# 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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A.L.S. Santos et al. (eds.), *Proteins and Proteomics of Leishmania and Trypanosoma*,
Subcellular Biochemistry 74, DOI 10.1007/978-94-007-7305-9_10,

## 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.

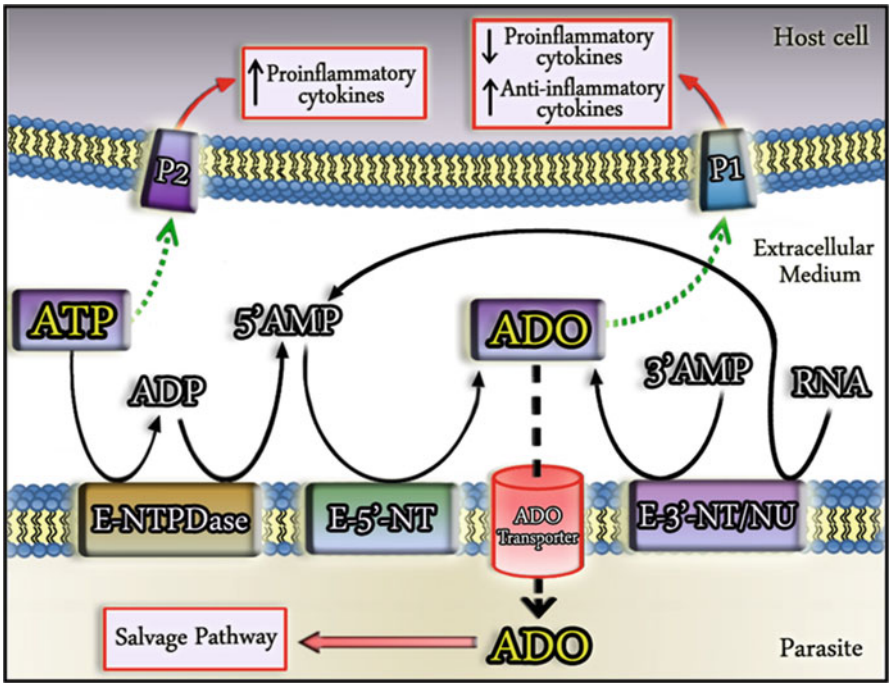


**Fig. 10.1 The action of ecto-nucleotidases in parasite cell surface**. Schematic representation of the reactions catalyzed by the ecto-nucleotidases, emphasizing their substrates and products. The sequential hydrolysis of ATP to adenosine plays a role in both purine acquisition and suppression of host-immune 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

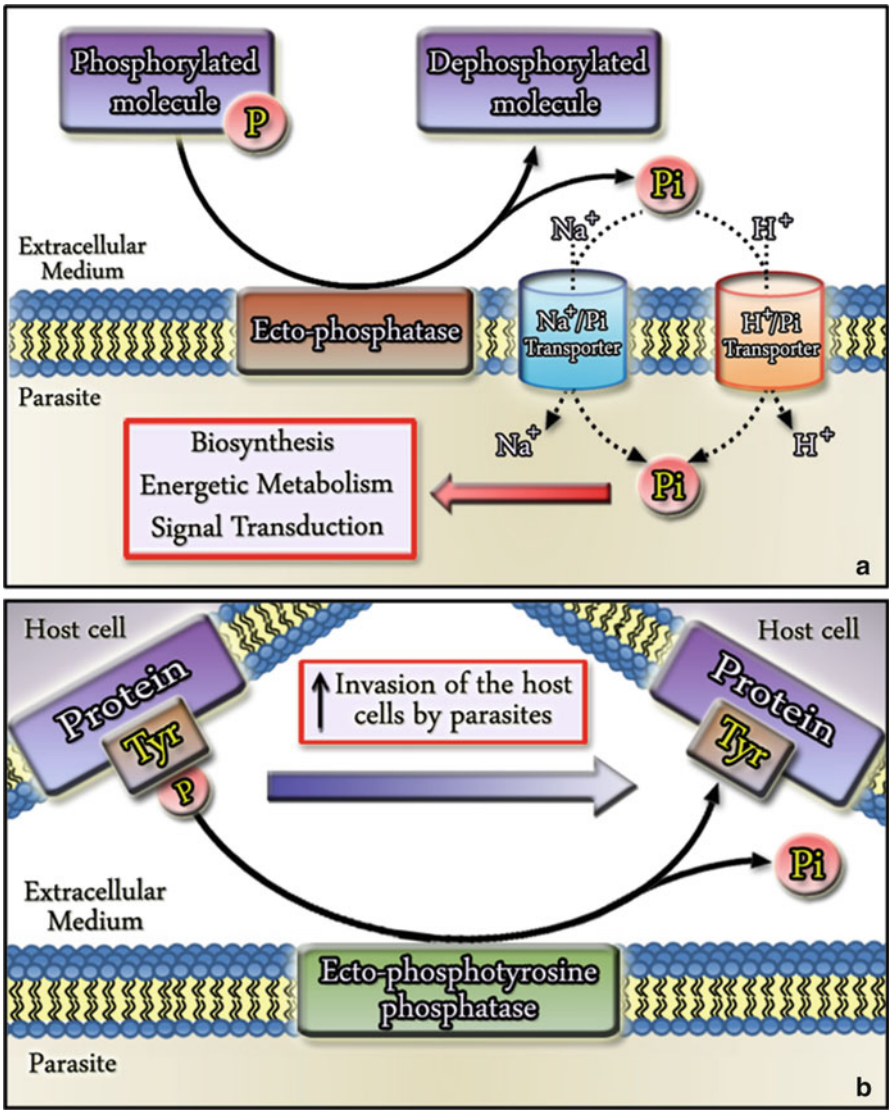


**Fig. 10.2 The action of ecto-phosphatases in parasite cell surface**. Ecto-phosphatases are able to hydrolyze a wide variety of phosphorylated substrates and the released extracellular Pi can be transported into cell by co-transporters of $Na^+$ or $H^+$. Once inside the cell, Pi is routed to the biosynthesis 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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