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Molecular analysis of early host cell infection by *Trypanosoma cruzi*

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TABLE OF CONTENTS

1. ABSTRACT

Trypanosoma cruzi, the causative agent of Chagas heart disease, infects heart and other cells leading to cardiac arrest frequently followed by death (1). The disease affects millions of individuals in the Americas and is posing health problems because of blood transmission in the US due to large Latin American immigration (2-3). Since the current drugs present serious side effects and do not cure the chronic infection (4), it is critically important to understand the early process of cellular infection at the molecular and structural levels to design novel inhibitors to block *T. cruzi* infection. In this review, the authors critically analyze the molecular and cellular basis of early *T. cruzi* infection and discuss the future directions in this area. The candidate *T. cruzi* invasive genes and host genes involved in the process of early infection are just beginning to be understood. The trypanosome invasive proteins are excellent targets for intervention. The progress made in the cell biology of *T. cruzi* infection will also facilitate the development of novel cell-based therapies to ameliorate the disease.

2. *T. CRUZI* MOLECULES INVOLVED IN INVASION

The surface coat of *T. cruzi* is comprised of mucins, trans-sialidases, trans-sialidase like molecules, polysaccharides and lipids anchored to the outer phospholipid layer of the plasma membrane by glycosylphosphatidylinositol (GPI), and integral membrane proteins. Some of these surface molecules such as mucins, trans-sialidases, trans-sialidase like molecules, and integral membrane proteins have been implicated in the invasion of mammalian cells. *T. cruzi* proteases and other trypanosome molecules have also been implicated in the infection process.

2.1. Mucins

The surface of *T. cruzi* is covered with mucins (5-6), which contribute to host invasion, parasite protection, and to the establishment of persistent infection. Their importance is supported by the fact that 850 mucin-

encoding genes represent 1% of the parasite genome and 6% of all predicted *T. cruzi* genes. The synchronized expression of a large repertoire of mucins containing variable regions in the mammalian stages of *T. cruzi* suggests a possible role in hindering the host immune response. Mucins are glycoproteins that contain a large variety of *O*-linked oligosaccharides. It is thought that mucins act at the interface between the parasite and the host to protect the trypanosome from the innate immunity system of the vector and the mammalian host thereby facilitating the invasion of target cells. In accordance to this notion, it is import to note that mucins were first discovered in mammals, where they serve to protect the digestive and respiratory epithelia (7) and orchestrate interactions between cells expressing mucins and receptors and/or other cellular lectins (7).

The major surface glycoproteins of *T. cruzi* are mucin-like molecules. The core polypeptides of these glycoproteins are only 50–200 amino acids in length, and their sequences are rich in Ser and Thr residues, which are acceptor sites for the addition of *O*-linked oligosaccharides. Carbohydrates account for up to 60% of the mucin molecular mass, conferring a strong hydrophilic character to the parasite (6, 8-9); their sugar residues may participate in interactions between insect-derived metacyclic forms, blood trypomastigotes and mammalian cells (10-11). The amino acids Cys, Phe, Trp and Tyr, which can alter the physicochemical properties of mucins, are underrepresented or absent (12). Their particular size, the absence of Cys-rich multimerization domains and the predicted extended structure, suggest that *T. cruzi* mucins may be similar to the class of mammalian mucins that are involved in lymphocyte trafficking (7), which would facilitate trypanosome interaction with host cells to promote entry.

To date, the structure of parasite *O*-linked oligosaccharides has been determined for the epimastigote mucins of five different *T. cruzi* strains (8, 13-18). Although there are inter-strain variations in these *O*-linked carbohydrate structures, structural and immunological evidence suggests that the presence of galactofuranose is restricted to strains of one *T. cruzi* phylogenetic group, group I (18-20). Antibodies to galactofuranose-containing epitopes present in *T. cruzi* mucins inhibit parasite invasion (21). Furthermore, a set of monoclonal antibodies that recognizes a *T. cruzi* 45-kDa mucin was used to characterize this molecule and study its role in trypanosome adhesion to heart myoblasts (22). The 45-kDa surface mucin is expressed only in invasive trypomastigotes, but not in noninvasive epimastigotes or amastigotes, and is released by the trypanosome in culture medium. The monoclonal antibody B5, that recognizes a critical epitope on the 45 kDa mucin, inhibits the attachment of trypomastigotes to heart myoblasts preventing trypanosome entry, suggesting that this 45-kDa mucin is used by invasive forms of this organism to adhere to heart myoblasts (22). The structure of O-linked oligosaccharides of invasive trypomastigote mucins has not been elucidated and the specific epitopes within these O-linked oligosaccharides that may be involved in host-cell interactions are unknown.

T. cruzi mucins (TcMUC) are generally diverse and are encoded by a complex family of mucin-like genes (23). The groups of repetitive and non-repetitive genes are designated TcMUC I and TcMUC II, respectively. The TcMUC I family presents point mutations, small deletions and insertions focused in a short stretch that is located just after the predicted signal peptide (24).

A characteristic feature of TcMUC II genes is that a number of them are linked in the genome to TS (trans-sialidase) genes (25-26), also implicated in host cell invasion. Due to the fact that polycistronic transcription drives gene expression in trypanosomatids (27), this genomic linkage might play a role in coordinating the expression of the TS and mucin genes that are functionally coupled. Whole-genome analysis has revealed another gene family linked to TcMUC II genes, encoding small variable GPI-anchored surface proteins named MASPs (mucin-associated surface proteins), which present structural similarity to TcMUC II proteins (28). MASP expression also seems to be up-regulated in mammalian stages of *T. cruzi* (29). A *T. cruzi* small mucin-like (TcSMUG) gene family has also been described (30).

Despite their complexity and heterogeneity across different parasite isolates (31), *T. cruzi* mucins can be divided into two major types. A group of mucins is present in the insect stages whereas another is present in the mammalian stages.

Mucins from epimastigotes and culture derived-metacyclic trypomastigotes ranging from 35–50 kDa have a similar amino acid and carbohydrate make up (8, 21, 31). The GPI anchor of epimastigote and culture derived-metacyclic trypomastigote mucins is different (14). Differences between GPI anchors in these forms may be important in the regulation of mucin shedding and initial host cellular infection.

This group of mucins protects epimastigotes from proteases present in the intestinal tract of the insect vector (19). Culture derived-metacyclic trypomastigote mucins have additional roles in the attachment and invasion of mammalian host cells. Accordingly, mucins purified from culture derived-metacyclic trypomastigotes, but not from epimastigotes, bind to mammalian cell lines; antibodies directed to their carbohydrate components or core peptides inhibit host-cell invasion (21-22, 32). Moreover, culture derived-metacyclic trypomastigote mucins can trigger Ca^{2+} mobilization in host cells (33), which is associated with early *T. cruzi* invasion (34). Sialidase treatment of purified mucins enhances mucin attachment to cells and intracellular Ca^{2+} mobilization, which correlates with the enhancement of infection by culture derived-metacyclic trypomastigotes after sialidase treatment (35). This is consistent with previous reports showing that removal of sugar residues from the surface of blood trypomastigotes and insect-derived metacyclic trypomastigotes by other glycosidases enhances *T. cruzi* infection (10-11). These findings indicate that removal of sialic acid and other sugar residues from mucins inhibit the invasion process of blood trypomastigotes, culture derived-

metacyclic trypomastigotes and insect-derived metacyclic trypomastigotes (10-11).

The mucins from cell-derived trypomastigotes (tGPI-mucins) range in size from 60–200 kDa. They share the sialic-acid-containing epitope Ssp-3, which is important for mammalian-cell attachment and invasion (36). Although little is known about the *O*-oligosaccharide structure of these mucins, they differ from culture derived insect form mucins in that they present terminal Gal(1,3)Gal epitopes (37). The tGPI, oligosaccharide and peptide regions of these mucins have been characterized (9, 38). In contrast to mucins from cell culture derived insect forms, mucins from mammalian forms contain unsaturated fatty acids at the *sn-2* position of the glycerol moiety. This difference may be implicated in their abilities to induce cytokines (9). These GPI anchors may interact with Toll-like receptor 2 on the surface of macrophages to generate cytokines (39), which would facilitate chronicity. The stage-specific GPI-anchor structure of *T. cruzi* mucins has been documented (39-40) and their role in invasion needs to be determined.

T. cruzi switches from a mucin coat of rather homogeneous polypeptide composition to a highly heterogeneous one when it changes from the insect into the mammalian host. Whether the parasite will use this surface coat switching mechanism to possibly interact with different types of mammalian cells is unknown. Moreover, whether each parasite expresses different TcMUC molecules or different subpopulations of parasites express different subsets of TcMUC genes to attach and enter host cells is also unknown. The presence of a mosaic mucin coat could also allow the trypomastigotes to modulate or evade the mammalian-host immune response to gain entry.

Alternatively, the variable TcMUC apo-mucins of trypomastigotes may function as a large repertoire of ligands that facilitate the attachment and entry into a large variety of cell types and mammalian hosts that this parasite infects. The fact that tGPI-mucins function as ligands is experimentally supported (36) by the fact that mucins bind to galectin-3 (41), a beta-galactosidase binding lectin, which in turn binds to the surface of mammalian cells to mediate parasite attachment leading to entry (41-42). On the other hand, additional evidence suggests that mucin binding to cellular receptors involve their carbohydrate moieties.

2.2. Trans-sialidases and trans-sialidase like molecules

Other glycoproteins are also present on the *T. cruzi* surface coat, but in much lower numbers. Of prime importance in the biology of the parasite and host-parasite interactions are the trans-sialidase (TS) molecules and TS-like molecules. TS activity has been postulated to enable *T. cruzi* to elude its lack of *de novo* synthesis of sialic acid (43). The major acceptors of sialic acids are the mucins in *T. cruzi* (6) and sialylation of the surface acceptor molecules is crucial for the viability and propagation of the parasite (7).

TS molecules transfer sialic-acid residues from host glycoconjugates to parasite mucins. TS activity is unique in that it does not use CMP-sialic acid as the monosaccharide donor, it is located on the parasite surface instead of at the Golgi apparatus, and it is more efficient in transferring rather than hydrolyzing terminal sialic acids between glycoconjugates. In addition to its role in sialic-acid salvage, TS and TS-like molecules (anchored to the parasite membrane or shed into the bloodstream) can bind to mammalian cell receptors to mediate trypanosome binding and entry (44-52) as well as to undermine host defense mechanisms (53-54).

There is a large super family of TS genes in *T. cruzi*, with only 70 members coding for enzymatically active products (44). A second group of 70 genes encode products that, despite being 95% identical to active TSs, are enzymatically inactive due to a mutation in a key Tyr residue. They maintain their ability to bind to sialic acids and terminal galactose residues, indicating a possible role for these molecules in parasite binding to substrates or cell surfaces (44) to mediate infection. A third group contains hundreds of genes and codes for molecules displaying a sialidase-motif signature and an overall 30% homology to active TS (44). Although the crystal structure of *T. cruzi* TS has been elucidated (55), its validation as a target for the development of new interventions to block infection is complex, due to the number of genes and the fact that TS are not affected by sialidase inhibitors; however, a recent study has shown that an alternative substrate, lactitol, only partially inhibits both parasite sialylation and host-cell infection (56). Whether suicide substrates of sialidases can inhibit both sialylation and the early process of infection is unknown.

The surface associated TS mediate the sialylation of O-linked oligosaccharides of mucins of cell-derived trypomastigotes. After cleavage of its glycosylphosphatidylinositol (GPI) anchor by the action of a phosphatidylinositol-phospholipase C (PI-PLC), TS and TS-like molecules are shed into the bloodstream to up-regulate the early infection in phagocytic and nonphagocytic cells (50-51) and to exert other biological effects on several cell types.

The candidate trypomastigote ligands for structural and atomic analysis are TS, TS-like molecules (36, 53, 57), mucins (58), heparin binding protein (59), and surface casein kinase II substrates (60) that bind to nonphagocytic cells, kinases and to extracellular matrix proteins (52-61) and to host cell receptors on non-phagocytic cells in order to invade them (52-57). It is thought that trypanosome trans-sialidases transfer sialic acids to trypanosome mucins and serve as acceptors of sialic acids during infection.

One *T. cruzi* ligand that is expressed in all strains of *T. cruzi* trypomastigotes and is used by the parasite to attach and enter macrophages as well as non-phagocytic cells is the trypomastigote gp83 molecule (50-51). The host surface p74 functions as a receptor for *T. cruzi* gp83 ligand to mediate trypomastigote attachment to host cells leading to entry (47). Since this trypanosome ligand is released from the parasite by the action of a parasite PLC in order to signal macrophages and non-phagocytic cells, special

attention has been given to its description and characterization. Previous reports have shown that the *T. cruzi* surface gp83 (45, 50-52) is expressed only in invasive trypomastigotes (48), is more expressed in highly than weakly infective trypomastigote clones (46) and that blocking this molecule with anti-gp83 antibodies reduces trypanosome binding and invasion (48). Purified soluble gp83, from PLC-treated *T. cruzi* trypomastigote membranes, binds to myoblasts, fibroblasts and macrophages to mediate trypanosome entry (62). Heart myoblasts display a single class of receptors for the gp83 present at 4 x 10^4 per myoblast with a K_d of 8 nM (62). Monovalent Fab fragments of the mAb 4A4 (4A4 –Fab) specific for the gp83 ligand inhibit its binding to myoblasts, fibroblasts and macrophages, block the trypanosomes from attaching to and entering these cells and neutralize *T. cruzi* infection in Balb/c mice (62). This is the first demonstration that gp83 is a ligand that *T. cruzi* uses to attach to phagocytic and non-phagocytic cells to promote entry. Both the mAb 4A4 and polyclonal antibodies specific for r-gp83 are able to immunoprecipitate the membrane gp83 from the trypanosome and the released gp83 (51).

The released gp83 is a GPI anchored membrane glycoprotein cleaved by the trypanosome PLC. It is reasoned that if trypomastigotes use gp83 to bind to heart myoblasts, fibroblasts and macrophages, then 4A4-Fab, which blocks the binding of this molecule to these cells, should inhibit the attachment and internalization of trypomastigotes into host cells. Indeed, 4A4-Fab effectively blocks trypomastigote attachment to heart myoblasts, Vero cell fibroblasts and macrophages in a concentration dependent manner. A significant reduction in trypanosome entry into these cells is detected at 4 hours. 4A4-Fab effectively inhibit trypomastigote binding and internalization into mammalian cells at nanogram amounts, whereas an irrelevant Fab (I-Fab) fails to inhibit binding and internalization of trypanosomes (62). 4A4-Fab neutralizes *T. cruzi* trypomastigote infection in Balb/c mice. Intravenous (i.v.) injection of 4A4-Fab into Balb/c mice 2 hours before i.v. challenge with a lethal dose of blood trypomastigotes followed by three i.p. doses of 4A4-Fab dramatically reduces parasitemia compared to control mice receiving I-Fab. After 27 days, the mortality of mice receiving I-Fab is 100%, whereas no mortality is observed in the group of mice receiving 4A4-Fab. At the termination of the experiment (118 days) no mortality was observed in these mice. Parasitemia levels and mortality with I-Fab are similar to PBS controls. These results indicate that 4A4-Fab strongly neutralizes the infectivity of *T. cruzi in vitro* and *in vivo* by blocking the surface gp83 ligand of invasive forms of this organism (62). Immunization of mice with the r-gp83 ligand significantly protects animals against the challenge with lethal doses of blood trypomastigotes. Thus, the gp83 ligand is a prime target for molecular intervention and is also a candidate for vaccine development against *T. cruzi* infection. This is the first study that provided direct evidence that *T. cruzi* uses the surface gp83 as a ligand to bind receptors on myoblasts, fibroblasts and macrophages to mediate trypanosome attachment to these cells and initiate *in vitro* and *in vivo* infection.

A subset of the TS super gene family, Tc-85, has also been implicated in cell infection since antibodies to this molecule partially block parasite internalization (63) and the subset binds to laminin (61) and cytokeratin 18 (57). The role of TS in mammalian cell invasion has yet to be fully clarified since opposing results have been reported. On the basis that polyclonal antibodies that block TS activity enhance invasion of host cells by trypomastigotes *in vitro*, it was proposed that TS negatively modulates *T. cruzi* infection (64). It was also found that compared to the minor subset of trypomastigotes recognized by anti-TS antibodies (TS^+), the TS^- population shows enhanced ability to enter host cells. Anti-TS monoclonal antibodies and various cell types and parasite isolates, confirmed the previous results, reinforcing the hypothesis that TS down regulates *T. cruzi* infection (65). Furthermore, pure TS^+ and TS^- populations were tested for their abilities to invade cells (66). It was found that TS^+ trypomastigotes are highly invasive, whereas TS^- parasites are extremely inefficient in invading epithelial cells and fibroblasts. Moreover, addition of TS to non-penetrating TS^- trypomastigotes convert them to a highly invasive phenotype (66).

Immunocytochemical studies localized TS on the surface of trypomastigotes and associated with the flagellar pockets, suggesting that the enzyme is also secreted (67). The shed acute phase antigen (SAPA), presents TS activity and is secreted in enzyme form (68). Whether the secreted form of TS and the enzyme expressed on the trypomastigote surface act simultaneously during interaction with host cells, or the action of one of them predominates depending on the circumstances, is not clear. Several reports indicate that trypomastigotes release the gp83 ligand to activate the MAP kinase pathway and PKC in the host to promote infection (50-52). Alternatively, after binding of trypomastigotes to target cells through another molecule, the secreted TS may transfer sialic acid from the mammalian cell membrane to parasite mucins. It was suggested that these transference reactions could disrupt the binding of sialoadhesins, allowing the parasites to detach and find a new binding site in order to proceed towards their internalization (69).

It was also suggested that mucins and sialyl residues apparently are not primary requirements for trypomastigote invasion (70), although monoclonal antibodies to sialic acid-containing epitopes inhibit parasite adhesion to host cells (70). On the other hand, the involvement of target cell sialic acids in *T. cruzi* internalization was reported by different groups, using sialic acid-deficient CHO (Lec2) cells. Trypomastigotes enter Lec2 cells to a much lower extent than parental CHO cells, but sialylation by TS restores parasite adhesion and invasion (70-72).

It has been generally accepted that culture derived-metacyclic trypomastigotes function as a model for insect vector-derived metacyclic trypomastigotes. Almost all findings about mucins and TS molecules was performed using epimastigotes and culture-derived metacyclic trypomastigotes, and there is no indication that the same mucins or TS molecules expressed in culture derived-

metacyclic trypomastigotes are also expressed in insect-derived metacyclic trypomastigotes, or may be involved in the attachment to mammalian cells leading to entry. Furthermore, it is not known whether the glycosylation of mucins or TS molecules is the same in these two invasive metacyclic forms. However, limited studies performed with insect vector-derived metacyclic trypomastigotes in the infection process support the contention that sugar moieties of mucins and TS molecules regulate the infection of the insect vector borne metacyclic trypomastigotes (10-11, 73-75). The functional differences between mucins and TS molecules of culture derived-metacyclic trypomastigotes and insect-derived metacyclics are largely unknown. The limiting number of insect derived-metacyclic trypomastigotes that can be obtained from the insect vector to validate the culture metacyclic model and to study the natural infection at the molecular level continues to pose great challenges in the current functional genomic, proteomic and nanotechnology era.

3. OTHER *T. CRUZI* MOLECULES INVOLVED IN THE INFECTION

3.1. Casein Kinase II substrate (TC1)

The cloning and characterization of the first cell surface casein kinase II (CKII) substrate (Tc-1) of *T. cruzi*, expressed only in invasive trypomastigotes, phosphorylated by host cell CKII, and involved in the early process of cellular infection was recently reported (60). Northern blot analysis shows a 4.5-kb transcript that is expressed in invasive trypomastigotes but not in noninvasive epimastigote forms of *T. cruzi*. Southern blot analysis indicates that Tc-1 is a single-copy gene. Analysis of the translated amino acid sequence indicates that the Tc-1 gene has a putative transmembrane domain with multiple cytoplasmic and extracellular CKII phosphosites. Exogenous human CKII is able to phosphorylate serine residues on both recombinant Tc-1 and Tc-1 of intact trypomastigotes. This phosphorylation is inhibited by the CKII inhibitors heparin and 4,5,6,7,-tetrabromo-2-azabenzimidazole (60).

This surface 62-kDa protein is expressed only in infective trypomastigotes and is uniformly distributed on the surface of trypomastigotes. Antibodies to Tc-1 effectively block trypomastigote invasion of host cells and consequently reduce parasite load. Pre-incubation of either trypomastigotes or myoblasts with CKII inhibitors block *T. cruzi* infection.

It is important to note that other intracellular microorganisms such as viruses have exploited the uniqueness of the host's CKII constitutive activity by using CKII to phosphorylate proteins involved in viral infection (76). It is possible that the CKII substrate on the surface of the intracellular protozoan *T. cruzi* may play a similar role in the infection process. CKII's enzymatic activity is always turned on, and its substrates are involved in very essential cell functions including viral infection and cell survival. Basically, cells cannot survive without CKII activity and substrates for the enzyme fall into many critical functional categories including cell cycle regulation, protein synthesis, metabolic activity, signaling, DNA/RNA synthesis, and viral infection (77).

Tc-1 appears to be the first described transmembrane surface protein of *T. cruzi* involved in *T. cruzi*-host cell interactions that leads to infection, since other molecules that are apparently implicated in the infection process are glycosylphosphatidylinositol membrane-anchored molecules, which are released from the trypanosome by action of the trypanosome PLC. The fact that Tc-1 is expressed only in invasive trypomastigotes but not in noninvasive epimastigotes or amastigotes indicates that the Tc-1 gene is developmentally regulated in the cell cycle of parasites. Furthermore, the fact that *T. cruzi* infection is inhibited when Tc-1 is blocked by specific antibodies suggests that the Tc-1 gene may be an invasive gene in the parasite that regulates the pathogenesis of early *T. cruzi* infection.

It is suggested that *T. cruzi* exploits the host CKII to phosphorylate the extracellular domain of Tc-1, which may be important for cellular invasion. The CKII sites in the cytoplasmic region of Tc-1 would be phosphorylated by the parasite's own CKII, which in turn might activate a series of signal cascades that may be important for parasite invasion or parasite biology.

Since there are no homologs of Tc-1 in humans, this gene and its encoded protein could be excellent targets for molecular intervention in Chagas' disease and might lead to the development of novel drugs for chemotherapy. Furthermore, the fact that Tc-1 is highly immunogenic and the fact that antibodies raised against Tc-1 neutralize *T. cruzi* infection of mammalian cells suggest that Tc-1 could also be a candidate for vaccine development.

3.2. LYT1

The LYT1 protein of *T. cruzi* is required for efficient *in vitro* infection (78). The LYT1 gene product was characterized and shown to be involved in cell lysis and infectivity. LYT1 is a single-copy gene that encodes a protein involved in a lytic pathway. Mutational analysis demonstrated that deletion of LYT1 results in attenuation of infection, which is associated with diminished hemolytic activity. Reintroduction of LYT1 restores infectivity in null mutants, confirming the critical role of LYT1 in infection.

LYT1 is dispensable in epimastigotes, but *LYT1* null parasites are infection deficient, display accelerated *in vitro* development, and have diminished hemolytic activity at acidic pHs. How one protein influences such seemingly diverse biological processes is unclear. One possibility is that the LYT1 protein is a factor, which regulates the expression of multiple genes encoding proteins of diverse function. Consistent with this idea is the presence of a possible nuclear localization sequence (NLS) that is homologous to the classical NLS of the simian virus 40 large T antigen. Another possibility is suggested by the occurrence of multiple potential 3′ splice acceptor sites, which if used, would lead to the expression of different LYT1 protein derivatives. One derivative would carry a possible amino-terminal signal sequence. The second would lack this element as a result of *trans* splicing within

the protein coding sequence, leading to translation initiation from the ATG codon at position +85 within the protein coding sequence. Consequently, it is possible that two forms of the protein are produced: one secreted, consistent with a role in hemolysis, and a second nuclear, consistent with a role in development (79). Further studies are needed to clarify how LYT1 influences several biological processes in the parasite and during the infection process.

3.3. Penetrin

Another surface molecule of *T. cruzi* trypomastigotes with affinity for extracellular matrix components is penetrin, a 60 kDa protein that selectively binds to heparin, heparin sulfate and collagen, and promotes fibroblast adhesion and penetration (59). Recombinant penetrin, expressed in *Escherichia coli* and localized on its surface, induced bacterial attachment to and penetration into Vero cells in a proteoglycan- and collagen-inhibitable manner (59). Assays to evaluate the role of host cell heparin and heparan sulfate glycosaminoglycans in *T. cruzi* invasion show that proteoglycan-deficient mutants of Chinese hamster ovary (CHO) cells are poor targets for trypomastigote penetration (80). Penetrin has not been further characterized and its molecular structure remains unknown.

3.4. Peptidyl-prolyl cis-trans isomerase

A secreted *T. cruzi* protein with peptidyl-prolyl cis-trans isomerase activity susceptible to inhibition by the immunosuppressant FK506 and related drugs was characterized (81). It was shown that the addition of the recombinant protein to simian epithelial or HeLa cells enhances parasite invasion. The monomeric protein has a peptidyl-prolyl cis-trans isomerase core encompassing the characteristic rotamase hydrophobic active site, and its mechanism of action may involve triggering of host cell signaling, with or without the contribution of rotamase activity (82). The molecular structure of this enzyme as well as its precise role in parasite infection are unknown.

3.5. Lectin-like 67-kDa glycoprotein

A 67 kDa lectin-like glycoprotein, binding to desialylated human erythrocyte membranes in a galactose-dependent manner and recognizing receptors in mouse cardiac tissue and human cardiac aortic endothelium was reported (83). It was also reported that antibodies to the 67-kDa lectin-like molecule inhibit cellular invasion by *T. cruzi* (83). Again, the molecular structure of this trypanosome lectin-like protein is unknown and the precise mechanism by which this molecule participates in the infection process is unknown.

4. *T. CRUZI* PROTEASES

4.1. Prolyl oligopeptidase

A member of the prolyl oligopeptidase (POP) family of serine proteases, with specificity for human collagen types I and IV, has been identified in cell-free extracts of trypomastigotes, amastigotes and epimastigotes (84-85). The 80 kDa enzyme, denominated POP Tc80, also hydrolyses fibronectin and appears to be implicated in host cell invasion. Selective and irreversible inhibitors of POP Tc80 were found to block trypomastigote entry into non-phagocytic mammalian cells (85-86). The hydrolytic activity and the ability of several trypomastigote molecules to bind laminin, fibronectin, collagen, heparin, and heparan sulfate may be important for the transit of the parasite through the extracellular matrix towards target cells.

4.2. Cruzipain

Cruzipain is the major *T. cruzi* proteinase. It is a gp57/ 51 cysteine proteinase, which is active in pH ranges of 5 to 7.5. The enzyme is expressed in all developmental forms of different *T. cruzi* isolates (87-88). The involvement of cruzipain in host cell invasion and intracellular development was suggested by using peptidyl diazomethane derivatives, a class of irreversible inhibitors of cysteine proteinase (89). A beneficial role by inhibiting cruzipain in mice infected with *T. cruzi* was observed by treating infected mice with peptide-fluoromethyl ketones (90). The inhibitors that inactivate cruzipain arrest intracellular replication and intercellular transmission *in vitro* (91).

The participation of cruzipain in host cell invasion by trypomastigotes is associated with its ability to generate bradykinin (92). The involvement of the B type bradykinin receptor (B_2R) in *T. cruzi* infection was studied using human umbilical vein endothelial cells (HUVECs) or CHO cells over expressing B_2R (CHO-B_2R). Captopril, an inhibitor of bradykinin degradation by kininase II, enhances trypomastigote entry into HUVECs and CHO-B_2R, but not into mock-transfected CHO cells, whereas the B_2R antagonist HOE 140 or a monoclonal antibody to bradykinin blocks these effects. Cruzipain enhances parasite invasion and triggers Ca^{2+} mobilization in CHO-B_2R in a manner inhibitable by HOE 140 or the cruzipain inhibitor E-64, indicating that the enzyme may play a role in producing the kinin agonist from cell-bound kininogen. The kinin-mediated signal transduction route is not ubiquitous and its activation depends on the cell type and the parasite isolate used (92).

It has also been shown that MGTA, an inhibitor of kininase I, selectively decreases trypomastigote infectivity for B_1R-expressing cells and that addition of B_1R or B_2R antagonists to host cells co-expressing these receptors inhibit parasite infectivity to a similar extent (93). Both antagonists had no additive effects on the infection of both cardiomyocytes and HUVECs.

Processing of kininogens presumably takes place within the secluded spaces formed by the juxtaposition of parasites (92-93). Cruzipain appears to be modulated by both the host and *T. cruzi* components. Heparin sulfate enhances cruzipain activity by 6-fold. On the other hand, a tight-binding cysteine proteinase inhibitor, chagasin, was identified in *T. cruzi* (94). It is localized in the flagellar pocket and cytoplasmic vesicles of trypomastigotes, and its expression is inversely correlated with that of cruzipain.

4.3. Oligopeptidase B

A soluble factor secreted by trypomastigotes, is the sole component of this *T. cruzi* developmental form

reported to directly trigger Ca^{2+} response in host cells. This soluble factor is generated by the action of a 120 kDa alkaline peptidase on precursors present only in infective trypomastigotes (95). The purified peptidase is devoid of Ca^{2+} signaling activity on its own and is also present in non-infective epimastigotes (95).

The trypomastigote peptidase was found to be a cytosolic enzyme closely related to members of the prolyl oligopeptidase family of serine endopeptidases, and is called *T. cruzi* oligopeptidase B (96). Oligopeptidase B null mutant trypomastigotes are defective in mobilizing Ca^{2+} from thapsigargin-sensitive stores in mammalian cells, and in establishing infection *in vitro* and *in vivo* (97). It was proposed that the Ca^{2+} agonist generated by oligopeptidase B is exported from the parasite and binds to a receptor on the surface of target cells, activating PLC and generating IP, which binds to its receptor on the membrane of the endoplasmic reticulum and promotes Ca^{2+} release.

4.4. Metalloproteinases

Surface antigens with metalloprotease activity, homologous to *Leishmania* gp63, were identified in *T. cruzi* (98). Affinity-purified antibodies to these antigens inhibit host cell invasion by fifty percent. The molecular characterization, structural analysis and the precise role of the surface metalloproteinases in the early process of infection are unknown.

5. PUTATIVE HOST CELL RECEPTORS FOR *T. CRUZI*

5.1. Cytokeratin 18

Tc85-11A, a member of the TS multigene family, presents adhesive properties to laminin and to the cytoskeletal protein cytokeratin18, which is expressed in monkey kidney epithelial cells (57, 61). The binding region of Tc85-11, VTVXNVFLYNR, is co-localized at the carboxyl terminal. Anti-cytokeratin antibodies partially inhibit the *in vitro* infection, suggesting that these molecules participate in the process of infection. Experiments to knock out the Tc85-11 gene to validate its function in the process of infection pose challenges because it belongs to the large TS gene family. On the other hand, no studies have been performed with cytokeratin 18 KO animal models or silencing the expression of cytokeratin 18 *in vitro* and *in vivo* by stable RNAi to validate the proposed interaction. The fact that cytokeratin 18 is one of many protein isoforms may pose similar challenges to validate its function in parasite infection. The 85-kDa proteins of trypomastigotes also bind to fibronectin (99), a host molecule involved in the infection process that also interacts with amastigotes to modulate the infection of *T. cruzi* by human monocytes and macrophages (100).

It would seem likely that cruzipain (92, 101) and other proteases (84) would be operative in breaking successive protein-protein interactions, thus facilitating parasite progression in the host. It is possible that several TSs may function as ligands using different domains to interact with host cell surface receptors during the initial steps of infection. It is thought that the catalytic site of the TSs do not participate in invasion, since antibodies against the catalytic site of the enzyme do not inhibit parasite internalization into host cells (64).

5.2. P74

A 74-kilodalton surface glycoprotein from heart myoblasts that specifically binds to *T. cruzi* trypomastigotes to mediate trypanosome attachment to heart myoblasts was reported (102). Pre-incubation of trypomastigotes with the soluble 74 kDa glycoprotein strongly inhibits binding and internalization of trypomastigotes into heart myoblast monolayers in a concentration dependent and saturable manner. Pre-incubation of heart myoblast monolayers with antibodies specific to the purified 74 kDa glycoprotein also strongly inhibits trypomastigote binding and internalization into heart cells in a concentration dependent manner (102). It was recently found that this recombinant molecule binds to the trypomastigote gp83 ligand and that antibodies to both proteins block this interaction and trypanosome attachment and entry (unpublished observations). These results support the notion that the surface 74 kDa glycoprotein is a target molecule on heart myoblasts to which *T. cruzi* binds in order to invade them. The molecular characterization of this putative receptor for *T. cruzi* and *in vitro* and *in vivo* validation of the role of this gene in the process of early infection is under way.

5.3. Galectin-3

Galectin-3 is a beta galactoside binding human lectin that plays important roles in the early critical steps of *T. cruzi* infection of human cells (42). Binding of galectin–3 to the surface of human coronary artery smooth muscle cells is granular, distributed around the cellular membrane, polarized, and more pronounced at the cellular ends (42). The receptors for human galectin-3 are distributed in patches on the surface of the cells and are more abundant at the terminal regions of the cells. The binding of galectin-3 to trypomastigotes is also seen as granular, restricted to some areas of the membrane of trypanosomes, and polarized (42). The binding of galectin-3 to the surface of both *T. cruzi* trypomastigotes and human coronary artery smooth muscle (HCASM) cells is completely inhibited by 5 mM lactose but not by 5 mM sucrose, indicating that galectin-3 binds to the surface of both trypomastigotes and HCASM cells in a lectin-like manner (42). Galectin-3 increases five times the normal adhesion of trypomastigotes to HCASM cells; this effect is specific since it is inhibited by lactose in a concentration-dependent and saturable manner. Cells stably transfected with galectin-3 antisense show a dramatically reduced expression of galectin-3 compared to cells transfected with vector alone and a significant reduction in the numbers of bound trypanosomes compared to cells transfected with the vector alone. The addition of exogenous galectin-3 to cells transfected with galectin-3 antisense restores the initial capacity of these cells to bind to trypanosomes.

Galectin-3 is expressed on the surface of and secreted from human HCASM cells; exogenous galectin-3 enhances the binding of *T. cruzi* to human HCASM cells.

Cellular expression of galectin-3 is required for *T. cruzi* adhesion to human cells. The fact that exogenous human galectin-3 specifically binds to the surface of HCASM cells and to *T. cruzi* suggests that galectin-3 bridges trypanosomes and cells, resulting in an enhancement of the binding of *T. cruzi* to human cells. These results are consistent with the notion that galectin-3 can serve as an adapter for different types of molecules in other biological systems (103). Galectin-3 can bind glycoconjugates by means of its carbohydrate recognition domain region and can bind other molecules through its non-lectin half, therefore serving as a cross-linker between glycoconjugates and other molecules (103). Galectin-3 binds to the membrane of *T. cruzi*-containing surface glycoproteins. One surface glycoprotein of invasive *T. cruzi* trypomastigotes that plays a role in this interaction is the 45 kDa mucin of trypomastigotes which specifically interacts with galectin-3 (22, 41).

Galectin-3 is also implicated in the association of *T. cruzi* trypomastigotes with laminin (41). Binding of trypomastigotes to laminin is enhanced by galectin-3 and this enhanced binding of trypanosomes is inhibited by lactose. Co-immunoprecipitations indicate that galectin-3 binds to the 45, 32 and 30 kDa trypomastigote surface proteins and this binding is also inhibited by lactose (41). The monoclonal antibody B5 that recognizes the trypomastigote 45 kDa surface mucin blocks trypomastigote attachment to heart myoblasts (22). This is a mechanism where a human parasite, *T. cruzi*, utilizes human galectin-3 to effectively interact with laminin. A working model proposes that galectin-3, released by human cells, forms bridges between *T. cruzi* and laminin. Galectin-3 molecules interact with *T. cruzi* 45 (mucin), 32 and 30 kDa surface proteins on one hand and with laminin on the other, via their carbohydrate recognition domains and are joined together by the R-domains (104) in a concentration dependent manner. Since nearly all the tissues which *T. cruzi* infects are surrounded by basement membranes of which laminin is a major constituent, its ability to effectively interact with laminin is critically important for passage through the membrane barrier. These studies suggest that this is a trypanosome trapping mechanism, which enables the organisms to accumulate in the basement membrane prior to invasion of heart myoblasts, making galectin-3 a candidate molecule, which enhances the pathogenesis of *T. cruzi*.

Galectins have long been suspected of modulating cell to extracellular matrix interactions in a novel fashion (104). Data suggest that one mechanism used involves the ligation of mammalian cells to extracellular matrix proteins, which also interact with galectin-3 such as laminin and elastin (104). The other mechanism involves the interaction of galectins with the polylactosamine residues of integrins, resulting in the modulation of cellular adhesion to extracellular matrix proteins (105). Whereas most of these studies were done in mammalian systems, it has been suggested that galectins expressed by *Entamoeba* may be critical in their interactions with host cells (106). It is likely that in parasitic organisms, the binding of the organisms to the extracellular matrix proteins or cell surface glycoconjugates may be the primary adhesion mechanisms mediated by galectins.

Galectin-3 is also expressed in B cells from *T. cruzi*-infected mice (107) and is up regulated by *T. cruzi* infection of mice (108). The fact that galectin-3 is secreted by macrophages and by other cells, including human CASM cells, suggests that released galectin-3 modulates infection. The concentrations of galectin-3 that increase trypanosome adhesion to HCASM cells *in vitro* are similar to the concentrations of galectin-3 present in fluids *in vivo* (109). Furthermore, the concentrations of galectin-3 in fluids *in vivo* increase approximately 300-fold during microbial infection (110). These observations suggest that the parasite may have adapted to the host and that it takes advantage of a host inflammatory molecule, galectin-3, to bind to host cells. Since, it has been recently reported that Chagas' disease cardiomyopathy is in part a vasculopathy (111) it is suggested that these findings may contribute in part to determining the cause of this pathology.

6. EXTRACELLULAR MATRIX

6.1. Laminin gamma-1

Recent evidence indicates that *T. cruzi* modulates the extracellular matrix network to facilitate infection of human cells (52). The *T. cruzi* gp83 ligand increases the level of laminin gamma-1 transcript and its expression in mammalian cells, leading to an increase in cellular infection. Stable RNA interference (RNAi) of host cell laminin gamma-1 knocks down the levels of laminin gamma-1 transcript and protein expression in mammalian cells, causing a dramatic reduction in cellular infection by *T. cruzi* (52). Thus, host laminin gamma-1, which is regulated by the parasite, plays a crucial role in the early process of infection.

Previous studies have shown that parasite molecules bind to immobilized laminin (61) and that human galectin-3 enhances this interaction (41), suggesting that the trypanosome interacts with laminin. Laminin gamma-1 is the most abundant isoform of laminin in humans (112). The hypothesis that *T. cruzi* gp83 binds to human cells to regulate the expression of laminin gamma-1, which is required for *T. cruzi* infection, is experimentally supported by the fact that pre-exposure of *T. cruzi* gp83 to human coronary artery smooth cells up-regulates *T. cruzi* infection and expression of laminin gamma-1 and that knocking down the expression of laminin gamma-1 by RNAi dramatically reduces *T. cruzi* infection of human cells.

Host laminin gamma-1, which is regulated by the parasite ligand, plays a crucial role in the early process of *T. cruzi* infection of heart cells. The regulation of gp83 expression by the parasite as well as the gp83 released by the trypanosome PLC play an important role in the modulation of early signaling events leading to initial parasite infection. The regulation of infection by the gp83 ligand represents a parasite escape mechanism in which invasive trypomastigotes release gp83 to efficiently gain entry into human heart cells.

T. cruzi must navigate through the basal lamina, which contains laminin gamma-1, and surrounds individual muscle cells such as HCASM cells before infecting these cells. The fact that a *T. cruzi* trypomastigote ligand increases laminin gamma-1 transcript levels in human coronary artery smooth muscle cells, correlates with the finding that laminin is deposited in the hearts of patients infected with Chagas' disease (113). This suggests that the regulation of laminin gamma-1 in heart cells by gp83 might explain, in part, the cause of this pathology.

The fact that the *T. cruzi* gp83 ligand remodels the extracellular matrix (ECM) by up-regulating the expression of laminin gamma-1, together with the report that *T. cruzi* presents laminin receptors on its surface (61), indicates that the parasite exploits laminin gamma-1 to navigate through the ECM to facilitate infection. Thus, the *T. cruzi* gp83 ligand is a virulence factor that modifies laminin gamma-1 expression in the ECM to contribute to the pathogenesis of *T. cruzi* infection in human heart cells.

6.2. Thrombospondin-1

T. cruzi is also able to increase the levels of thrombospondin-1 (TSP-1) expression in host cells during early infection. Stable RNA interference of host cell TSP-1 knocks down the levels of TSP-1 transcripts and protein expression in mammalian cells causing inhibition of *T. cruzi* infection (114). Addition of TSP-1 to these cells restores infection. Thus, host TSP-1, regulated by the parasite, plays a crucial role in early infection.

T. cruzi must navigate the ECM in order to infect mammalian cells. Amongst the glycoproteins of the ECM, thrombospondins constitute a set of unique multidomain proteins that are synthesized, secreted and incorporated into the ECM by many cell types (115-116). Thrombospondins are unique members of the ECM in that they have been described as 'matricellular' proteins because they modulate cell function but they do not play a direct role in the structure of the ECM. Most thrombospondin types are expressed in myocytes, smooth muscle cells, fibroblasts, and endothelial cells, among others that *T. cruzi* infects. Most tissues express at least one type of thrombospondin (115).

It was suggested that *T. cruzi* might have receptors for thrombospondin (117). The role that host TSP isoforms may play in the process of microbial infections is just beginning to be elucidated. Since nearly all cells that *T. cruzi* infects are surrounded by basement membranes, of which several TSP isoforms are important constituents, the ability of the parasite to effectively regulate and interact with TSP-1 is also critically important for its passage through the membrane barrier.

The kinetics of change in gene transcript profiles of TSP-1, TSP-2, TSP-3 and TSP-4 was evaluated by real time PCR during the early process of infection of HCASM cells by *T. cruzi*. It was shown that TSP-1 transcript levels increases approximately 6-fold at 60 min, followed by an increase of 2.7-fold at 120 min during infection of HCASM cells by *T. cruzi*. The transcript levels of TSP-2, TSP-3 and TSP-4 do not change significantly during the same period of time of *T. cruzi* infection of HCASM cells.

TGF beta-1 induced over-expression of TSP-1 in HeLa cells and causes a significant increase in both trypanosome binding to cells at 2 h and parasite load at 72 h. Thus, there is a direct correlation between over expression of TSP-1 in HeLa cells with an increase of infection of these cells. The over expression of TSP-1 in human cells induced by TGF beta-1 is consistent with previous reports (118-119). The enhancement of *T. cruzi* infection of mammalian cells caused by pretreatment of cells with TGF beta-1 is also consistent with a previous report (120). Pre-exposure of human epithelial cells, in which the TSP1-1 gene was silenced by stable RNAi, blocked *T. cruzi* infection and exogenous recombinant TSP-1 restores the infection of these cells.

RNAi of TSP-1 significantly decreases the protein levels in human cells. In cells in which TSP-1 is silenced by RNAi, the expression of other ECM proteins such as fibronectin and laminin and/or galectin-3 are not altered nor were their abilities to adhere to substrate, supporting the specificity of these studies.

RNAi of TSP-1 dramatically reduced the number of trypomastigotes that attach to human cells and reduced parasite loads at 72 h as compared to cells transfected with vector alone or scrambled antisense TSP-1. These results indicate that RNAi of TSP-1 substantially reduces the transcript and its encoded protein, rendering the human cells to be substantially less susceptible to *T. cruzi* infection and indicating that TSP-1 is required for the process of *T. cruzi* infection. The residual *T. cruzi* infection seen indicates that the parasite uses other TSP-1 independent mechanisms to infect cells.

T. cruzi infection causes extensive fibrosis and severe heart cardiomyopathology, which is in part vasculopathy, leading to cardiac arrest that is frequently followed by death (1). The fact that *T. cruzi* trypomastigotes increased TSP-1 transcripts in HCASM cells suggest that TSP-1 may also contribute in part to the pathology caused by *T. cruzi*.

Other groups have shown that *T. cruzi* uncharacterized antigens up-regulate laminin, fibronectin, and type I collagen (121). The fact that *T. cruzi* remodels the ECM by also up-regulating the expression of TSP-1 indicates that the parasite exploits TSP-1 in addition to laminin to navigate through the ECM to facilitate infection. TSP-1 is required for the infection process of *T. cruzi* as evidenced by RNAi of that specific isoform.

The *T. cruzi* up regulation of host TSP-1 expression to facilitate the infection of human cells represents a new mechanism that contributes to the pathogenesis of *T. cruzi* infection.

6.3. Fibronectin

The involvement of fibronectin in target cell invasion by trypomastigotes was suggested from

experiments in which the peptide RGDS, corresponding to the fibronectin cell attachment site, was found to bind to the trypomastigote surface and to inhibit trypomastigote internalization (99). The trypomastigote ligand for fibronectin was purified by affinity chromatography, and identified as an 85 kDa protein that interacts with cells bearing fibronectin molecules, such as human monocytes, neutrophils and 3T3 fibroblasts (122). However, molecular genetic approaches have not been used to validate the role of fibronectin in the process of infection.

7. NEW GENETIC TOOLS TO STUDY THE FUNCTION OF *T. CRUZI* GENES IN INFECTION

7.1. pTcINDEX expression vector for *T. cruzi*

T. cruzi is an early diverging eukaryote that displays many unusual biochemical features. The completion of the *T. cruzi* genome project has highlighted the need to extend the range of techniques available to study gene function with particular emphasis in trypanosome attachment and entry. The inability to use RNAi in *T. cruzi* and to use this approach to rapidly study gene function in *T. cruzi* attachment and entry and other fundamental biological processes of parasite biology is a significant limitation to study the first steps of pathogenesis. Very limited numbers of genes in *T. cruzi* have been knocked out by homologous recombination to study their function in the process of infection and most of the *T. cruzi* genes suggested to play roles in the process of host cell invasion have not been validated. However, a recent development to study gene function in *T. cruzi* biology has been described (123) to overcome this problem. It is expected that the pTcINDEX expression vector for *T. cruzi* (123) will represent a valuable addition to the genetic tools available for *T. cruzi* research. This vector system is sufficiently flexible in that it should have widespread uses including inducible expression of tagged proteins, generation of conditional knockout trypanosome cell lines and the application of dominant-negative approaches to validate the role of candidate invasive genes in the process of *T. cruzi* infection.

7.2. Microarrays

Oligonucleotide and cDNA microarrays have emerged as indispensable research tools for studying global gene expression profiles in cells, including studies of host cell-microbial interactions. The application of genome-scale approaches to study *T. cruzi*-host interactions at different stages of the infective process is now possible with the completion of the *T. cruzi*, human and murine genome projects. Investigators have begun to exploit DNA microarray technology to analyze host transcriptional responses to *T. cruzi* infection (124-126) and dissect developmental processes in the complex *T. cruzi* life-cycle (127-128). Validation of microarray data by Real-Time PCR is an essential step for confirmation of gene expression results.

The microarray platforms available with the whole human and murine genomes will rapidly facilitate the evaluation of gene expression profiles during *T. cruzi* infection of different types of human cells and the expression profiles in tissues and organs in the murine experimental model of Chagas disease. Collectively, information generated from these and future studies will provide valuable insights into the molecular requirements for establishment of *T. cruzi* infection in the host and highlight the molecular events coinciding with disease progression.

Microarray technology will also allow rapid identification of the molecular signature caused by *T. cruzi* during the early process of infection of heart cells as well as other phagocytic and non-phagocytic cells that *T. cruzi* infects. It will also permit comparison of gene expression profiles in different cell types that *T. cruzi* infects. Microarray technology combined with powerful bioinformatics gene expression tools will allow the identification of new pathways and gene clusters required for *T. cruzi* infection. The generation of microchips with the whole *T. cruzi* genome will be instrumental and will rapidly facilitate the gene expression profiling during *T. cruzi* attachment and entry as well and the identification of *T. cruzi* genes expressed during trypanosome entry that are important for the understanding of the early infection process from the molecular point of view of the trypanosome and the host cell.

Until now the response of the whole *T. cruzi* genome during early, intermediate and late infection has not been analyzed. Microchips containing the whole *T. cruzi* genome will also be important to identify the *T. cruzi* genes that are regulated during the intracellular phase in non-phagocytic and phagocytic cells. The trypanosome genes and their gene products regulated in the intracellular environment during the early infection process are prime targets for intervention. The accessibility of high-throughput microchip technologies with the *T. cruzi* whole genome will represent a significant advancement towards the identification of novel drug targets and vaccine candidates for the treatment and prevention of Chagas' disease.

Investigators have used part of the human genome in microarrays to study the gene expression profiling in human foreskin fibroblasts infected with *T. cruzi* (124). Results from these studies suggest that the immediate/early response to *T. cruzi* infection involves minimal modulation of host cell transcription (124). Other investigators have evaluated gene expression profiles in a murine model of chronic chagasic cardiomyopathy using microchips with part of the murine genome (125). From these studies, the regulated genes were interpreted as potential contributors to the pathogenesis of chagasic heart disease (125). More recently microarray studies also implicated that genes from Chagas susceptibility loci that are differentially expressed in *T. cruzi*-resistant mice are candidates accounting for impaired immunity (126). It is unknown whether strains of *T. cruzi* may generate the same or different types of gene expression profiles in the same cell types that *T. cruzi* infects. The generation of a database of gene expression profiles of several types of human cells infected with *T. cruzi* will be instrumental to the elucidation of the molecular signature caused by *T.*

cruzi in the cell. It is expected in the coming years that this molecular signature will be elucidated. These findings will be very important for molecular intervention in *T. cruzi* infection.

7.3. RNAi

The discovery of RNA interference (RNAi) - gene silencing by double-stranded RNA has facilitated the rapid study of the function of genes in biological processes. RNAi is already being used in basic science as a method to study the function of genes and it may lead to novel therapies in the future. RNAi was recently used to reduce the expression of host genes involved in early *T. cruzi* infection of human epithelial cells (52, 114). RNAi of extracellular matrix genes, laminin gamma-1 and thrombospondin 1 blocks *T. cruzi* infection (52, 114). These are the first reports showing that knocking down the expression of human genes by RNAi inhibits the infection of an intracellular parasite. This is a powerful technique that, combined with microarray gene expression data, will facilitate the rapid discovery of the roles of critical host genes and their encoded proteins play in the process of infection.

8. CELLULAR RESPONSES TO EARLY *T. CRUZI* INFECTION

Invasion of host cells by *T. cruzi* is a mechanism of immune evasion that is essential for the survival and replication of the parasite in several tissues and organs including the heart. The exact cellular mechanisms by which trypomastigotes become internalized and retained within non-phagocytic cells have only recently begun to be understood. Several reviews have documented in detail the significant progress made in some aspects of the cell biology of *T. cruzi*- host interactions with specific emphasis in cellular responses (129-130). Therefore, it is not the intent of this section to provide a full discussion of cellular responses to early infection, but to present a brief provocative discussion.

Briefly, *T. cruzi* invades host cells without any detectable extension of the plasma membrane or the formation of spacious phagosomes. A tight association between trypomastigotes and the host-cell membrane is observed during parasite entry, and *T. cruzi* is seen inside this tight vacuole for a considerable period of time before escaping into the cytosol (131-132). Invasion is not inhibited by cytochalasin D (133), a drug that blocks actin polymerization and phagocytosis. Host-cell lysosomes are recruited to the site of trypomastigote entry and gradually fused with the plasma membrane (134). This lysosome-mediated entry process is dependent on a calcium-signaling pathway that is specifically triggered by trypomastigotes in non-phagocytic cells (135-137). Lysosome recruitment is not the only mechanism by which *T. cruzi* can enter non-phagocytic cells (138). Trypomastigotes can also invade cells enveloped by invaginated plasma membranes, generating an intracellular vacuole that subsequently fuses with lysosomes (139). This pathway is also completely independent of host-cell actin polymerization. Disruption of the host-cell cortical actin cytoskeleton facilitates plasma-membrane-mediated entry. It was suggested that *T. cruzi* has two entry pathways (140-141). One is plasma membrane-mediated and the other is lysosome-mediated; they can be distinguished by their differential sensitivity to PI-3 kinase inhibitors such as wortmannin, which abolishes the lysosome-mediated pathway, whereas a significant fraction of the plasma-membrane-mediated pathway remains (139). It was suggested that cell invasion mediated exclusively by plasma-membrane invagination, in the absence of PI-3 kinase activation, is not viable. In the absence of lysosomal fusion, trypomastigotes gradually exit from host cells. Lysosomes also seem to serve as intracellular anchors, crucial for the retention of trypomastigotes inside host cells, to facilitate a successful infection (130). Although significant progress has been made in this area, the knowledge of the parasite molecules that are responsible for the early cellular responses to *T. cruzi* are very limited and require extensive investigation.

The previous limitation of genetic tools to identify and validate *T. cruzi* genes and their coded proteins responsible for the described cellular effects has presented difficulties for the identification and validation of trypanosome invasive genes. It is expected that the new genetic tools discussed in this review will fulfill the gap in the roles that trypanosome and host genes require for cellular invasion by *T. cruzi*.

9. OTHER SIGNALING EVENTS DURING *T. CRUZI* INVASION

T. cruzi activates signal transduction events during cellular entry that appear to be needed for the initial establishment of cellular infection. Trypomastigotes or their isolated membranes, but not non-infective epimastigotes, induce repetitive cytosolic-free Ca^{2+} transients in fibroblasts and parasite entry is inhibited by depletion of host cell cytosolic-free Ca^{2+} or pretreatment with channel blockers (135). A soluble trypomastigote fraction induced Ca^{2+} response in a variety of cell types including cardiac myocytes (34, 142). IP3, generated upon PLC activation, mediated intracellular Ca^{2+} mobilization triggered by a trypomastigote soluble factor (143).

The host cell Ca^{2+} response induces the recruitment of lysosomes to the site of *T. cruzi* penetration (130). Lysosome redistribution and trypanosome invasion of cells is inhibited upon treatment with microtubule-binding drugs, or after microinjection with antibodies to kinesin, indicating that lysosome transport is mediated by microtubule/kinesin (144). The fused lysosomes are predominantly pre-docked at the plasma membrane, Ca^{2+} being primarily responsible for fusion and not recruitment of lysosomes to the cell surface (145). By fusing with the plasma membrane, lysosomes would contribute to formation of the parasitophorous vacuole (130).

Elevation in intracellular free Ca^{2+} concentration triggers lysosome fusion and exocytosis (146). Ca^{2+}-dependent exocytosis of lysosomes is cAMP-regulated and is enhanced by isopreternol, a beta-adrenergic agonist that activates adenylyl cyclase through the

heterotrimeric G protein G_s (137). Sinaptotagmin VII, a ubiquitously expressed sinaptotagmin isoform that is localized on the membrane of lysosomes in different cell types and regulates exocytosis of these organelles, appears to mediate *T. cruzi* invasion. Trypomastigote entry was impaired in CHO cells loaded with antibodies that recognize the Ca^{2+}-binding domain of sinaptotagmin VII and inhibit the Ca^{2+}-triggered exocytosis of lysosomes (147).

Targeted lysosome exocytosis may not be the predominant mechanism by which trypomastigotes gain access to non-professional phagocytic cells. A minimal fraction of the invading trypomastigotes associate with host cell lysosomes whereas the majority of parasites induce plasma membrane invagination and the trypomastigote-containing vacuoles gradually acquire lysosomal markers (139). The newly forming *T. cruzi* compartments first interact with an early endosome compartment and subsequently with other late endosomes, before interaction with lysosomes (148).

The actin cytoskeleton is implicated in the intracellular retention of *T. cruzi* (140). A significant fraction of the internalized parasites is not retained inside host cells for productive infection by blocking lysosome-mediated trypomastigote invasion through phosphoinositide 3-kinase inhibition (141).

Phosphoinositide (PI)-3 kinases, protein kinase and phosphatases are also implicated in the mechanisms of *T. cruzi* invasion. Infection of macrophages by trypomastigotes stimulates the formation of the lipid products of PI 3-kinases; treatment with wortmannin, an inhibitor of PI 3-kinases, impairs parasite internalization (149). Human macrophages and non-phagocytic cells become less susceptible to *T. cruzi* infection upon treatment with wortmannin (150). Host cell PI 3-kinases activated by trypomastigotes early in the cell invasion process regulate lysosome-dependent parasite entry.

The *T. cruzi* gp83 ligand activates the MAP kinase pathway and PKC in macrophages to enhance *T. cruzi* infection of these cells (50-51) whereas, *T. cruzi* infection of HUVECs and vascular smooth muscle cells activates the MAP kinase pathway (151). Activation of parasite PTK is required for trypomastigote entry into nonphagocytic cells (152). Other protein kinases also participate in *T. cruzi* entry into host cells. It was found that infection of transiently transfected 3T3 cells containing an inactive mutant protein kinase B is less susceptible to invasion, as compared to the active mutant-transfected cells (150).

On the other hand, protein tyrosine phosphatases appear to be involved in the initial infection. Invasion of trypomastigotes induces tyrosine dephosphorylation of several proteins in heart myoblasts, and the parasite internalization is greatly reduced in the presence of protein tyrosine phosphatase inhibitors and in the presence of excess phosphotyrosine, but not of phosphoserine or phosphothreonine (151).

The signaling pathway mediated by transforming growth factor-beta (TGF-beta) receptor may be activated during trypomastigote invasion of mammalian cells. There is a requirement for the TGF-beta pathway in *T. cruzi* invasion of epithelial cells. Trypomastigotes attach to TGF-beta receptor-deficient epithelial cell lines, but are unable to penetrate them. *T. cruzi* infection is restored by transfection with TGF-beta receptor genes, and treatment with TGF-beta greatly enhances parasite internalization. A TGF-beta -responsive reporter gene is induced in TGF-beta -sensitive cell lines by trypomastigotes but not by noninvasive epimastigotes. Therefore, it is suggested that *T. cruzi* might directly trigger activation of the TGF-beta -signaling pathway required for invasion. The putative TGF-beta -like factor from trypomastigotes has never been characterized (120).

A 200 kDa TS of *T. cruzi* (153) appears to mimic nerve growth factor (NGF) by activating the nerve growth factor receptor TrKA *in vitro* (154-155). Its was also reported that *T. cruzi* infects PC12 cells by activating TrKA and that injection of an inhibitor of TrkA auto phosphorylation, or TrKA into mice attenuates *T. cruzi* infection (156). It was suggested that this activation also facilitates neuron survival (154). However, infections with a 200 KDa TS dominant negative or transgenic trypanosomes and mice in which the TrKA gene was silenced are required to support these contentions.

10. FUTURE DIRECTIONS

Although there is growing evidence from previous studies to suggest that *T. cruzi* surface molecules participate in the initial process of cardiomyocyte infection, the predominant molecular mechanism for their interactions is not well defined, and the cellular receptors that interact with such molecules have not been characterized. While progress in the cell biology of the infection process has also been made, the identification of a common molecular signature caused by *T. cruzi* in heart and other cells will help develop novel strategies to prevent Chagas heart disease.

The involvement of most of the suggested critical trypanosome and host molecules that participate in the early process of the infection has not been validated using molecular genetic approaches such as gene knock out or transgenic animal models, or *T. cruzi* gene knock out or transgenic trypanosomes. Furthermore, the structural and atomic analysis of *T. cruzi* surface ligands and the co-crystallization of these ligands and their host receptors that mediate trypanosome attachment to non-phagocytic and phagocytic cells leading to entry are critically important to understand this interaction at the molecular and atomic levels and to develop small inhibitors to block the first steps of infection. These studies are just beginning and it is expected that in the long term they will molecularly dissect this interaction. Furthermore, the use of high throughput technology to identify inhibitors of ligand-receptor interactions is beginning to be used and it is expected that it will bring novel drugs to block the first step of infection in Chagas heart disease.

There is documented information that *T. cruzi* triggers early signal transduction events during cellular entry, however very little is known about the trypanosome molecules that cause these events in heart cells leading to *T. cruzi* entry. Knock outs of host signal transduction genes, silencing them by RNAi, use of mouse embryo fibroblasts expressing, non-expressing or over-expressing signal transduction molecules or use of dominant negative cell lines may be a rapid approach to confirm or validate the role of signaling molecules in the early process of cellular invasion. Furthermore, the generation of trypanosome gene knock-out cell lines, transgenic trypanosomes and dominant negative trypanosomes using recently developed tools will be instrumental to elucidating the molecules in invasive trypomastigotes that trigger critical signal transduction events in heart cells leading to trypanosome entry. The studies of early *T. cruzi* entry into cardiomyocytes using approaches such as microarray platforms containing the whole human and *T. cruzi* genomes, host RNAi, new generation of expression vectors in *T. cruzi*, proteomics, combined with appropriate bioinformatics analysis will open windows to study systems biology during early *T. cruzi* infection of cardiomyocytes. These studies are also very much needed not only to understand the global molecular pathogenesis of *T. cruzi* infection of heart cells, but to understand the molecular basis of cardiovascular disease important for the development of both novel *T. cruzi* and cell- based therapies for Chagas heart disease.

The fact that two putative receptors for *T. cruzi* have been suggested by *in vitro* studies is promising, however the function of these putative receptors for *T. cruzi* needs to be validated *in vivo* using animal modes not expressing, expressing or over-expressing the genes coding for these putative receptors for *T. cruzi*.

Two attempts to address the role of two trypanosome genes in the process of infection by homologous recombination were documented. However, none of these genes encodes *T. cruzi* ligands that mediate attachment to heart cells to promote entry. *T. cruzi* gene silencing by RNAi to study the function of genes in the process of infection cannot be performed because the RNAi interference system in *T. cruzi* does not operate. Furthermore, since most of the candidate *T. cruzi* subsets of molecules such as mucins and TS implicated in invasion belong to large multi gene families, the approach of knocking them out by homologous recombination to study their functions in the attachment and entry process of heart cells poses limitations. Alternative approaches that need to be used include the generation of conditional knockout trypanosome cell lines and the application of dominant-negative approaches to validate the role of candidate invasive genes in the process of *T. cruzi* infection of heart cells.

The approach of RNAi to silence extracellular matrix genes such as laminin gamma-1 and thrombospondin 1 that are regulated during early *T. cruzi* infection has provided very impressive results to evaluate the function of cellular genes required in the early process of the infection of heart myoblasts and coronary artery smooth muscle cells. It is expected that future studies will use similar RNAi approaches to validate the involvement of other genes regulated by *T. cruzi* in the heart cells that are implicated in cardiovascular disease.

It is also important to identify the *T. cruzi* genes that are regulated when *T. cruzi* invades heart cells. We know almost nothing about the *T. cruzi* genes that may be important in the molecular pathogenesis of cardiac and other cells, which may contribute to cardiovascular disease in Chagas heart disease. The genes regulated by recently internalized *T. cruzi* during early infection of heart cells may cause the heart to arrest, facilitating the establishment of the parasite. It is important to identify these trypanosome genes as well as understand their roles in cardiomyocyte infection and pathology. We expect that the recent completion of the *T. cruzi* genome project and the generation of microchip platforms containing the whole *T. cruzi* genome will facilitate studies that will also be relevant to understand the molecular pathogenesis of cardiomyocyte infection that may lead to the cardiovascular pathology seen in Chagas heart disease. The information gained in this area will also be important to target those *T. cruzi* genes and their coded proteins to block parasite intracellular establishment and development in heart cells.

Even though the complete *T. cruzi* genome has been sequenced and partial annotations of the genome took place (28) this new information has not been used vigorously to identify the invasive genes and the critical genes that are regulated during the early process of infection. No global gene-networking analysis of the genes involved in the invasion process of the parasite and host cells has been established. The work of functional genomics and systems biology during early infection of mammalian cells by *T. cruzi* is in infancy. Future progress in this area will facilitate the full understanding of the participation of the parasite and host cells during early infection.

Neither DNA microarrays and proteomic analysis of *T. cruzi* clonal populations with different degrees of infectivity nor the molecular host response to different clonal populations and to strains of *T. cruzi* with different infectivity has been performed to understand how molecular determinants are involved in the infection. Furthermore, systems biology of host responses to the whole *T. cruzi* and to critical surface molecules that participate in the initial cellular infection has not been done. Is the molecular signature caused by early *T. cruzi* infection the same, similar or different across various cell types? What are the molecular signatures caused by TS, TS-like molecules, mucins, casein Kinase II substrate and other molecules involved in the process of infection of cardiomyocytes and other cells?

Since *T. cruzi* infects almost all cells in our body except red blood cells, the questions “does *T. cruzi* utilize the same types of surface receptors in all cells, does *T. cruzi* use different classes of receptors depending on the type of host cell and what are the innate immune molecules

that facilitate, promote, and regulate the early process of cellular infection" need to be addressed. Most of the answers to the questions raised in this review will not only facilitate the understanding of the mechanisms of *T. cruzi* attachment, entry, intracellular development, multiplication and egress from infected cells, but also the understanding of how *T. cruzi* evades the host to establish early infection. Once answered, these questions will also facilitate the understanding at the molecular and atomic levels that is critically important to establish prime targets to intervene against *T. cruzi* infection and to facilitate the manipulation of the host to ameliorate infection. We enthusiastically expect that the future directions highlighted in this review will significantly advance the molecular understanding of how *T. cruzi* infects heart and other cells to contribute to cardiovascular disease. The new knowledge gained will be useful to develop strategies to prevent this devastating heart disease. Furthermore, the use of the proposed integrated and powerful approaches will also facilitate underlining the precise mechanisms of cardiomyocyte and other cell signaling at the molecular level during early infection that are critical for the development of molecular interventions in Chagas heart disease.

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Abbreviations: TS: trans-sialidases; GPI: glycosylphosphatidylinositol; TcMUC: *T. cruzi* mucins; MASPs: mucin associated surface proteins; TcSMUG: *T. cruzi* small mucin-like gene family; tGPI-mucins: GPI-mucins from cell derived trypomastigotes; Ssp-3: sialic acid containing epitope; PI-PLC: phosphotidylinositol-phospholipase C; SAPA: shed acute phase antigen; PKC: protein kinase C; MAP: mitogen activated protein; CKII: casein kinase II; Tc-1: *T. cruzi* CKII substrate; LYT1: lytic pathway protein; NLS: nuclear localization signal; CHO: Chinese hamster ovary cells; POP: prolyl oligopeptidase; B_2R: B type bradykinin receptor; HUVEC: human umbilical vein endothelial cell; CMP-sialic acid: cytidine monophospho-sialic acid; MGTA: DL-2-Mercaptomethyl-3-guanidinoethylthiopropanoic acid; IP: inositol phosphate; HCASM: human coronary artery smooth muscle; RNAi: RNA interference; ECM: extracellular matrix; TSP-1: thrombospondin- 1; TGF Beta-1: transforming growth factor beta-1; PI-3 kinase: phosphoinositide-3-kinase; NGF: nerve growth factor

Key Words: *Trypanosoma cruzi*, Chagas disease, Host cell invasion, *T. cruzi* ligands, *T. cruzi* receptors, Proteases, Cell signaling, Laminin gamma-1, Thrombospondin-1, Trans-sialidase, Trans-sialidase-like molecules, Mucins, Galectin-3, Casein kinase II substrate, Genetic tools, Review

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