps to elucidate cellular signaling mechanisms in trypanosomatids. The analysis of the genome gives us clues about cell signaling in a given organism, such as its repertoire of protein kinases, its phosphatases, and the protein domains able to interact with phosphorylation sites, among other features. However, the comprehensive identification and quantification of phosphorylation sites, as well as the linkage between protein kinases or phosphatases and their targets cannot be identified from the genome; this correlation would be an outstanding landmark in the knowledge about trypanosomatids biology.

In this chapter, we present a review about the control of gene expression and the signaling-related genome of trypanosomatids, as well as the most used methodologies involved in phosphoproteomics. In addition, we provide a historical overview of phosphoproteomic studies for trypanosomatids and discuss some challenges and future perspectives in the field.

2 The Control of Gene Expression in Trypanosomatids Occurs Post-Transcriptionally

It is well known that transcription represents an initial step towards the control of gene expression. For prokaryotes and most eukaryotes, the combination of DNA promoter regions, transcription factors, and specific RNA polymerases are responsible for an important step in the control of cellular mRNA levels. However, this is not applicable to trypanosomatids. In these parasites, conventional promoters for RNA polymerase II have not been identified, suggesting no influence of transcriptional control on the expression of protein-coding genes. Also, the individual transcription of each mRNA, observed in most eukaryotes, does not occur in trypanosomes. Instead, mRNAs are transcribed as a prokaryote non-related polycistronic units (Sather and Agabian 1985) and subsequently, the units within polycistronic mRNAs are individualized, and independently subjected to post-transcriptional control. Moreover, the mRNA processing in trypanosomatids is different from that of higher eukaryotes, in which the RNA splicing occurs predominantly in *cis*. In trypanosomatids, *cis*-splicing is uncommon, giving place to RNA splicing in *trans*, in which a short RNA sequence (splice leader, also referred as miniexon) is transcribed from a specific locus, undergoing processing and added to the 5′ end of transcripts (Van der Ploeg et al. 1982; Agabian 1990). Despite the apparent absence of typical polymerase II promoters, it has been shown that polymerase II transcription of an entire polycistronic unit initiates upstream of the first gene of the polycistron, in strand-switch regions (Martinez-Calvillo et al. 2003). Specific histone acetylation has been identified as a marker for these regions in *L. major* (Thomas et al. 2009). Specific histone modifications have also been shown to be involved with the chromatin state and may alter DNA accessibility by the replication machinery (Nardelli et al. 2009).

The absence of transcriptional control results in ubiquitous mRNA transcription in trypanosomatids. However, mRNA processing and translation are still steps amenable to the control of gene expression. After exportation from the nucleus, mRNAs are available for translation, and sent to storage or degraded, according to cell regulation. Different factors contribute to mRNA stability in trypanosomes, such as RNA binding proteins and small silencing RNAs, whose function in the control of gene expression is still unclear. Finally, after mRNA translation, the synthesized proteins can undergo post-translational modifications (PTM), such as phosphorylation, glycosylation, ubiquitilation, auto-cleavage, and others. These modifications can, for example, activate, inactivate, or label to degradation proteins.

Therefore, it is reasonable to consider mRNA stability control and PTMs as key processes for gene expression modulation in trypanosomatids (Clayton 2002; Kramer 2012).

3 Signaling-Related Genome

Genomes of three pathogenic trypanosomatids, *Trypanosoma cruzi*, *Trypanosoma brucei* and *Leishmania major*, also known as TriTryps, were published in 2005 (Berriman et al. 2005; El-Sayed et al. 2005; Ivens et al. 2005), allowing researchers to mine information about several signaling pathways. In the same year of these genome publications, Parsons and co-workers, using the genome information, published a comparative analysis of the protein kinase (PKs) repertoire (kinome) of the TriTryps (Parsons et al. 2005). This analysis revealed a set of protein kinases comprised of about 2 % for each genome, representing almost twofold the number of *Plasmodium falciparum* PKs (Table 15.1) and suggesting an even greater

Table 15.1 Comparison of the kinase and phosphatase repertoires in different genomes

Group/Family	*T. brucei*	*T. cruzi*	*L. major*	*P. falciparum*	*H. sapiens*
Protein kinases					
AGC	12(8)[a], 22(13)[b]	12(7)	11(6)	14(16)	82(17)
CAMK	14(9)[a], 28(16)[b]	13(8)	16(9)	20(23)	95(20)
CK I	5(3)[a], 5(3)[b]	8(5)	7(4)	1(1)	12(3)
CMGC	42(27)[a], 47(28)[b]	42(25)	45(25)	19(22)	68(14)
Other	39(25)[a], 20(12)[b]	42(25)	40(22)	27(31)	21(4)
STE	25(16)[a], 28(16)[b]	31(18)	34(19)	1(1)	61(13)
Tyrosine kinase	0(0)[a], 0(0)[b]	0(0)	0(0)	0(0)	91(19)
TKL	0(0)[a], 0(0)[b]	0(0)	0(0)	5(6)	48(10)
Unique[c]	19(12)[a]	23(13)	26(15)	–	–
Total ePK	**156**[a], **170**[b]	**171**	**179**	**87**	**581**
Protein phosphatases					
Protein tyrosine phosphatase	2(2)	2(3)	3(3)	3(9)	43(31)
Dual-specificity phosphatase	20(23)	19(24)	22(25)	1(3)	43(31)
Lipid phosphatase	7(8)	3(4)	4(5)	0(0)	21(15)
Serine/Threonine phosphatase	56(65)	54(69)	58(66)	30(88)	33(24)
Total PP	**86**	**78**	**88**	**34**	**140**

Kinases from: (Parsons et al. 2005) (*T. brucei*[a], *T. cruzi* and *L. major*), (Nett et al. 2009b) (*T. brucei*[b], *P. falciparum* and *H. sapiens*). Phosphatases from: (Brenchley et al. 2007) (*T. brucei, T. cruzi* and *L. major*), (Wilkes and Doerig 2008) (*P. falciparum*), (Alonso et al. 2004a), (Cohen 2004), (Alonso et al. 2004b), (Stern et al. 2007) (*H. sapiens*)

[c]The unique PKs were assigned only for trypanosomatids. ePK – eukaryotic protein kinase. In parenthesis, the percentage representation of each group/family

importance of phosphorylation in these parasites. In the same study, many other considerations were postulated. Among them, the absence of receptor-linked tyrosine and tyrosine kinases, as well as the absence of protein domains that interact with phosphotyrosine, such as SH2 domains were observed, an intriguing finding since tyrosine phosphorylation has been well documented in trypanosomatids (further discussed below). Also, several canonic PK groups were identified, such as AGC, CAMK, CK, CMGC and STE, some of which were over-represented in comparison to humans. This was the case in the CMGC and STE groups, comprised of MAP-kinases, cyclin-dependent kinases, and dual-specificity kinases, among other families. The large number of MAP-kinases and STEs, components of MAP-kinase cascades, might be related to the need of these parasites to quickly respond to environmental cues. Another group worthy of note is that of the unique PKs, which represent from 12 % to 15 % of the total kinome of each trypanosome genome (Table 15.1). The members of this group could not be assigned to any other PK group, due to the lack of a complete subdomain structure, as well as the catalytic residues in their sequences. Indeed, the unique is the least conserved PK group among the three analyzed trypanosomes (Parsons et al. 2005). More recently, Nett and co-workers reviewed the kinome of *T. brucei*, showing considerable differences in the number of genes in some groups, such as AGC and CAMK (Table 15.1) (Nett et al. 2009b).

The required tight regulation of cellular processes promoted by phosphorylation is achieved by the combined activity of PKs, responsible for protein phosphorylation and protein phosphatases (PPs), with dephosphorylation functions. An analysis of the trypanosome phosphatase complement (phosphatome) was performed by Brenchley and co-workers (Brenchley et al. 2007). As was seen in the kinome, the trypanosome phosphatome is composed of protein groups classified according to their substrate specificity. Similar to what occurs in the kinase groups, the proportion of PP groups in trypanosomes differs from that observed for higher eukaryotes. The serine/threonine phosphatase (STP) group is the group most represented in the analyzed trypanosomes, corresponding to 65, 66, and 69 % of the *T. brucei*, *T. cruzi* or *L. major* phosphatomes, respectively (Table 15.1). In contrast, a human phosphatome contains only 24 % of STPs. Dual-specificity phosphatases are the second major group within the trypanosomatid phosphatomes that dephosphorylate phospho-serine, phospho-threonine and phospho-tyrosine substrates, representing approximately 24 % of the trypanosomatid phosphatomes, versus 31 % in humans. Following the absence of tyrosine-specific kinases, tyrosine-specific phosphatases (PTP) represent only 2–3 % of each phosphatome. This PTP proportion resembles that of *Arabidopsis thaliana* (2 %), but differs greatly from humans and *Saccharomyces cerevisiae*, 31 and 16 %, respectively. Moreover, trypanosomatid PTPs have many substitutions/deletions in their amino acid sequence, resulting in a low homology compared to humans.

After analyzing features of the trypanosomatid kinome and phosphatome, several differences to other eukaryotes become evident, ranging from amino acid sequences and domain architectures to the proportion of PK/PP groups found in these organisms. The genome analysis may prioritize important protein groups as potential candidates for functional analysis, helping to direct further functional experiments. Altogether,

the signaling-related genome information highlights specific trypanosomatid PKs and PPs as targets for in depth functional analysis, taking advantage of their divergence from other organisms, as well as their application as potential targets for therapeutic purposes. In this context, phosphoproteomic studies can be of great value, as will be discussed in the following sections.

4 Sample Preparation for Phosphoproteomic Analysis

4.1 General Aspects

A successful phosphoproteomic study needs to deal with the labile phosphate groups. In order to preserve the integrity of the phosphorylated sites in addition to protease inhibitors it is essential to use phosphatase inhibitors as a measure for preventing phosphate removal from proteins. Since different phosphatase inhibitors have distinct specificity, cocktails containing inhibitors to suppress both serine/threonine phosphatases and tyrosine phosphatases are used. Given the dynamic nature of protein phosphorylation, signaling responses triggered by stress caused in the sample isolation procedure need to be minimized so that they are not mistaken for the true response resulting from stimuli evaluated in the study. To conclude, it is essential prepare the sample dealing with the characteristics of this post-translation modification (Thingholm et al. 2009) as described below. Different techniques can be used for phosphoproteomic approaches, a description of some methods can be found in the next sections and a list of methods apply to trypanosomatids can be found in Table 15.2. It is worth mentioning that only a few works combined pre-fractionation to phosphopeptide enrichment.

4.2 Phosphoprotein and Phosphopeptide Enrichment

Advances in phospho-enrichment and bioinformatics approaches combined with the availability of high resolution hybrid mass spectrometers have enabled a rapid development in the phosphoproteomics field.

The low abundance and substoichiometry of phosphorylated proteins in addition to inefficient ionization of phosphopeptides when compared to their nonphosphorylated counterparts, as well as losses caused by their adsorption to metal and plastics, make it necessary to enrich molecules containing phosphate moiety in order to increase the sensitivity of MS analysis. To this end, a wide variety of strategies have been developed, which allow the enrichment to be performed on either the phosphoprotein or phosphopeptide level.

Before choosing a phospho-enrichment approach, it is important to consider some aspects, such as the aim of the study, sample characteristics, amount of starting material, and the advantages and disadvantages that each enrichment method offers.

Table 15.2 Phosphoproteomics studies performed for trypanosomatids

Organism	Model	Phospho-enrichment method	Proteomic approach	Peptide fragmentation method	Phosphoproteins	Phosphosites	Reference
T. brucei	Quantitative procyclic X bloodstream	SCX, TiO_2	LC-ESI-MS/MS	MS/MS (MSA)	2,551	10,096	(Urbaniak et al. 2013)
T. cruzi	Epimastigotes, TGF-β response	Phosphoprotein-specific Pro-Q Diamond stain	2DE-MALDI-MS/MS	MS/MS	–	–	(Ferrao et al. 2012)
T. cruzi	*Metacyclogenesis*	TiO_2	LC-ESI-MS/MS	MS/MS (MSA)	753	2,572	(Marchini et al. 2011)
L. donovani	Axenic promastigotes and amastigotes	Phosphoprotein purification kit (Qiagen), TiO_2	2DE-MALDI-MS/MS	MS/MS	2	3	(Morales et al. 2010)
L. donovani	Axenic amastigotes	Phosphoprotein purification kit (Qiagen), TiO_2, SCX	LC-ESI-MS/MS	MS/MS	126	181	(Hem et al. 2010)
T. brucei	Procyclic form	Immunoprecipitation of Tyr-phosphorylated peptides	LC-ESI-MS/MS	MS/MS	34	46	(Nett et al. 2009a)
T. brucei	Bloodstream form	SCX, TiO_2	LC-ESI-MS/MS	MS/MS	491	1,204	(Nett et al. 2009b)
T. cruzi	Epimastigotes	SCX, IMAC		MS/MS, MS/MS/MS, MS/MS (MSA)	119	220	(Nakayasu et al. 2009)
L. donovani	Axenic promastigotes and amastigotes	Phosphoprotein purification kit (Qiagen), Phosphoprotein-specific Pro-Q diamond stain	2DE-MALDI-MS/MS	MS/MS	–	–	(Morales et al. 2008)

2DE *MALDI* Matrix-assisted laser desorption/ionization, *MS* Mass spectrometry, *LC* liquid chromatography, *MSA* Multi stage activation
Only proteins with phosphorylation site identification were considered

Currently, there is no gold-standard technique, and the general agreement is that a comprehensive characterization of a phosphoproteome rely on the use of multidimensional strategies, combining pre- or post-fractionation approaches with phospho-enrichment methods and phosphoproteomic-specific mass spectrometry.

4.2.1 Fractionation Methods

In order to decrease the sample complexity and to increase the number of phosphosites identified, additional to the regular C18 liquid chromatography fractionation methods are used previously or subsequently to enrichment in phosphoproteomics studies. The most extensively employed fractionation methods are ion exchange chromatography, comprising of strong anion exchange (SAX) and strong cation exchange (SCX); hydrophilic interaction chromatography (HILIC), and electrostatic repulsion-hydrophilic interaction chromatography (ERLIC).

The SCX separation principle is based on the differences of charge state between phosphorylated and nonphosphorylated peptides in solution. Under acidic conditions (pH 2.7 or lower), tryptic phosphopeptides have a net charge of 1+, whereas the nonphosphorylated tryptic peptides are double positively charged. As a consequence of the weaker interaction of phosphorylated peptides with the SCX column they are expected to elute earlier than the nonphosphorylated peptides; although some studies have noticed that phosphopeptides are also present in other SXC fractions (Trinidad et al. 2006; Villen et al. 2007). SCX can be used either online or off-line, alone or combined with immobilized metal affinity chromatography (IMAC) or TiO_2 enrichment methods (Ballif et al. 2004; Beausoleil et al. 2004; Lim and Kassel 2006; Villen et al. 2007; Olsen et al. 2010; Dephoure and Gygi 2011; Monetti et al. 2011).

The SAX method is based on the retention of negatively charged phosphopeptides in a positively charged stationary phase. This methodology can be used alone, or more frequently, combined with IMAC or TiO_2 as a pre- or post-fractionation step (Nuhse et al. 2003; Han et al. 2008; Ficarro et al. 2009; Nie et al. 2010; Ficarro et al. 2011).

The HILIC chromatography is an orthogonal analytical method to RP, which partionate molecules based on their polarity. Phosphopeptides bind to a polar stationary phase under high concentration of organic solvent and are eluted by increasing the aqueous buffer proportion (McNulty et al. 2011; Wu et al. 2011).

The ERLIC separation method combines properties of ion exchange chromatography with HILIC and the selectivity of this method can be controlled by changing pH, salt gradient, or organic proportion of the mobile phase (Alpert 2008). A recent study compared the performance of online ERLIC ESI-MS/MS with the current most employed RP ESI-MS/MS resulting in 57 % more peptides (de Jong and Griffin 2012). This combination showed very efficient for enrichment of both singly and multiply phosphorylated peptides, since singly phosphorylated peptides are effectively fractionated by SCX while multiply phosphorylated peptides are fractionated by ERLIC (Zarei et al. 2012).

4.2.2 Antibody-Based Enrichment

Specific antibodies can be used to detect phosphorylated proteins in Western blotting assays and to isolate proteins and peptides by immunoaffinity chromatography. Although less abundant in nature, phosphorylation on tyrosine residues has been far more studied by antibody-based approaches than phosphorylation on serine and threonine residues. This is partially because highly specific and selective antibodies recognizing phosphotyrosine are available, while the low specificity of anti-phosphoserine and anti-phosphothreonine antibodies has limited the number of studies targeting these residues. However, the large amount of starting material required to immunoprecipitate is a major drawback of phosphotyrosine immunoprecipitation (Villen et al. 2007).

Immunoprecipitation using phosphotyrosine-specific antibodies has been successfully employed at both the protein and peptide level, enabling the identification of hundreds to thousands of phosphotyrosine sites in distinct cell types (Rush et al. 2005; Rikova et al. 2007; Villen et al. 2007; Kettenbach and Gerber 2011). Nett and coworkers used phosphotyrosine antibody to enrich phosphopeptides for a better understanding of the phosphorylation of tyrosine in *T. brucei* (Nett et al. 2009a) (Table 15.2).

4.2.3 Metal Affinity Enrichment

Metal Oxide Affinity Chromatography (MOAC)

The MOAC strategy is based on the high affinity of metal oxides towards phosphate ions. The use of different metal oxides such as titanium, aluminum, and zirconium for phosphopeptide enrichment purposes has been reported. Among these, TiO_2 is the most used and, therefore, is the focus of this section (Pinkse et al. 2004; Wolschin et al. 2005; Kweon and Hakansson 2006).

Due to its amphoteric ion-exchange properties, phosphopeptides bind to TiO_2 under acidic conditions and are eluted under alkaline pH (Pinkse et al. 2004). The major drawback of this technique was, initially, the nonspecific binding caused primarily by peptides rich in acidic residues. To circumvent this problem, organic acids (e.g. 2,5-dihydroxybenzoic acid (DHB), phtalic acid, glycolic acid and lactic acid) have been used in the loading step, to decrease unspecific adsorption of non-phosphorylated peptides. These compounds act as competitive binders, exhibiting intermediate affinity to TiO_2 microspheres (more greater than acidic peptides and less than phosphorylated peptides) increasing the enrichment specificity (Larsen et al. 2005; Jensen and Larsen 2007; Sugiyama et al. 2007). Furthermore, the use of stronger acids, such as trifluoroacetic acid (TFA), in the loading step also decreased nonspecific binding of acidic peptides (Thingholm et al. 2006).

This robust enrichment approach has been widely employed in large-scale studies to identify and quantify phosphosites (Olsen et al. 2010; Lundby et al. 2012) and was the choice of four articles for trypanosomatids. Two of these studies, performed

for *L. donovani* (Hem et al. 2010; Morales et al. 2010), used 2DE to separate phosphorylated protein previously enriched by a phosphoprotein purification kit (Qiagen), followed by protein digestion and phosphopeptide enrichment by TiO2. The other two applied SCX followed by MOAC or MOAC directly, respectively *T. brucei* (Nett et al. 2009b) and *T. cruzi* (Marchini et al. 2011) (Table 15.2).

Immobilized Metal Affinity Chromatography (IMAC)

Currently, in addition to TiO_2, IMAC is one of the most utilized enrichment techniques in phosphoproteomic experiments. The IMAC approach is based on the electrostatic interaction between the negatively charged phosphate group and different trivalent metal ions (e.g. Fe^{3+}, Ga^{3+}, Al^{3+}, Zr^{3+} and Co^{3+}), immobilized in columns through different linkers, such as nitrilotriacetic acid (NTA), iminodiacetic acid (IDA) and tris-(carbomethyl)-ethylendiamine (TED) (Andersson and Porath 1986; Posewitz and Tempst 1999; Gaberc-Porekar and Menart 2001; Nuhse et al. 2007).

The strong affinity of peptides rich in acidic residues to IMAC columns impedes the selectivity of this method. However, changes in the pH, concentration of organic solvent and derivatization of carboxylic groups of acidic residues decrease nonspecific binding, improving the specificity of the method (Ficarro et al. 2002; Kokubu et al. 2005; Tsai et al. 2008). The IMAC enrichment is largely used, mainly in combination with pre-fractionation approaches (Huttlin et al. 2010; Dephoure and Gygi 2011). As an example for trypanosomatids, this methodology was applied to *T. cruzi*, were, the authors used pre-fractionation of peptides with SCX, followed by IMAC, phosphopeptide enrichment of each fraction (Nakayasu et al. 2009) (Table 15.2).

4.3 Final Considerations

Methods combining distinct fractionation and/or enrichment approaches were also developed. The SIMAC (Sequential Elution from IMAC) approach was designed combining the positive aspects of both IMAC and TiO_2 to effectively enrich singly and multiply phosphorylated peptides from complexes samples (Thingholm et al. 2008; Engholm-Keller et al. 2011; Engholm-Keller et al. 2012).

Some studies have shown that each enrichment method or combination of methods possesses distinct specificity towards a particular segment of the phosphoproteome. A comparison between Phosphoramidate Chemistry, IMAC and TiO_2 showed an overlap of approximately 35 % between the three approaches (Bodenmiller et al. 2007). A large-scale phosphoproteomics study compared 3 different prefractionation strategies – SCX, ERLIC and HILIC– previous to TiO_2 enrichment (Zarei et al. 2011), the combination of SCX with TiO_2 enabled the highest number of phosphopeptide identification, whereas the use of ERLIC associated to TiO_2 showed the best performance to enrich multiply phosphorylated peptides. An overlap lower than 10 % between the three approaches was found.

As previously mentioned, the trend in the field for other species has been the combination of pre- and/or post-fractionation (SCX, SAX, HILIC, ERLIC), enrichment methodologies (IMAC, TiO_2) and mass spectrometry setups, since there is no single method capable of covering the entire phosphoproteome. Therefore, in order to obtain the most comprehensive possible characterization and a closer picture of a complete trypanosomatid phosphoproteome, the same principles should be applied.

5 Mass Spectrometry Identification of Phosphorylated Sites

5.1 MS Fragmentation Methods for Phosphopeptides

To precisely determine the site of phosphorylation, the sequence of phosphopeptide must be known and the specific modified amino acid must be assigned. In a typical experiment to identify peptides by mass spectrometry (MS), MS^1 refers to the initial mass-to-charge-ratio (m/z) spectrum collected for all molecular ions present in a sample, called precursor ions, which can be selected for further analysis by fragmentation. To uncover its sequence a peptide must be energized and disrupted into fragments through tandem MS (MS/MS or MS^2). During MS^2, selected precursor ions from the MS^1 stage are dissociated into fragments (product) and the fragment ion masses are measured, providing further information about the molecular structure of the precursor ion. A second mass analyzer is often required, hence the term 'tandem MS'. According to the nomenclature proposed by Roepstorff and Fohlman (1984), if the charge is retained by the N-terminal of the product ion, the fragment ion is classified as ion a, b or c. If it is retained by the C-terminal of the product ion, it is classified as ion x, y, or z. In shotgun proteomics experiments (peptide-based proteomics using LC-MS/MS), the peptide sequence, as well as site-specific information, are provided by such fragment ion spectra, generated through MS/MS. There are several MS/MS fragmentation strategies applicable to the analysis of phosphopeptides (Salih 2005; Bakalarski et al. 2007; Boersema et al. 2009; Grimsrud et al. 2010; Leitner et al. 2011). Here, we present the basic concepts of the most vantages for the characterization of phosphopeptides.

Collision-induced dissociation (CID) is the most widely used fragmentation strategy for sequencing (Hunt et al. 1986; Sleno and Volmer 2004; Wells and McLuckey 2005) peptides with and without phosphorylation. When undergoing low-energy CID, peptides collide with inert gas molecules causing a large amount of energy to deposit into the peptide backbone which, ultimately, leads to the dissociation of the most labile bonds, typically at the amide bonds between amino acid residues. After CID, the resulting fragment ions are predominantly b- or y-type product ions, containing the N- or C-terminus of the peptide, respectively. In MS^2 CID spectra, the sequence of a precursor peptide can then be deduced based on the series of b- or y-type product ions generated. In the case of phosphopeptides, however, CID usually causes the dissociation, or neutral loss, of a phosphate group

(H_3PO_4, −98 Da) from pSer and pThr residues, which are more labile bonds than the amide bonds joining the amino acid residues in the peptide backbone. Therefore, upon CID many pSer- and pThr-peptides exhibit a neutral loss of H_3PO_4 as the dominant fragmentation pathway and show low efficiency in breaking the phosphopeptide backbone compared to non-phosphorylated peptides. Consequently, when using CID for pSer- and pThr-peptides, product ions resulting from neutral loss dominate the MS/MS spectra, while product ions derived from backbone fragmentation may be present in very low intensities or might be completely suppressed, thus, making it difficult to deduce the sequence of the precursor peptide. This is in contrast with pTyr-peptides, for which the modification remains stable (Steen et al. 2001; Palumbo et al. 2008; Johnson and White 2012).

Nonetheless, new methods have been developed to take advantage of this distinctive fragmentation behavior of pSer- and pThr-peptides and to improve their fragmentation upon CID. In a strategy called neutral loss-triggered MS^3, the neutral loss of a phosphate group can be detected in real time during MS/MS, triggering (in a data-dependent manner) the selection of the neutral loss product for further fragmentation by MS^3 that provides additional sequence information . Alternatively, in a strategy called multi-stage activation (MSA) or pseudo-MS^n (Schroeder et al. 2004), the precursor ion and one or more neutral loss product ions can be subsequently activated, without an intermittent isolation step, and used to form a more informative composite spectrum, which contains both MS/MS and MS^3 information with significant enhancement in the intensity of the backbone fragment peaks. A more recent type of fragmentation, termed higher-energy C-trap dissociation (HCD), provides beam type collision cell CID MS/MS with detection of fragment ions at high resolution (Olsen et al. 2007). As a result of the faster energy deposition and the occurrence of consecutive dissociation, HCD MS/MS spectra of phosphorylated peptides contain less abundant neutral loss product ions, and more interpretable sequence information, thus allowing for interpretation of the peptide sequence without MS^3 or MSA fragmentation.

Recently, two new techniques, electron capture dissociation (ECD) (Zubarev et al. 2000; McLafferty et al. 2001) and electron transfer dissociation (ETD) (Syka et al. 2004; Mikesh et al. 2006) have arisen as promising fragmentation modes to characterize phosphopeptides, which differ from CID in the mechanism of dissociation and predominant product ions generated. In both techniques the mechanism of dissociation is the same, i.e. electron capture neutralization of a charge site on a multiple-charge ion resulting in formation of a radical ion, the difference being in the source of electrons. ECD involves the direct introduction of low energy electrons to trapped gas phase ions. The mechanism of ECD is still under debate but appears to be a fast process (nonergodic) in which the radical reactions initiated by the electron may be responsible for the cleavages. In ETD, the peptides are placed in contact with a radical gaseous anion, such as fluoranthene, which transfers an electron to the peptide cation, causing it to fragment. The cleavage, in both techniques, occurs at the amide bond close to the radical site (between the peptide bond nitrogen and the α-carbon along the peptide backbone), generating primarily c- and z-type product ions, making ECD and ETD MS/MS spectra highly complementary to CID

MS/MS spectra. In contrast to CID, fragmentation by ECD/ETD is sequence independent and, more importantly to phosphopeptide sequencing and phosphosite localization, leaves many post-translational modifications intact. Also, fragmentation by ECD/ETD allows sequencing of longer peptides (20-30 residues, while CID fragmentation is limited to 20 or fewer residues) and generation of product ions occurs in a more predictable and homogeneous manner than in CID.

5.2 Localization of Phosphorylation Sites

Even though phosphorylated peptides are effectively fragmented, and thereby identified by MS^2 and MS^3, the correct localization of phosphorylation sites can be difficult to achieve, especially when multiple serine, threonine and tyrosine residues are present within a single peptide or for multiply phosphorylated peptides. To circumvent this problem and unambiguously position the phosphorylation event in a site-specific manner, the fragments surrounding each site must be identified in the MS^2 or MS^3 spectrum. Consequently, different approaches to complement the traditional search engines and manual inspection have been developed.

Matthias Mann's group published one probabilistic method specific for PTM (Olsen et al. 2006), which is based on a previous work for MS^2 + MS^3 peptide identification (Olsen and Mann 2004) and currently implemented on the MSQuant (http://msquant.sourceforge.net) (Mortensen et al. 2010) and MaxQuant (www.maxquant.org) (Cox and Mann 2008) software suites. Briefly, this method is probability-based and uses the four most intense fragment ions for every 100 m/z window. When dealing with MS^2 spectra, fragment ions are expected to retain the phospho-group (+80 Da) and for neutral loss-triggered MS^3 the fragments lose H_2O (18 Da). For each mass range, the algorithm looks for matches of all combinations of phosphorylation sites to the putative b- and y-ions.

Another probability-based method for localization of phosphorylation sites was published by Steven P Gygi, and implemented on Ascore (http://ascore.med.harvard.edu) (Beausoleil et al. 2006). Briefly, in this method a cumulative binomial probability is obtained using the 100 m/z windows, where all possible b- and y-type ions are the number of trials and the number of successes are the matched ions for each possibility (possible phosphorylated site) versus peak depth (peaks per 100 m/z).

Another important point of consideration involves how to correctly manage rare events, such as, for example when one type of modification is much less frequent than another analyzed in the same sample. In phosphoproteomics experiments, this occurs with tyrosine phosphorylation that is substechiometric when compared to serine and threonine phosphorylation. In these cases, treating all modifications as "equally probable" might overestimate the occurrence of such rare modifications. Therefore, to avoid generating false positive hits, Gnad and co-workers (2009) suggest that an independent FDR (False Discovery Rate) be used for each one.

6 Phosphoproteomics of Trypanosomatids

Due to the importance of protein phosphorylation in the control of signal transduction, especially considering the key role of post-transcriptional control in protozoan parasites, much effort has been made in the search for phosphorylated proteins on these organisms, including *Leishmania* and *Trypanosoma*. However, due to technical limitations, only a few have been initially identified, including RNA polymerase II (Chapman and Agabian 1994) and the acidic ribosomal P proteins (Gomez et al. 2001). With respect to identifying phosphorylation sites using traditional approaches, until very recently only a couple of examples have been described for Kinetoplastida, such as on Histone H1 Ser12 for *T. cruzi* (da Cunha et al. 2005).

Phosphoproteomics helped to increase the number of phosphorylated proteins, as well as phosphorylation sites described for these organisms. Up to now, studies have been performed on *Leishmania donovani*, *Trypanosoma brucei* and *Trypanosoma cruzi*, and over 1,500 phosphoproteins and 4,200 phosphorylation sites have been described for trypanosomatids (Table 15.2, only proteins with phosphorylation site identification were counted), increasing rapidly ever since.

6.1 Leishmania donovani

The first phosphoproteomic study targeting trypanosomatids was performed for axenic promastigote and amastigote forms of *L. donovani* (Morales et al. 2008). Using a combination of IMAC phosphoprotein enrichment, fluorescent phosphoprotein staining of 2DE-separated proteins and MALDI-TOF-TOF, the authors identified phosphorylated proteins, involved in stress and heat shock response, RNA/protein turnover, metabolism and signaling. Several of these proteins were detected in multiple forms, mainly due to differences in their phosphorylation state, as confirmed by shifting of 2DE spots upon phosphatase treatment. Although this study consisted essentially of a qualitative 2DE analysis, and did not describe any phosphorylation sites, few of the phosphorylated proteins identified showed differences in abundance/distribution of isoforms between stages, or were detected in their phosphorylated form exclusively in amastigotes, suggesting the existence of stage-specific phosphorylation in *L. donovani*.

Later, a gel-based quantitative analysis (2D-DIGE) of affinity enriched *L. donovani* phosphoproteins identified and quantified phosphorylated proteins, among which 38 % displayed stage-specific regulation (Morales et al. 2010). Some of the findings from these articles are described here. This study also revealed that amastigote-specific phosphoproteins were mostly chaperones, including HSP90 (HSP83), HSP70, stress-induced ST1/HOP that have phosphorylation sites identified and cyclophilin 40, *L. donovani* orthologous of tetratricopeptide repeat (TPR) domain-containing peptidylprolyl-isomerase-like protein LinJ19_V3.1560. A couple of phosphorylation sites were identified for HSP90 (Thr223 and Ser256) and HSP70 (Thr498). The *L. donovani* phosphorylated threonine residues are either conserved

or replaced by serine residues in human HSP90 and HSP70, and thus may be regulated by phosphorylation in higher eukaryotes as well. More interestingly, HSP90 Ser526 is exclusive of *L. donovani* and, although occupied by a threonine in *Trypanosoma*, is substituted by an aspartic acid in humans and mice, suggesting that in higher eukaryotes, HSP90 may be locked into a conformation that mimics constitutive phosphorylation. These findings indicate that regulation of HSP90 functions through post-translational modifications may substantially differ between parasite and host despite the highly conserved sequence of this protein from *L. donovani* to humans. It was also demonstrated that in *L. donovani*, similar to other eukaryotes, chaperone phosphorylation is linked to formation multiprotein complexes. STI/HOP-containing chaperone complexes interact with ribosomal clients in an amastigote-specific manner, and STI1 residues S15 and S481 are essential for parasite viability.

More recently, gel-based qualitative and quantitative analyses have been extended by the use of quantitative MS-based proteomics, to characterize the phosphoproteome and monitor signaling events in *L. donovani* axenic amastigotes (Hem et al. 2010). In this approach, two complementary experiments were performed, and 2-D Nano-LC-ESI-MS/MS was used to identify affinity enriched *L. donovani* phosphoproteins and TiO_2-enriched phosphopeptides. In some samples, isobaric tag for relative and absolute quantification (iTRAQ) labeling was used for quantification. The analysis of IMAC-enriched phosphoprotein extracts led to the identification of 126 phosphoproteins, and was complemented by the identification of 181 unique phosphorylation sites (distributed in 126 distinct phosphoproteins) in the analysis of TiO_2-enriched phosphopeptides. Similar to the distribution observed in higher eukaryotes, the phosphorylation events identified corresponded to 86 % pS, 12 % pT, and 2 % pY residues. The data showed significant enrichment of proteins whose biological functions are associated with protein turn-over, stress response, and signal transduction. More interestingly, conservation analysis across related trypanosomatids and higher eukaryotes revealed *L. donovani*-specific phosphoresidues in highly conserved proteins that share a significant sequence homology to orthologs of the human host. The identification of *L. donovani*-specific phosphoresidues supports the previous findings that the *L. donovani* stress response may be largely regulated at the post-translational level by protein modification in a parasite-specific manner.

The existence of specific phosphorylation sites in the major *L. donovani* chaperones at the pathogenic stage opens several possibilities for further studies and points to these proteins as promising targets for the development of novel anti-parasitic drugs, via inhibition of their respective protein kinases. Although very promising, experimental characterization of these phosphorylation sites is still lacking and whether or not they can be validated as drug targets remains to be established.

6.2 Trypanosoma cruzi

The phosphoproteome of *T. cruzi* was first characterized in a proteomic fashion in 2009 by Nakayasu and coworkers (Nakayasu et al. 2009), through a proteomic approach combining SCX fractionation, ion metal-affinity chromatography

(IMAC) phosphopeptide enrichment and LC-ESI-MS/MS (MS/MS, MS/MS/MS and MSA) coupled in a low-resolution mass spectrometer. The authors identified 192 unambiguous phosphorylation sites in 119 distinct proteins of *T. cruzi* epimastigotes.

Phosphorylation sites were distributed in 148 (65.5 %) serine, 57 (25.2 %) threonine, and 8 (3.5 %) tyrosine residues. The adjacent regions of several phosphorylation sites matched known consensus phosphorylation motifs, pointing to the potential kinases responsible for these modifications. The most represented motifs were CAMK2, CK1, PKA, CK2, GSK3, ERK and CDK1, known to be related to Ser/Thr-kinases. In addition, few Tyr-kinase motifs, such as ALK, SRC, and EGFR were also matched by the identified phosphorylation sites.

The phosphorylated proteins identified by Nakayasu and co-workers in *T. cruzi* epimastigotes were related to several basic cellular functions, such as cell structure, motility, transportation, metabolism, DNA/RNA/protein turnover and signaling, suggesting a role of protein phosphorylation in the control of these processes. More interestingly, some of the phosphoproteins belonged to groups involved in pathogenesis of the parasite (e.g. *trans*-sialidases and dispersed gene family, DGFs) and, therefore, could also be regulated by phosphorylation.

To characterize the phosphoproteome of *T. cruzi* in more depth, Marchini and co-workers (Marchini et al. 2011) analyzed cells in different stages during the process of metacyclogenesis (differentiation of the parasites from non-infective epimastigotes to infective metacyclic trypomastigotes), which resulted in an increase of more than 13-fold in the total number of phosphosites previously available for this parasite. In this study, phosphopeptides were enriched using TiO_2 chromatography and analyzed by LC-MS/MS, using MSA for better fragmentation. In total, 753 phosphoproteins and 2,572 phosphorylation sites were identified.

The distribution of phosphorylation sites was 2,162 (84.1 %) on serine, 384 (14.9 %) on threonine and 26 (1.0 %) on tyrosine residues. As in other eukaryotic phosphoproteomes, it was also determined that the phosphorylated amino acid residues of *T. cruzi* are located almost exclusively in turn and loop regions of the proteins surface, making them more accessible than their non-phosphorylated counterparts. Regarding conservation among other eukaryotes, the phosphorylated *T. cruzi* proteins are more conserved than non-phosphorylated proteins. On the other hand, in the prokaryotic domain this difference disappears and phosphorylated proteins are just as conserved as those that are non-phosphorylated.

The phosphorylated proteins identified were enriched for different functional groups, including some related to the morphological changes as the repositioning of organelles during the differentiation of the parasite. Despite the fact that the data obtained by Marchini and co-workers (Marchini et al. 2011) was not quantitative, it was possible to observe similar biological processes regulated in a timely manner across metacyclogenesis. For instance, proteins involved in calcium ion binding, protein kinase activity and protein modification appeared at all-time points, while processes related to cytoskeleton, motor activity and nucleotide binding were enriched at later stages of differentiation. Evidence was also found of phosphorylation

in several pathways involved in signal transduction, particularly related to protein kinase activity.

The data showed evidence of phosphorylation of about 22 % of the kinome (42 protein kinases in total), covering 39 ePKs (e.g. PKA, CAMK, CK1, CDK, DYRK, GSK, CDKL, MAPK, MAPK-like, CaMKK, NEK, PEK and ULK) and 3 aPKs (e.g. FRAP and RIO2). Interestingly, the *T. cruzi* kinome S/T/Y phosphorylation site distribution shifted towards the phosphotyrosines (6 % of all the phosphorylation sites found on PKs are pYs), showing a sixfold enrichment compared to the rate found for the total phosphoproteome, and protein kinases (including DYRK and GSK), accounting for more than 30 % of the phosphotyrosine sites identified. Among the phosphorylated *T. cruzi* protein kinases, 13 presented phosphorylated residues in their activation loop and could be under regulated by this modification. These included 6 of the 15 described MAPK or MAPK-like kinases showing signs of activation, as evidenced by the concomitant presence of both tyrosine and threonine phosphorylation on their activation loop.

As described by Nakayasu et al. (2009), some of the phosphorylation sites of *T. cruzi* are located in regions matching known consensus protein kinase motifs. To expand on this analysis, Marchini and co-workers (2011) used a different approach, one in which the conservation of the amino acids close to the phosphorylated residues was used to directly extract patterns from the data and reveal several phosphorylation motifs. Despite the absence of a direct link between the substrate and the protein kinase, the phosphorylation sites could be grouped based on the similarity of their surrounding amino acid sequences, and the proteins contained in each phosphorylation motif group were found to be enriched for specific functions: (1) RxxpSxS (motor activity), (2) RxxSxxpS (motor activity, cytoskeleton and cytoskeleton organization and biogenesis), (3) pSxS (kinase activity and cytoskeleton), (4) pSxxG (calcium ion binding), (5) SxxpS (protein kinase activity) and (6) pTxxxxxxxxR (protein kinase activity, protein modification process, amino acid and derivative metabolic process, signal transduction, nucleotide binding, embryonic development and reproduction).

Another study, performed by Ferrão and co-workers (Ferrao et al. 2012), focused on the role of transforming growth factor beta (TGF-β) in *T. cruzi* biology. Using a combined approach of 2DE analysis and mass spectrometry protein identification, the authors analyzed *T. cruzi* epimastigotes treated with TGF-β for different times (0, 1, 5, 30 or 60 min) with regard to changes in protein phosphorylation and/or expression, and reported a global and kinetic view of epimastigote proteins responsive to TGF-β stimulus. In this study, however, due to the technical limitations of the chosen proteomic approach, no phosphorylation sites were identified.

In total, 42 protein spots showing more than 2-fold up- or down-regulation after TGF-beta treatment were identified, including 20 protein spots showing consistent phosphorylation changes in one or more time points after treatment. Most of the regulated proteins identified were involved in metabolic processes (39 %), heat shock response (24.4 %), translation (12.2 %), proteolysis (7.3 %), cytoskeleton composition (4.9 %), signal transduction (4.9 %), and oxidative stress regulation (2.4 %), while others were hypothetical proteins (7.3 %). Proteins that presented changes in phosphorylation were related to metabolism (40 %), heat shock response

(25 %), cytoskeleton (10 %), translation (10 %), signal transduction (5 %), proteolysis (5 %), and also included some hypothetical proteins (10 %).

The response of *T. cruzi* epimastigotes to TGF-β triggered changes in both directions (up- or down-regulation) and seemed to occur very rapidly while several proteins presented changes in phosphorylation and/or expression in the first few minutes after treatment. For instance, phosphorylation of 10 and expression of five of the identified proteins was rapidly increased after 1 min of treatment with TGF-β. In addition, eight protein spots showed reduced phosphorylation and 23 presented down-regulated expressions in response to the addition of TGF-β, 30 % of which were down-regulated in the first minute.

Proteins with the highest phosphorylation increase in response to TGF-β at each time point were: mitochondrial precursor Hsp70 (1 min); beta-tubulin (5 min); IgE-dependent histamine-releasing factor (IgE-HRF, 30 min), also known as translationally controlled-tumor-protein (TCTP) and glycosomal malate dehydrogenase (GMD, 60 min). The most dephosphorylated proteins observed at each time point were: glycosomal malate dehydrogenase (GMD, 1 min); prostaglandin F2-alpha synthase (Pf2αS, 5 min); hypothetical protein (30 min), and mitochondrial precursor Hsp70 (60 min). The proteins with the highest up-regulation in protein expression at each time point were: cyclophilin A and DnaK (1 min); cruzipain (5 min); hypothetical protein (30 min) and tryparedoxin peroxidase (TRYP, 60 min). Among the down-regulated proteins, the most repressed at each time point were: Pf2αS (1 min); methylthioadenosine phosphorylase (MTAP, 5 min); enolase (30 min) and Hsp70 (60 min).

Treatment with TGF-β also induced the proliferation of *T. cruzi* epimastigotes, as well as stimulated the differentiation of trypomastigotes into amastigotes (amastigogenesis). Although evidence to confirm this hypothesis is still lacking, the authors speculate, based on data previously reported in the literature, that the stimulation of epimastigote growth by TGF-β may be partially attributed to positive regulation of proteins such as TRYP, tubulin, and TCTP, which have already been described as participants in the proliferation process and prevention of apoptosis.

6.3 Trypanosoma brucei

Nett and co-workers (2009a) applied a phosphotyrosine-specific proteomics approach to the procyclic form of *T. brucei*. To increase the level of phosphorylation in tyrosine residues, procyclic trypanosomes were treated with hydrogen peroxide before cell lysis, and tyrosine-phosphorylated proteins were identified using a combination of immunoaffinity purification of phosphotyrosine-containing peptides and LC-MS/MS. In total, 34 tyrosine-phosphorylated proteins were identified, including some of unknown function or involved in several functions, such as energy metabolism, protein synthesis, RNA metabolism, most related to protein kinase activity. The latter included 19 protein kinases and 2 metabolic kinases (phospho-enolpyruvate carboxykinase and 6-phospho-1-fructokinase).

The majority of tyrosine-phosphorylated protein kinases identified belonged to the CMGC kinase group, and one member of the NEK kinase group was also identified. Of the CMGC kinases, 11 putative *T. brucei* homologues of *Leishmania* MAPKs were identified, including the characterized TbMAPK2. A homologue to LmjMPK15 (Tb10.329.0030), a member of the *T. brucei* CDK kinase family, was also detected. Full phosphorylation of the highly conserved TXY motif, located in the activation loop of MAPKs, was detected on eight of the identified putative TbMAPKs, indicating that they were present in an activated state in procyclic *T. brucei*. Although the TbMAPK upstream activators remain unknown, the expression of phosphorylated STE kinases, described for bloodstream *T. brucei* in the previous study (Nett et al. 2009b) points to the existence of a canonical signal transduction via MAPK in this parasite. The phosphorylation sites previously identified within the activation segment of both *T. brucei* GSK3 isoforms (Tb927.7.2420 and Tb10.61.3140) and a DYRK homologue (Tb11.02.0640) (Nett et al. 2009b) were also confirmed, strengthening the conclusion that known kinase activating sites and signaling pathways are conserved in this organism. In addition, all the three CRK isoforms (CRK1, CRK2 and CRK3) were also found to be phosphorylated on the same tyrosine residues reported in the previous global phosphoproteomic study of bloodstream parasites, indicating that the phosphorylation of such residues is not stage specific.

It was also demonstrated that, in both *T. brucei* life cycle stages (bloodstream and procyclic), tyrosine-phosphorylated proteins were mostly concentrated in regions associated with cytoskeletal structures (basal body and flagellum) and in the nucleolus of the parasite. The concentration of tyrosine-phosphorylated proteins in specific organelles of *T. brucei* supports the well-known idea that the function of signaling molecules is regulated by their precise location in the cell. On the other hand, it also demonstrates that, for this parasite, the localization pattern of tyrosine-phosphorylated proteins is remarkably different from that of higher eukaryotes, such as human, in which they are found concentrated at the plasma membrane at focal adhesion points. This difference could be explained not only by the absence of conventional receptor tyrosine kinases in *T. brucei,* but also by the differences in pathways regulated by the post-translational modification in this parasite.

Although more targeted studies are necessary to determine which specific tyrosine-phosphorylated proteins are associated with these organelles, some assumptions can be made. For example, the location of tyrosine-phosphorylated proteins in the basal body and along the axoneme of the flagellum to its distal tip suggests an association of signaling proteins with microtubules, similar to what has been demonstrated for mammalian cells. It was previously shown that in *Chlamydomonas reinhardtii*, tyrosine-phosphorylated GSK3 was essential for the assembly and maintenance of the flagellum, presumably through the regulation of intraflagellar transport (Wilson and Lefebvre 2004). Therefore, the concentration of tyrosine-phosphorylated proteins in the flagellum of *T. brucei*, together with the identification of phosphorylation sites in the activation loop of TbGSK3, suggest not only that this kinase is active in the parasite, but also that tyrosine phosphorylation

may be involved in the regulation of vital processes for this organism, such as flagellum formation and basal body segregation. With respect to the nucleolus, detection of tyrosine phosphorylation could be due to the presence of the protein NOPP44/46, as has been previously reported for *T. brucei* (Das et al. 1996). However, evidence to confirm this hypothesis is still lacking.

Thus far, there are two global phosphoproteomic studies available for *T. brucei*, both from Michael A. J. Ferguson's group. In the first study, Nett and co-workers (2009b) profiled the cytosolic phosphoproteome of the bloodstream form of the parasite. In the second study, Urbaniak and co-workers (2013) compared the phosphorylation sites of the bloodstream and procyclic life forms. The latter is also the first study to obtain a truly quantitative phosphoproteomic dataset for Kinetoplastida. The methodology chosen for both studies was similar: a two-step phosphopeptide enrichment approach (SCX fractionation followed by TiO2 chromatography) coupled with peptide identification by LC-MS/MS (using CID with MSA for peptide fragmentation).

The first global phosphoproteomic study, performed by Nett and co-workers (2009b) identified 491 phosphoproteins and described 1,204 phosphorylation sites in *T. brucei*. The general distribution of these phosphorylation sites corresponded to 906 phosphoserine (75 %), 259 phosphothreonine (21.5 %) and 39 phosphotyrosine (3.5 %) residues. The phosphoproteome included proteins involved in different processes, such as signal transduction, processing of DNA and RNA, protein synthesis and degradation and, to a lesser extent, in metabolic pathways. Forty-four of the identified phosphorylated proteins were protein kinases (one aPK, and 43 ePKs). The phosphorylated ePKs belonged to seven major groups, including 18 CMGCs, 5 CAMKs, 7 AGCs, 6 STEs, 3 NEKs, 1 CK1 and 3 members of the "other" kinase group. In addition, the receptor for activated protein kinase C-1 (RACK1) was also found to be phosphorylated. For the sub-set of protein kinases, 100 phosphorylation sites (63 pS, 24 pT and 13 pY) were identified. About half of the ePKs presented multiple phosphorylation sites, and most of the phosphotyrosines (12 of 13) were found on members of the CMGC kinase group, including cyclin-dependent kinases, MAP kinases, glycogen synthase 3 kinase (GSK3), and DYRKs.

Several sets of kinase recognition motifs were matched to *T. brucei* phosphoproteins. The predicted motifs with null or low occupancy in the *T. brucei* dataset were mainly those related to kinases not identified in the parasite. On the other hand, almost every motif corresponding to *T. brucei* kinases showed strong positive hits. For example, cell division cycle 2-related protein kinases (CRK1, CRK2 and three CRK3) were found to be phosphorylated on tyrosine residues (Y16, Y57, and Y34, respectively), and were preceded by phosphorylated serine residues, which could correspond to the conserved phosphorylation sites of human CDK1 by Wee1 dual-specificity tyrosine kinases. Since Wee1 homologues are also encoded by *T. brucei*, this could indicate that, in this parasite, CRKs are regulated by a mechanism similar to that regulating human CDK1. Also, phosphorylated tyrosine residues were found in the activation loops of several *T. brucei* kinases, including putative DYRKs and GSK3β and MAPKs. For GSK3β, in

addition to the detection of a phosphorylated tyrosine residue in the activation loop, a phosphorylated serine residue at the N terminus was identified, which could correspond to the inhibitory site of its human counterpart. Similarly, the identification of members of the STE kinase family and the *D. discoideum* ERK1 homologue kinase in a phosphorylated state suggests the existence of an ERK1/2 signaling pathway in *T. brucei*. However, since tyrosine kinase-like kinase group and G protein-coupled receptors as well as proteins with phosphotyrosine-binding domains are absent from the *T. brucei* genome, this pathway must be regulated by a completely different mechanism in this parasite.

The observation that known activation and deactivation phosphorylation sites are conserved in bloodstream *T. brucei* suggests that at least part of the corresponding known signal transduction pathways might be conserved as well. However, evidence confirming this conclusion and the identification of upstream receptors or downstream effectors of these pathways are still lacking. In addition, 25 % of the manually validated kinase sites were not predicted by the programs, which may indicate the presence of *T. brucei* kinases with novel substrate specificity and thus offer the perspective of identifying new motifs for uncharacterized kinases.

The study by Urbaniak and co-workers (2013) used bloodstream and procyclic *T. brucei* life forms cultivated under SILAC (stable isotope labeling by amino acids in cell culture) (Ong et al. 2002) labeling conditions, resulting in the first study to truly achieve quantification of phosphorylation sites for kinetoplastida. A total of 2,551 protein groups were identified as phosphorylated, which contained 10,096 phosphorylation sites. The phosphorylation sites were distributed in 8,087 phosphoserine (80 %), 1,720 phosphothreonie (17 %), and 289 phosphotrosine (3 %). Among the phosphorylation sites identified, 8,275 could be quantified between both forms analyzed.

In addition to being the largest phosphoproteomic dataset available for kinetoplastida so far, the real importance of this study relies on the meaningful biological information extracted from the phosphorylation site quantification obtained. Some proteins showed upregulation of specific phosphorylation sites in the procyclic form of the parasite, such as (i) PAD2, a carboxylic acid transporter associated with cellular differentiation (three phosphorylation sites with an average nine, eightfold increase) (Dean et al. 2009); (ii) ubiquitin carboxy-terminal hydrolase (10 phosphorylation sites with an average of 12-fold increase) and (iii) C-14 sterol reductase, involved in the biosynthesis of ergosterol, principal sterols in the procyclic form (2 phosphorylation sites with an average of 13.9-fold increase. Other proteins, on the other hand, showed higher phosphorylation levels in bloodstream forms. The RNA recognition motif protein RBP10, reported to promote bloodstream mRNA expression showed 4 phosphorylation sites with an average of 27.8-fold increase.

Besides the specific examples described above, regulated phosphorylation sites were also found for some groups of proteins, such as ZFPs (Zinc Finger Proteins) and RBPs (RNA Binding Proteins), which are expected to be involved in post-transcriptional regulation of gene expression. In the study by Urbaniak and co-workers (2013), 31 of the 49 predicted ZFPs and 13 of the 47 predicted RBPs were identified, among which 11 and five showed specific regulation of phosphorylation respectively.

7 Future Perspectives

The use of localization scores and phospho-specific fragmentation strategies were crucial developments in addressing phosphorylation site localization, replacing the need for manual validation of spectra in large-scale phosphoproteomic experiments. Despite these advances, however, the field of mass spectrometry continues to develop, opening up new possibilities in phosphoproteomics. One of the new strategies being explored is called High/High mass spectrometry, the use of the high resolution for both MS^1 and MS^2. Scanning the fragment ions on High resolution increase the confidence of identification and localization of phosphorylation sites (Michalski et al. 2011). Alternatively, the higher speed of second generation two chamber linear ion traps deliver much more information (Jedrychowski et al. 2011) within the same analysis time, yet with low mass resolution. Although none of these alone is able to guarantee a complete coverage of the phosphoproteome, both represent good options in order to increase the depth of phosphoproteomic datasets.

An interesting perspective involves the correlation of phosphorylation sites and their respective effector protein kinases. To address this issue, some studies have used phosphoproteomic high throughput experiments, together with kinase inhibitors and knockout (Bodenmiller et al. 2010; Kunz et al. 2012).

8 Conclusion

The approaches described in this chapter and the phosphorylation datasets generated per se can offer great possibilities for further biological characterization. The initial phosphoproteomic articles from Kinetoplastida are of great importance to accurately pin point phosphorylation sites, however dynamic information is still scarce. The quantification of phosphorylation sites would help to understand how each phosphosite is turned on and off for each different biological situation. The combination of quantitative proteomics and phosphorylation site quantification with classical biochemical approaches enables several other endeavors, such as the search for phosphorylation binding proteins and biological characterization of phosphorylation sites identified by mutation as important for a given condition. Approaches such as these would lead to a better biological picture of cell signaling.

Despite these advances, high throughput identification of proteins equal in sequence, differing only by their phosphorylation sites, is a situation that still remains unsolved by the current approaches and is rarely assessed by peptide focused proteomics. Defining the set of phosphorylation sites present in any given protein and occurring at the same time is still a challenge. This information could reveal the phosphosites at the intact proteins and clarify the real protein complexity. However, in order to obtain this information, development of large-scale, top down proteomics strategies with the capability of detecting very low stoichiometric events is essential.

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