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Signaling Pathways in *Trypanosoma cruzi* that Modulate Host Cell Interaction

Maria Júlia Manso Alves* and Walter Colli

Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, Brazil

Abstract: Members of the Gp85/trans-sialidase (Gp85/TS) superfamily and mucins play an essential role in the invasion of host cells by *T. cruzi* trypomastigotes. Together, they constitute a large portion of the genome; approximately 700 and 433 genes encode Gp85/TS glycoproteins and mucins (as do a similar amount of pseudogenes), respectively. Gp85/TS proteins bind to a variety of host cell receptors and extracellular matrix components and binding of TS to host cells is independent of their enzymatic activity. Because mucins are the main substrate for TS, their interaction with host cells has been described as carbohydrate-dependent. Complex signaling cascades operate during the infection process within both parasite and host cells, but most research into signaling events has been limited to those of host cells. Much less information about the parasitic side is available; these pathways will be the subjects of intense research in the near future. Analyses of protein kinases and phosphatases in the parasite genome show pathways common to other organisms, but also parasite-specific pathways that should be exploited as candidates for drug targeting.

Keywords: Trypanosoma, kinases, phosphatases, invasion, signaling pathways.

1. INTRODUCTION

Trypanosoma cruzi, the etiological agent of Chagas disease, exhibits biological, immunological and pathological diversity. Since the initial identification of the parasite and description of the disease by Carlos Chagas in 1909 [1], a large spectrum of clinical manifestations have been described, ranging from cases that are asymptomatic to those with severe cardiac or digestive complications. Efforts have been made to correlate clinical manifestations with distinct biological *T. cruzi* strains and different parameters have been proposed for classifying the parasite. Recently, two groups, *T. cruzi* I and II, (with 5 sublineages in group II), associated with the sylvatic and domestic cycles of the parasite, respectively, were identified. However, an analysis of a larger number of samples has pointed to the need for further revision [2]. Clear associations between biological and pathological parameters and genetic classification have not yet been determined. Drawing these types of correlations for a protozoan like *T. cruzi (*which reproduces clonally rather than sexually) has proven to be complicated.

The life cycle of *T. cruzi* includes vertebrate and invertebrate hosts (reduviid insects such as *Triatoma infestans*, *Rhodnius prolixus*, *Panstrongilus megistus*), with an obligatory intracellular stage in the vertebrate. The trypomastigote is the classical non-replicating infective form; metacyclic trypomastigotes (MT) are found in the invertebrate host, and blood trypomastigotes are present in the mammalian host. More than a hundred species of mammals are infected by *T. cruzi*. In humans, an acute phase that may last for 2 months is followed by a long chronic phase, which is asymptomatic in the majority of cases. After several years, 20-35% of the infected individuals will develop irreversible lesions in the heart, intestine or peripheral nervous system [3].

Whereas the basis for this manifestational diversity is not clear, both the host genetic background [4, 5] and the capacity of the parasite to deal with the host immune system contribute to the disease outcome. In a typical infection, macrophages are one of the first cells to be infected following phagocytosis of the parasite. Some of the trypomastigotes survive the oxidative burst, in contrast to engulfed epimastigotes, which are normally killed. Interestingly, during the differentiation process of epimastigotes into trypomastigotes, several antioxidant defense proteins are up-regulated [6]. In addition, when 10 strains of *T. cruzi* were compared, a good correlation was found between antioxidant enzyme contents and parasitemia levels; apparently, high antioxidant levels may contribute to the virulence of the parasite [7].

In order to establish a successful infection, trypomastigotes must adhere to the host cell surface and trigger events required for cell entry. These steps are followed by the formation of the parasitophorous vacuole and the escape of the parasite to the cytoplasm of the host cell. Two mechanisms, either dependent or independent on membrane-recruited lysosomes, have been described [8, 9]. In both, fusion of the lysosomes with the parasitophorous vacuole is essential. The observation that some trypomastigotes can cross host cells without establishing an infection adds a new level of complexity to the biology of the invasion process [10].

After a variable period of time, trypomastigotes escape to the cytoplasm and differentiate to amastigotes. The desialylation of lysosome membrane glycoproteins by the enzyme trans-sialidase, a member of the gp85/TS gene superfamily is important for this escape because it facilitates pore formation by the trypomastigote-secreted protein Tc-Tox [11]. Amastigotes in the cytoplasm divide for the next 4-5 days and then differentiate back into trypomastigotes, passing through a

*Address correspondence to this author at the Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo Av. Prof. Lineu Prestes 748, 05508-900. São Paulo, Brazil; Tel: (11) 30913810/ ext 231;
Fax: (11)30912186; E-mail: mjmalves@iq.usp.br

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transient [12], proline-dependent, intracellular epimastigote form [13]. It should be mentioned that intracellular forms of *T. cruzi* have a capacity to transport proline or glucose that fluctuates with the intracellular levels of the metabolite. Amastigotes are the exception; they possess the highest amount of proline despite a barely detectable level of glucose or proline transport [14], which suggests that endogenous protein is the source of the amino acid. Certainly, the metabolic condition of the host cell is important for parasite survival, as the parasite, a purine auxotroph, is unable to synthesize amino acids such as leucine, isoleucine and valine [15]. Although the host cell nucleus does not seem to play an essential role in parasite development and differentiation [16], the necessity of soluble factors secreted by infected or non-infected neighbor cells cannot be ruled out. In fact, a lower number of trypomastigotes was obtained in the enucleated cells when compared to the nucleated cells, reflecting a less favorable environment for nutrient acquisition. Gene expression in the host cell, including metabolic and signaling genes in fibroblast, endothelial and smooth muscle cells, is altered 24 hours post-infection by *T. cruzi*. Interestingly, down-regulation of genes involved in mitotic cell cycle and cell division was also observed, suggesting that infection by *T. cruzi* may impede host cell cycle progression at late [17], but not early, stages of infection (see *T. cruzi* cell movie at www.sbpz.com.br).

2. ROLE OF THE *T. CRUZI* LARGEST GENE FAMILIES IN HOST CELL INVASION

Host cell invasion by *T. cruzi* is the result of multiple molecular interactions, with the consequent activation of a number of signaling pathways, probably involving redundant mechanisms. The invasion mechanisms of extracellular amastigotes appear to differ from those of trypomastigotes and have been a subject of recent review [18]. Therefore, only trypomastigotes will be focused herein.

The role of calcium in the process of *T. cruzi* invasion is supported by data from different laboratories, with an increase in intracellular free calcium concentration detected in the parasite [19] and host cells within 200 s of interaction with trypomastigotes; a reduction in host cell calcium is correlated with a decrease in the number of parasites in the infected cells [8, 19]. Calcium is essential for the recruitment and fusion of lysosomes with the plasma membrane; it is also required for the rearrangement of actin microfilaments that may facilitate *T. cruzi* invasion by one of several proposed mechanisms [8, 20-23]. The other mechanism of entry, which is lysosome- and actin-independent, occurs with invagination of the host cell plasma membrane [22] and involves the PI3K signaling pathway. Trypomastigote membranes and, to a lesser extent, material constitutively released by trypomastigotes also induced intracellular calcium transients in the host cell. Membrane vesicles shed by trypomastigotes [24] somehow prime host cells for invasion, as they are able to enhance parasite entry into epithelial cells or macrophages and increase the number of amastigote nests in heart tissues [25]. Other molecules also enhance invasion of host cells, such as the glycoprotein Gp83 (released into the matrix by the action of glycosylphosphatidylinositol-phospholipase C following activation of the macrophage MAP kinase pathway) and PKC [23], or members of the Gp85/trans-sialidase (Gp85/TS) superfamily described below. It should be mentioned that Gp85/TS proteins are released in soluble or membrane-bound forms (vesicles) [24].

Many of the important studies characterizing the interaction of *T. cruzi* trypomastigotes with host cell receptors including bradykinin, thromboxane and protease receptors have recently been reviewed [23]. Only some of them will be highlighted here.

2.1. Gp85/Trans-Sialidase (Gp85/TS)

The Gp85/trans-sialidase (Gp85/TS) superfamily of GPI-anchored glycoproteins that are present on the parasite surface is subdivided into groups with and without trans-sialidase activity [26, 27]. Gp85/TS is the largest gene family described in the *T. cruzi* genome (CL Brener strain), constituted by more than 700 genes and almost 700 pseudogenes [28]. Approximately 140 genes encode trans-sialidase proteins. Of these, 70 genes code for enzymatically active proteins and the other half for inactive forms. These proteins are inactive owing to the mutation of a key tyrosine residue, but they still bind sialic acid and galactose substrates [27, 29].

TS proteins are capable of transferring sialic acid from host glycoconjugates to parasite macromolecules [27, 29, 30], such as the mucins that are abundant on the surface of *T. cruzi*. Enzymatic activity does not seem to be important for the invasion step; both active and inactive TS bind to a variety of host cells, mediating parasite entry [21]. Some forms are expressed only in trypomastigotes [21, 30]; the relationship between the levels of a few highly expressed TS members and infectivity of the parasite has been established [21, 23, 30]; sialylation of *T. cruzi* surface molecules confers resistance to complement [31]; sialylated compounds interact with the inhibitory sialic acid-binding protein Siglec-E (sialic acid-binding Ig-like lectin-E) predominantly expressed on cells of the immune system, inhibiting the activation of immune cells [32] TS induces apoptosis in cells of the immune system due to acquisition of sialic acid [33].

Tc85 proteins, a subgroup of Gp85/TS, are mainly expressed in tissue-cultured trypomastigotes (TCT); an equivalent GP82 was described in metacyclic trypomastigotes. Both are devoid of trans-sialidase activity and are transcribed in infective and non-infective stages. Expression of GP82 and Tc85, as described for other genes in trypanosomatids [34, 35] is regulated by post-transcriptional mechanisms [36, 37]. In fact, the mobilization of GP82 mRNA to the polysomes is positively or negatively regulated by proteins whose levels can themselves be mediated by an element present in the 3´-UTR of the transcripts [37].

At least some proteins behave as multi-adhesion molecules, as has been shown for Tc85-11, a cloned member of the Tc85 subset, which binds to laminin by the N-terminal domain [38, 39] and to cytokeratin-18 by the carboxyl subterminal VTV motif of the Gp85/TS superfamily (herein called FLY domain) [40]. FLY also binds to other uncharacterized molecules on both epithelial and non-epithelial cells [41]. A FLY-containing synthetic peptide activates the ERK1/2 signaling pathway, which Gp85/TS proteins participate in [42, 43] and also induces cytoskeleton rearrangement in the host cell, enhancing invasion by *T. cruzi* [41]. Up-regulation of *T. cruzi* entry into endothelial cells through a

FLY-independent region has also been described for one inactive TS protein, but the specific sequence involved was not identified [42].

In addition to participating in the invasion of host cells, non-catalytic regions of TS exert other effects, for example the activation of survival signal pathways including MAPK and PI3K/Akt signaling in neurons, glial, dendritic or epithelial cells [43-46]. TS binds to sialic acid-containing molecules on the surface of endothelial cells leading to NF-κB activation, expression of adhesion molecules (ICAM-1, VCAM-1 and E-selectin) and blockage of cell apoptosis normally induced by growth factor deprivation [42].

Gp82 is the principal member of the Gp85/TS superfamily involved in MT invasion of host cells, and its expression has been correlated with invasion capacity [31]. Gp82 binds to gastric mucin and seems to be an important element in infection by the oral route [31], wherein food contaminated with the insect stages of *T. cruzi* is ingested. This manner of transmission is responsible for the occurrence of recent microepidemics in Brazil and other American countries [47]. Gp82 triggers calcium mobilization in the host cell and Ca^{2+}-dependent cytoskeletal disorganization, which facilitates parasite invasion [31]. In MT, phosphorylation of a 175-kDa protein by a protein kinase activated during parasite invasion, and the generation of inositol 1, 4, 5-triphosphate (IP3) by phospholipase C (which promotes calcium release from IP3-sensitive stores), have been described [31]. Metacyclic trypomastigotes from distinct strains express different amounts of GP82, GP30 and GP90. It is claimed that GP82 (and GP 30) is the principal actor in the invasion process. Since strains that express high levels of GP82 and GP90 are poor invaders, the latter was proposed to be a negative modulator of the invasion. Eventually, the strain expressing high levels of GP90 would become infective provided that this protein is digested in the intestinal tract, thus explaining the relative effectiveness of the oral infection route [31].

2.2. Mucins

The second largest family of genes in the parasite encodes *T. cruzi* mucins, which are also GPI-anchored surface glycoproteins. Approximately 4 x 10^6 mucins cover the parasite surface [26-29]. The small core polypeptide (50 to 200 amino acids length) is rich in carbohydrates O-linked to serine and threonine [see 27, 29, 48] for a detailed review on the structure and function of mucins]. In the insect-derived stages (epimastigotes and MT), mucins have a more homogeneous polypeptide composition, showing a double or triple band in the 35 to 50 kDa range in electrophoretic separations, as compared to TCT mucins, which on PAGE reveal as a smear from 60 to 200 kDa. TCT – and other intermediate mammalian stages – express a third group of mucins, called small surface antigens [29], which increase the mucin heterogeneity present in trypomastigotes.

Other stage-dependent differences in mucin structure have described, such as the presence in trypomastigote mucins of the epitope Gal (α-1,3) Gal, the main target of antibodies response in Chagasic patients [49], or the lipid composition of the GPI-anchor that induces a proinflammatory response *via* MAPK and NF-κB cascades through activation of Toll-receptors [50].

In addition to the protective role of *T. cruzi* mucins play against the proteases of the insect digestive tract, they have been implicated in attachment to, and invasion of, the host. Galactofuranose, which mainly decorates oligosaccharide chains in mucins from epimastigotes [27, 48], is responsible for adhesion of epimastigotes to the intestinal tract. This adhesion step is implicated in the differentiation to MT, as has been shown for *T. cruzi* GIPL-1, a galactofuranose-rich glycophosphoceramide [51]. Several pieces of evidence support a role for carbohydrates such as mannose or galactose in the invasion of *T. cruzi* [23]. Carbohydrates from the 35-50 kDa mucins are important for invasion of various cell types [52, 53] and removal of sialic acid from the surface of the parasite by sialidase treatment enhances the invasion capacity of *T. cruzi* [54]. In addition, mucins from trypomastigotes trigger calcium mobilization, an essential step in the invasion mechanism.

It has been proposed that galectin-3 establishes a bridge between mucins and laminin, thus recruiting trypomastigotes to the extracellular matrix and increasing adhesion of trypomastigotes to cells. Interestingly, galectin-3 expression is up-regulated during infection [55].

GPI-mucin-like proteins from trypomastigotes, but not from epimastigotes, trigger MAPK and NF-κB cascades through Toll-like receptor activation [49, 50] and are therefore responsible for the recognition of microbial and viral molecules and the induction of tolerance by macrophages.

3. *T. CRUZI* AND INTERACTION WITH EXTRACELLULAR MATRIX

Binding of trypomastigotes to elements of the extracellular matrix such as laminin, fibronectin, heparan sulphate, thrombospondin, galectin-3 or collagen IV is partially, but not exclusively, achieved by mucins and members of the Gp85/TS family. In most cases, the *T. cruzi* ligand was not characterized [21, 56]. The interaction of a trypomastigote 85-kDa surface protein with the RGD sequence of fibronectin was one of the first descriptions of the binding of *T. cruzi* to extracellular elements [57]. The role of thrombospondin A and galectin-3 [58, 59], as well as laminin γ1 [60], in the invasion by *T. cruzi* was shown using RNAi, confirming previous observations [38]. Binding of *T. cruzi* to heparan sulfate proteoglycans through a 60-kDa heparin-binding protein known as penetrin [61, 62] was characterized as N-sulfation and glucuronic acid-dependent; heparin inhibited the invasion of cardiomyocytes by *T. cruzi* by 84% [63]. To our knowledge, neither the 60-kDa protein nor its signaling pathway was further characterized. It should be kept in mind that sequential changes to connective matrix components lead not only to parasite binding, but also to the heart tissue fibrosis characteristic of Chagas disease [55, 64, 65]. *In vitro* studies focusing on the remodeling of extracellular matrix proteins have had contradictory results, apparently dependent on experimental conditions [66, 67].

Transforming growth factor β (TGF-β) has been implicated in stimulation of fibrosis and parasitic cellular invasion and growth in Chagas disease, as well as in host immunity down-regulation [68]. Interestingly, the parasite is able to capture TGF-β from the host, an essential step for the differentiation of amastigotes to trypomastigotes [69]. The interac-

tion of TGF-β with its receptors on the host cell membrane leads to the phosphorylation of Smad-2 and Smad-3 in the cytoplasm, which subsequently form a complex with Smad-4. This complex translocates to the nucleus, inducing transcription of different genes such as those encoding for fibronectin, thrombospondin and collagen I, and probably contributing to heart fibrosis. Interestingly, a small inhibitor of TGF-β signaling (SB-431542) administered to experimental animals in the acute phase of infection was a potent inhibitor of parasitemia and heart injury and is being proposed for clinical use [70-72].

4. SIGNALING PATHWAYS IN *T. CRUZI* DURING HOST CELL INVASION

Until recently, knowledge of the signaling pathways activated during invasion by *T. cruzi* has been limited to those of the host cell. This scenario should change rapidly due to genome sequencing of trypanosomatids. In the particular case of the *T. cruzi* genome, genes encoding 190 protein kinases (19 being atypical protein kinases) and 86 phosphatases [72, 73] have been identified.

A detailed comparative analysis of the kinomes of *T. cruzi*, *T. brucei* and *L. major* showed the presence of a group of proteins involved in numerous pathways well established in other organisms, as well as key differences, as the lack of tyrosine kinases and tyrosine kinase-like groups in trypanosomatids. However, because protein tyrosine phosphorylation is described as a common modification of parasite proteins, activity of atypical tyrosine kinases or dual-specificity kinases have been proposed. Absence of common accessory domains present on human protein kinases (Ig, fn3, SH2 and SH3) or the lack of guanylyl cyclase receptor proteins in trypanosomatids are examples of other differences pointed out when trypanosomatids and human genomes are compared, regarding protein kinases.

The majority of the phosphatases in *T. cruzi* belong to the serine/threonine family; the parasite has a lower proportion of tyrosine phosphatases than other eukaryotic genomes [73]. This reduced number of tyrosine phosphatases is consistent with the lack of tyrosine kinases also observed.

Extensive reviews on protozoan MAP kinases [74], PI3-kinases [75] protein serine/threonine phosphatases [76], tyrosine phosphatases [77] and cyclic nucleotide signaling mechanisms [78] have been published recently. It should be pointed out that in contrast to the large number of kinases and phosphatases, only a restricted number of GTPase families are present in trypanosomes, with a complete absence of the heterotrimeric GTPases that are key elements in the signaling processes of other eukaryotes. So far, little is known about the contribution of small GTPases [79].

It is interesting to consider the idea that the flagellum may act as a sensory organ in the MAP kinase pathway [80]. This suggestion was based on the observations that various kinases are involved in the control of the flagellum length, and that a sensory function has been attributed to cilia and flagella in other organisms. Glycosomes and acidocalcisomes, which play important role in energy metabolism in trypanosomatids, are involved in the cellular responses triggered by nutrients. Differentiation in *T. brucei* is regulated by a phosphatase cascade, with the localization of a key unusual type of Ser/Thr phosphatase in the glycosome [81]. The role of acidocalcisomes is coupled to TOR (the target of rapamycin), an evolutionarily conserved atypical protein kinase that belongs to the phosphoinositide 3-kinase-related kinase family. In mammals, TOR signal pathway integrates intracellular and extracellular signals and is activated during various cellular processes [82]. TOR3, one of the three TOR kinases identified in *Leishmania major* is involved in the acidocalcisome biogenesis and infectivity, since *Tor3*$^{-}$ null mutants are unable to survive in macrophages and infectivity to mice is highly attenuated [83]. Two TOR kinases control cell growth in *Trypanosoma brucei* [84] and one of the two unusual TOR-like kinases identified, the cytosolic protein TOR-like 1 kinase, is involved in polyphosphate level and acidocalcisome maintenance [85].

Despite the annotation of a great number of kinases and phosphatases in the genome, very little is known about their role in *T. cruzi* signaling during invasion. One of the few examples is the phosphorylation of a 175-kDa protein during the invasion of MT into host cells by a protein kinase, as pointed out above. Recently, it was shown that treating *T. cruzi* metacyclic or tissue culture trypomastigotes with inhibitors or with specific anti-sense oligonucleotides of calcineurin B inhibits parasite invasion. Although no elucidation of the enzyme-mediated signaling pathways has yet been achieved, this observation points to the relevance of phosphatases in parasite internalization [86]. Interestingly, the parasitic enzyme, in contrast to its human counterpart, lacks calmodulin-binding and auto-inhibitory domains [87].

5. CONCLUDING REMARKS

A plethora of molecules that pass between the parasite and the host are responsible for the establishment of infection. Of these, molecules involved in the adhesion step and the ensuing signaling pathways are particularly being considered as possible drug-target candidates. In addition to more classical approaches, the SELEX method (Systematic Evolution of Ligands by Exponential Enrichment), an oligonucleotide-based combinatorial library approach can be used to isolate high-affinity ligands (aptamers) for a wide variety of molecules, as was done for proteins from the extracellular matrix that interact with *T. cruzi* [88] and could be used as potential targets.

The genomic sequences of *Trypanosoma* and *Leishmania* species reveal protein kinases and phosphatases with peculiar characteristics, such as a paucity or absence of tyrosine kinases and phosphatases, or of the accessory domains typically responsible for protein-protein interaction (IgG, fn3, SH3 or SH2 domains) in mammalian signaling pathways. As in other human diseases, differences like these between parasitic and mammalian signaling point to good targets for drug discovery. In addition to the target-base approach employed for drug discovery, the high-throughput drug screening can provide new chemical structures on which to base an effective compound . Interestingly, the majority of 4,205 compounds selected from the almost 2 million tested against asexual blood stage of *P. falciparum*, might target kinases of the parasite [89]. If they are directed to a small number or to a large spectrum of kinases is, as yet, unknown. Although the criteria to select a good inhibitor of kinases or phosphatases is not under discussion here, it should be recalled that

specific modulation of enzymes or protein-protein interactions by small peptides is one possibility. Small peptides bind more specifically, can target more than one interaction site, are effective at lower concentrations and can be delivered as prodrugs by coupling to other molecules. However, rapid turnover due to low stability and high susceptibility to intracellular protease degradation may be disadvantageous, as pointed out in a recent review [90].

In summary, the differences in signal cascades between the parasite and the host ought to be exploited as a probable locale for drug targeting.

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Due to space limitations, recent reviews are cited preferentially over the original research papers.

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