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

Claudia M. d'Avila-Levy, Ellen C.F. Altoé, Lívia A. Uehara, Marta H. Branquinha, and André L.S. Santos

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

C.M. d'Avila-Levy (✉) • E.C.F. Altoé • L.A. Uehara
Laboratório de Biologia Molecular e Doenças Endêmicas, Instituto Oswaldo Cruz (IOC), Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, Brazil
e-mail: davila.levy@gmail.com

M.H. Branquinha • A.L.S. Santos
Laboratório de Investigação de Peptidases, Departamento de Microbiologia Geral, Instituto de Microbiologia Paulo de Góes (IMPG), Universidade Federal do Rio de Janeiro (UFRJ), Ilha do Fundão, Rio de Janeiro, Brazil

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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

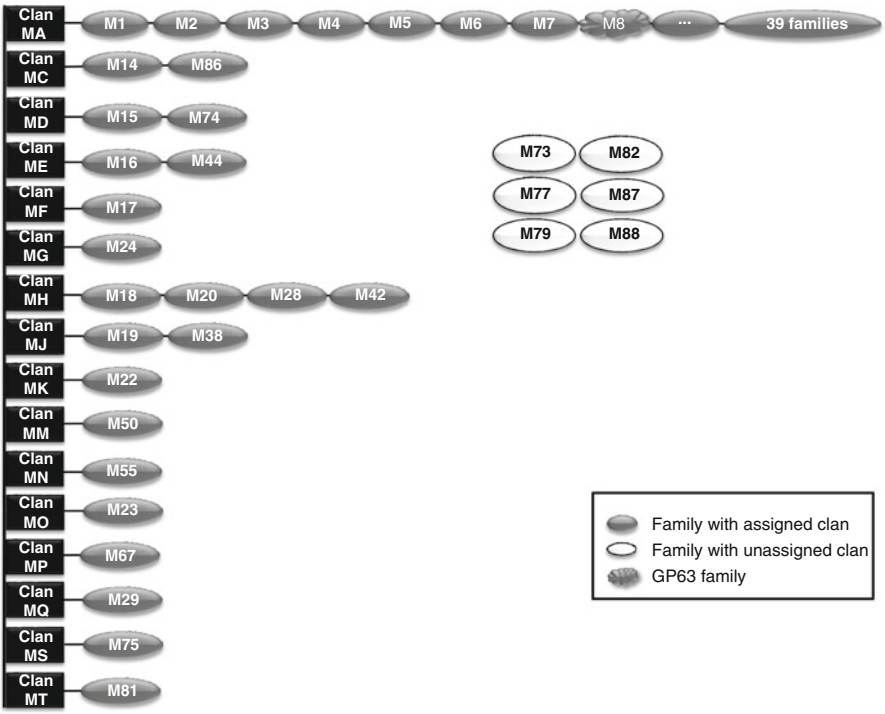

Fig. 11.1 Overview of the metallopeptidase clans and families according to the MEROPS Database (Rawlings et al. 2012). The clan (*black* boxes) contains enzymes that have arisen from a common evolutionary origin of peptidases and represents one or more families (*dark grey* circles) that show evidence 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).

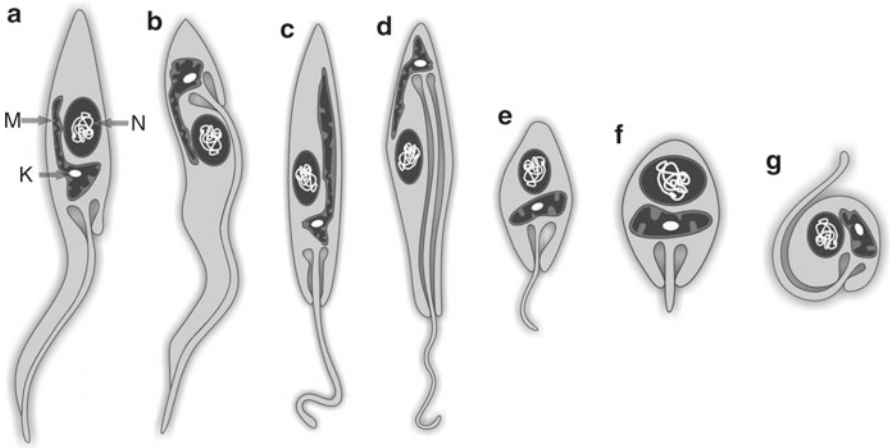

Fig. 11.2 Basic morphotypes of trypanosomatids that serve as genus-defining characteristics in the current taxonomy. Trypanosomatids forms are defined by cell shape, point of flagellum emergence and position of the kinetoplast (*K*) in relation to the nucleus (*N*). For epimastigote (**a**), promastigote (**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

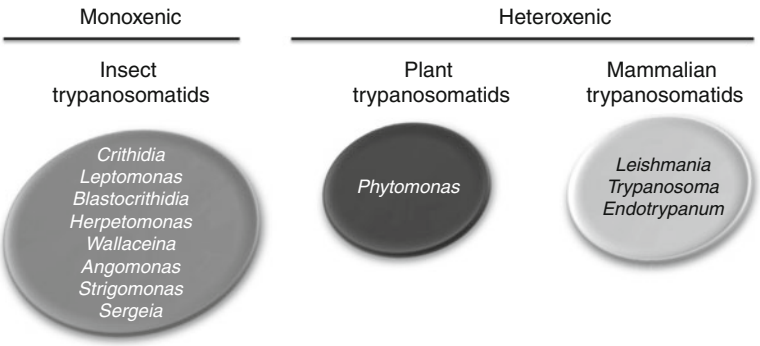

Fig. 11.3 The recognized genera in the Trypanosomatidae family. The development of monoxenic genera occurs in a single invertebrate host, although insect trypanosomatids can be found in plants and there are some reports that describe the presence of these trypanosomatids in mammalian hosts. The heteroxenic 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).

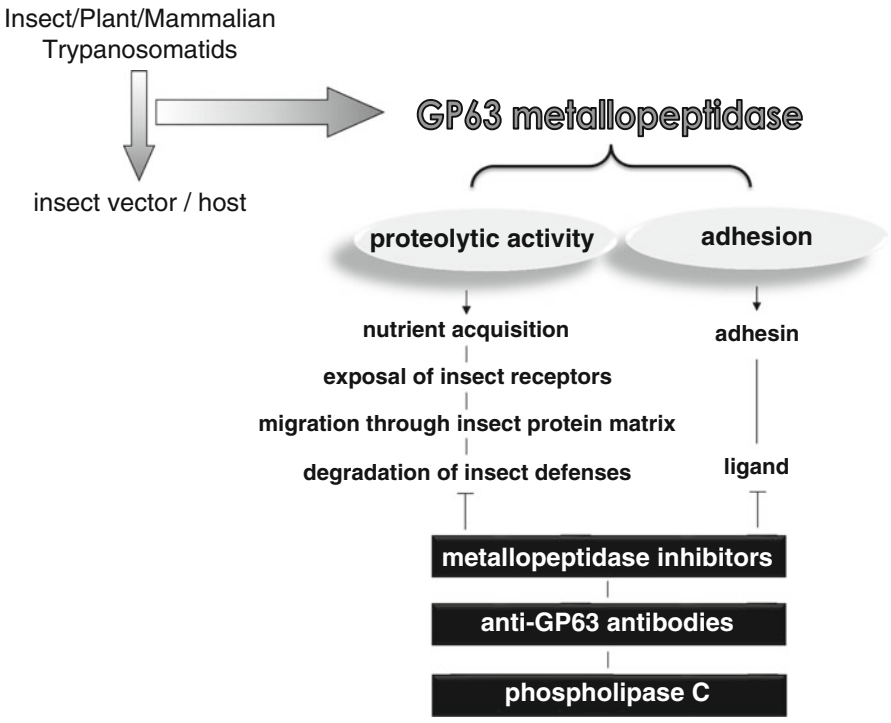

Fig. 11.4 Possible roles played by GP63 metallopeptidase produced by insect, plant and mammalian trypanosomatids (Table 11.1 and Fig. 11.1). The interplay between trypanosomatids and the insect vector or host can be mediated through GP63 molecules, which can act either as a peptidase or in the adhesion. 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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