Invasion of MDCK epithelial cells with altered expression of Rho GTPases by *Trypanosoma cruzi* amastigotes and metacyclic trypomastigotes of strains from the two major phylogenetic lineages

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Abstract

In order to invade mammalian cells, *Trypanosoma cruzi* infective forms cause distinct rearrangements of membrane and host cell cytoskeletal components. Rho GTPases have been shown to regulate three separate signal transduction pathways, linking plasma membrane receptors to the assembly of distinct actin filament structures. Here, we examined the role of Rho GTPases on the interaction between different *T. cruzi* infective forms of strains from the two major phylogenetic lineages with nonpolarized MDCK cells transfected with different Rho GTPase constructs. We compared the infectivity of amastigotes isolated from infected cells (intracellular amastigotes) with forms generated from the axenic differentiation of trypomastigotes (extracellular amastigotes), and also with metacyclic trypomastigotes.

No detectable effect of GTPase expression was observed on metacyclic trypomastigote invasion and parasites of Y and CL (*T. cruzi* II) strains invaded to similar degrees all MDCK transfectants, and were more infective than either G or Tulahuen (*T. cruzi* I) strains. Intracellular amastigotes were complement sensitive and showed very low infectivity towards the different transfectants regardless of the parasite strain. Complement-resistant *T. cruzi* I extracellular amastigotes, especially of the G strain, were more infective than *T. cruzi* II parasites, particularly for the Rac1V12 constitutively active GTPase transfectant. The fact that in Rac1N17 dominant-negative cells, the invasion of G strain extracellular amastigotes was specifically inhibited suggested an important role for Rac1 in this process.

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1. Introduction

*Trypanosoma cruzi* is a flagellated protozoan parasite and etiologic agent of Chagas’ disease, endemic throughout South and Central America. Recent studies with different *T. cruzi* isolates have grouped the parasite in at least two major phylogenetic lineages: *T. cruzi* I linked to the sylvatic cycle of the parasite and other mammalian hosts and *T. cruzi* II associated with the domestic cycle and human disease [1–3]. Besides the different genetic markers, *T. cruzi* I and II infective forms have distinct infectivity and engage characteristic signaling pathways upon invasion of cultured cells [4]. *T. cruzi* undergoes a biphasic life cycle, comprised of several distinct developmental stages in both the reduviid insect vector and the mammalian host. In the insect vector, the flagellated epimastigote proliferates in the midgut before differentiating into the nondividing, infective, metacyclic trypomastigotes found in the vector’s hindgut. Following its introduction into the mammalian body, the parasite infects host cells, differentiates into amastigotes, and begins to replicate in the cytosol of the infected cell. Amastigotes then develop into nondividing trypomastigotes that, upon cell lysis, may either initiate another round of infection or be taken up by the reduviid vector during a blood meal [5]. Amastigotes may occasionally be found extracellularly derived from the premature lysis of infected cells [6–9] or through extracellular differentiation of trypomastigotes [10,11], and can invade professional or nonprofessional phagocytes, where they survive and sustain the parasite’s life cycle [6–8,11–13].
Although intracellular amastigotes are larger and slightly more elongated than the extracellular forms [14], both share biochemical and ultrastructural similarities [10,12,15] and express similar antigenic stage-specific markers [10,16–18].

Moreover, it has long been recognized that trypomastigotes, the classical infective forms of *T. cruzi*, display marker complement resistance that has been associated with their infectivity [19,20]. Although previous results from the literature have provided conflicting evidence regarding the infectivity of *T. cruzi* intracellular amastigotes [9,12,21,22], results indicate that infective extracellular amastigotes resist antibody-independent complement lysis [23]. In spite of reports comparing surface antigen expression by *T. cruzi* amastigotes obtained through distinct procedures [18,24], there has been no direct comparison between intracellular and extracellular forms regarding their complement resistance and infectivity. Furthermore, despite intense studies on trypomastigote penetration, relatively little is known about the mechanism involved in the invasion of cultured cells by amastigotes. We have seen in a previous work, that G strain (*T. cruzi* I) extracellular amastigotes are able to infect HeLa cells by interacting with microvilli on the dorsal surface of these cells [13]. By contrast, trypomastigotes preferentially enter HeLa cells at the edges, showing that the different forms of the parasite interact with specific regions of the cell surface [13,25].

Evidence has been provided [26] that extracellular amastigotes utilize mannose receptors to infect professional phagocytes. Moreover, host cell surface glycoprotein sialylation is important for trypomastigote invasion [27] but has no effect on amastigote entry [28]. Work from our group also demonstrated the importance of host cell cytoskeleton integrity for extracellular amastigote and metacyclic trypomastigote (G strain) invasion of HeLa and Vero cells [29].

Adhesion of invasive microorganisms to mammalian cells is often accompanied by cytoskeletal rearrangements that favor their entry. In recent studies, evidence has shown that *T. cruzi* amastigote invasion of cultured cells involves distinct cytoskeletal rearrangements and signaling mechanisms when compared with metacyclic trypomastigotes [30]. Unlike trypomastigote invasion, which may trigger pseudopodium extension in HeLa cells [25], the entry of extracellular amastigotes in these cells involves the formation of a discrete cup-like membrane expansion around invading parasites [30]. By contrast, in Vero cells that are devoid of microvilli, amastigote invasion is associated with the expansion of membrane lamellae that surround invading parasites [30], suggesting that in each case, a particular set of microfilament-regulating GTPases might be specifically activated.

Rho-GTPases are molecular switches that control a wide variety of signal transduction pathways in eukaryotic cells. They cycle between two conformation states: one bound to GTP (active state) that turns into the GDP-bound inactive state, when they hydrolyse GTP to GDP. In the active (GTP-bound) state, GTPases recognize target proteins and generate a response until GTP hydrolysis returns the switch to inactivity. This general mechanism has been refined throughout evolution, and mammalian cells contain several hundred GTPase switches. They are best known for their pivotal role in regulating the actin cytoskeleton, but their ability to influence cell polarity, microtubule dynamics, membrane transport pathways and transcription factor activity is probably just as significant. When introduced into fibroblasts, constitutively activated (GTPase activity deficient) mutants of RhoA and Rac1 were found to induce the assembly of contractile actin and myosin filaments (stress fibers) and actin-rich surface protrusions (lamellipodia), respectively [31–33]. Later, Cdc42 was shown to promote the formation of actin-rich, finger-like membrane extensions (filopodia) [34,35]. Thus, RhoA, Rac1, and Cdc42 regulate three separate signal transduction pathways, linking plasma membrane receptors to the assembly of distinct filamentous actin structures. These observations have been confirmed in a wide variety of mammalian cell types as well as in yeast, flies and worms. In order to evaluate the relative importance of RhoA GTPases in host cell invasion by different *T. cruzi* infective forms of distinct strains, we used MDCK cell transfectants that express variants of RhoA, Rac1 and Cdc42 proteins [36]. Our results indicate that metacyclic trypomastigotes from G and Tulahuen strains (*T. cruzi* I) presented lower infectivity than Y and CL (*T. cruzi* II) strains for the different target cells, with no apparent specific requirement for GTPases. Regardless of the strain analyzed, intracellular amastigotes were not only susceptible to complement lysis but also showed very low infectivity towards the different transfectants. Extracellular amastigotes of all four strains were complement resistant and more infective than intracellular forms, and parasites from G strain infected transfected MDCK cells more efficiently than the other strains. Invasion was particularly high in Rac1V12 cells and was specifically reduced in the corresponding dominant-negative line Rac1N17, suggesting an important role for this protein in this invasion process.

2. Materials and methods

2.1. Cell lines and culture

Vero cells (African green monkey kidney fibroblasts, obtained from Instituto Adolfo Lutz, São Paulo, Brazil) were grown at 37 °C in a 5% CO₂ humid atmosphere, in RPMI-1640 medium supplemented with 10% fetal bovine serum, streptomycin (100 µg/ml) and penicillin (100 units/ml). These cells were used to maintain the *in vitro* *T. cruzi* cycle. The Madin Darby Canine Kidney (MDCK) cell line MDCK-Tet-OFF, which expresses the tetracycline repressor protein (stably transfected with the pTet-OFF plasmid, from
Clonetech) was provided by W. James Nelson (Stanford University, CA, USA) with different constructs: Rac1V12 (constitutively active); Rac1N17 (dominant negative); RhoAV14 (constitutively active); RhoAN19 (dominant negative); Cdc42V12 (constitutively active), and the control line that was transfected with empty plasmid [36]. MDCK Tet-Off cell lines were maintained at 37 °C in DMEM medium with 10% fetal bovine serum and doxycycline (20 ng/ml). For the expression of the recombinant proteins, the transfectants were grown in the absence of doxycycline for 48 h.

2.2. Parasites

In this study, two strains of each major T. cruzi phylogenetic lineage were used: T. cruzi I, G strain, isolated in the Amazon from a marsupial of the Didelphidae family [37] and Tulahuen strain, isolated in Chile from Triatoma infestans [38]; T. cruzi II, CL strain, isolated in southern Brazil from T. infestans in human dwellings where people were infected with T. cruzi [39], and Y strain, isolated from an acute chagasic patient [40]. Intracellular amastigotes were isolated from previously infected Vero cells and purified in discontinuous metrizamide gradients [41] and checked for viability by incorporation with 35S-methionine. Extracellular amastigotes were derived from the axenic differentiation of cell-derived trypomastigotes. Vero cell-derived trypomastigotes were isolated from culture supernatants of infected cells by centrifugation at 2500 × g for 5 min. The pellet was resuspended in liver infusion tryptose (LIT) medium [42] and incubated for 24–48 h at 37 °C, when at least 95% pure extracellular amastigotes were obtained [12,13]. Metacyclic trypomastigotes were obtained by differentiation of stationary-phase hemocultures of infected mice, in LIT medium [43]. Grace’s medium was also used to obtain cultures enriched in metacyclic trypomastigotes, which were purified by passage through DEAE-cellulose columns [37].

2.3. Complement-mediated lysis

In order to determine T. cruzi resistance to complement, 10 µl of a 10⁸ parasites/ml suspension in RPMI medium were incubated with 30 µl of freshly drawn guinea pig serum (devoid of lytic anti-a-gal antibodies [44]) as a complement source. After 30 min at 37 °C, 10 µl of the mixture were examined under phase-contrast microscopy, and viable refringent parasites were counted. Metacyclic trypomastigotes of all strains are complement-resistant forms (data not shown and [45]).

2.4. Cell invasion assay

MDCK cells were grown in the absence of doxycycline for 48 h to induce the expression of GTPase proteins. Host cell invasion assays were carried out as previously described [29], by seeding parasites onto each plastic Petri dish containing 13-mm diameter round glass coverslips with previously grown 1.0 × 10⁵ MDCK cells, at a 10:1 parasite/cell ratio. The dishes were immediately centrifuged at 2300 × g for 20 min at 37 °C. After centrifugation, unattached parasites were removed by washing the coverslips with phosphate-buffered saline (PBS) solution at 37 °C. Coverslips were fixed in 2% glutaraldehyde for 90 min at room temperature. After three washings in PBS, coverslips were incubated with 0.138 M ethanolamine, pH 8.3, for 30 min at room temperature to quench excess aldehyde groups [46], washed with PBS, and soaked in PBS containing gelatin (0.15%) and NaN₃ (0.05%, PGN solution).

2.5. Indirect immunofluorescence assay

The coverslips were inverted for 1 h onto 20-µl drops of anti-metacyclic trypomastigote monoclonal antibody (Mab) 3F6, which recognizes the 82-kDa surface glycoprotein (used for Y and CL strains), or Mab 1G7 directed towards the 90-kDa surface glycoprotein (for G and Tulahuen strains) [47]. Anti-amastigote Mab 1D9, which reacts with a carbodimide epitope on Ssp-4 [17], a major amastigote surface glycoprotein, was used to detect amastigote invasion [29]. Samples were then washed with PBS and treated in the same way with a PGN solution containing the appropriate FITC conjugate and 10 µM DAPI (4′,6-diamidino-2-phenylindole dihydrochloride, Molecular Probes, Eugene, OR, USA) to visualize internalized parasites [46]. After being washed, the coverslips were mounted with glycerol/0.2 M Tris–HCl pH 8.4 containing 1% p-phenylenediamine to minimize quenching. Quantification of parasite internalization was done under epifluorescence microscopy, as previously described [13,46], and was expressed by the following formula:

\[
\text{invasion index} = \frac{\text{number of intracellular parasites} \times \% \text{ of infected cells}}{\text{number of infected cells}}
\]

Triplicate coverslips were analyzed in each experiment, repeated at least three times. Invasion indexes were subjected to Student’s t-test. Fluorescently labeled samples were imaged on a Bio Rad 1024-UV confocal system [17]. In experiments carried out to check the expression of RhoA, Rac1, and Cdc42 GTPases, fixed cells were incubated with monoclonal antibody α-c-myc (Mab C9, Santa Cruz Biotechnology, Santa Cruz, CA, USA), since these transfectant proteins contained 10 additional amino acids at their N-termini, corresponding to the myc epitope tag. Phalloidin-rhodamine (Sigma Chemical Co., St. Louis, USA) was used to localize F-actin, and DAPI to visualize parasites’ kinetoplast and nuclei [30].
3. Results

3.1. Complement-mediated lysis of *T. cruzi* amastigotes

When exposed to complement, intracellular amastigotes of all four strains were readily lysed, whereas extracellular parasites were completely resistant (Table 1).

3.2. Regulated expression of GTP-binding proteins RhoA, Rac1, and Cdc42 in transfected MDCK cells

To ascertain the regulated expression of RhoA, Rac1, and Cdc42 GTPases, we carried out indirect immunofluorescence with an anti-c-myc Mab, since the recombinant proteins contain a c-myc tag epitope. We observed that the GTPases were homogeneously expressed in the constitutively active transfectants (Rac1\textsuperscript{V12}, RhoA \textsuperscript{V14} and Cdc42\textsuperscript{1V12}), while in the dominant-negative transfectants (Rac1\textsuperscript{N17} and RhoA\textsuperscript{N19}) and control cells (transfected with the empty plasmid), no expression could be detected (Fig. 1).

3.3. Infectivity of *T. cruzi* G, Tulahuen, Y and CL strains’ intracellular amastigotes in MDCK cells transfected with Rho GTPases

Intracellular amastigotes from G, Tulahuen, Y, and CL strains were tested for their capacity to invade MDCK cells transfected with RhoA, Rac1, and Cdc42 GTPases. The results showed that intracellular amastigotes presented low invasion indexes for all parasite strains, and no significant differences were observed among the transfected target cells (Fig. 2).

3.4. Distinct infectivity of *T. cruzi* metacyclic trypomastigotes and extracellular amastigotes towards MDCK cells transfected with Rho GTPases

Both metacyclic trypomastigotes and extracellular amastigotes from G, Tulahuen, Y, and CL strains were tested for their capacity to invade transfected MDCK cells. Metacyclic trypomastigotes from Y and CL strains invaded all the transfectants to comparable degrees, and the invasion indexes were higher than those of G or Tulahuen strain trypomastigotes (Fig. 3).

When we compared the infectivity of extracellular amastigotes of the four strains towards the transfected

<table>
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<th><em>T. cruzi</em> strain</th>
<th>% of lysed parasites (^a)</th>
<th>Intracellular amastigote</th>
<th>Extracellular amastigote</th>
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\(^a\) Lysed parasites were scored under phase-contrast microscopy. 
\(^b\) Control samples were incubated with heat-inactivated (56 °C for 1 h) guinea pig serum.

![Fig. 1. Expression of small GTP-binding proteins RhoA, Rac1 and Cdc42 in transfected MDCK cells. A, B: control cells; C, D: Rac1\textsuperscript{V12}; E, F: Rac1\textsuperscript{N17}; G, H: RhoAV14; I: J: RhoA\textsuperscript{N19}; K,L: Cdc42\textsuperscript{1V12}. In panels A, C, E, G, I, and K, MDCK cells were grown in the presence of doxycycline. In panels B, D, F, H, J, and K, MDCK cells were grown in the absence of doxycycline for 48 h. Cells were labeled with anti-c-myc (FITC, cyan) and DAPI (red). Magnification bars in \(\mu\text{m}\).]
MDCK cells, we observed that the number of parasites from G strain that invaded MDCK cells was about four times higher than that of Y or CL strains (Fig. 4). G strain extracellular amastigotes presented high invasion indexes on all transfectants examined, and invaded Rac1V12 (constitutively active) significantly more than the other transfectants (Fig. 4). Conversely, the dominant-negative transfectant Rac1N17 cell line was less susceptible to infection by G strain extracellular amastigotes than control cells and the Rac1V12 line (Fig. 4). These findings indicate that Rac protein appears to be related to cellular invasion by T. cruzi extracellular amastigotes (G strain).

4. Discussion

4.1. Infectivity of different T. cruzi infective forms towards MDCK cells: intracellular amastigotes, extracellular amastigotes and metacyclic trypomastigotes

We have shown here that T. cruzi amastigotes isolated from infected cells (intracellular forms) are much less infective in vitro to MDCK cells than extracellular amastigotes, confirming our previous observations using either Vero or HeLa cells as targets [48]. Extracellular amastigote infectivity correlates well with complement resistance, a well-known characteristic displayed by trypomastigotes [19,20]. Bloodstream amastigotes have been found circulating in infected mammals during the acute phase (probably extracellular forms) [49], and it has been shown that the aflagellated forms of the parasite are infective both in vitro and in vivo [7,9,10,21,22,26,50]. Although previous results from the literature are conflicting regarding the infectivity of T. cruzi intracellular amastigotes [9,12,21,22], there has been no dispute that extracellular amastigotes are not only infective [8,11–13] but also resist complement lysis [23]. Besides morphological [14] and immunochemical [24,18] variations, differences in infectivity and complement resistance between intracellular and extracellular forms suggest that circulating extracellular amastigotes may be involved in a complementary subcycle that sustains T. cruzi infection in mammalian hosts [51].

Studies from our group showed that extracellular amastigotes of the G strain are consistently more infective to both HeLa and Vero cells [29], as well as to CHO and sialic acid-deficient Lec-2 cells [28], than the corresponding metacyclic trypomastigotes. In this work, we noticed that for MDCK cells and their transfectants, metacyclic trypomastigotes of all strains are more infective than the corresponding extracellular amastigotes, supporting the notion that not only the infective form of the parasite but also the target cells are important for the outcome of the invasion process [29,48].

4.2. Cytoskeletal rearrangements in T. cruzi infective forms’ invasion: role of Rac1 in the invasion by extracellular amastigotes

In the previous work in our laboratory, evidence was presented that amastigote invasion involves distinct cytoskeletal rearrangements and signaling mechanisms when compared with that of metacyclic trypomastigote forms [29]. For instance, in Vero cells which are devoid of microvilli, amastigote invasion is associated with the expansion of...
membrane lamellae that surround invading parasites, and trypomastigotes invade these cells by their borders [29]. In contrast, the entry of extracellular amastigotes in HeLa cells involves the formation of a discrete cup-like membrane expansion around invading parasites [29] and sleeve-like pseudopodia around trypomastigotes [25], suggesting that for each case, a particular set of microfilament-regulating GTPases might be specifically activated. In order to test which of the main Rho GTPases might be performing a role in these processes, we tested RhoA, Rac1, and Cdc42 MDCK transfectants [24]. Interestingly, high or low expression of any of the GTPases had no effect on metacyclic trypomastigote invasion (Fig. 3). Our results showed, however, that extracellular amastigote invasion was enhanced in the over-expressing transfectant Rac1V12 cells and was specifically reduced in the corresponding dominant-negative line Rac1N17, suggesting an important role for Rac1 in this invasion process. The mobilization of mammalian cell GTPases is being disclosed as a widespread mechanism in microbial invasion. The involvement of Rac1 in MDCK cell invasion by T. cruzi amastigotes parallels the results described for Salmonella typhimurium apical invasion of MDCK cells, a process that depends on Rac1 expression and activation, and also shown to be reduced in Rac1N17 cells [52]. In the case of S. typhimurium, it appears that secreted bacterial components such as SopE2 and SptP [53,54] are involved in Rac1 recruitment that leads to surface ruffling [52]. Moreover, in polarized cells, both Cdc42 and Rac1 facilitate S. typhimurium invasion through the basolateral domain [52]. Cdc, Rho as well as Rac1 are also involved in Shigella flexneri invasion of HeLa cells transfect was with GTPase constructs similar to those used here [55]. Contrasting with bacterial invasion, neither T. cruzi metacyclic trypomastigote nor amastigote invasion seems to depend on Cdc42 (Figs. 3 and 4). This reinforces the notion that each microbe and infective form will engage a specific set of signaling components, from each host cell type, responsible for the formation of membrane expansions required for their invasion. In view of the morphological relatedness with Shigella and Salmonella invasion, it would be tempting to speculate that the membrane expansions formed during Vero cell invasion [56] might be related to Rac1-driven protrusions triggered by an as yet unidentified amastigote component.

4.3. Infectivity of T. cruzi infective forms of the two phylogenetic lineages

Evidence has been accumulating that T. cruzi exists in nature as a highly variable population. Over the last years, genetic markers have enabled researchers to group numerous parasite strains into two major phylogenetic lineages that display distinct biological properties. Our results indicate that metacyclic trypomastigotes from G and Tulahuen strains (T. cruzi I) presented lower infectivity than Y and CL (T. cruzi II) strains towards the different MDCK cells, with no apparent specific requirement for GTPases. These results are in line with the known infectivity of metacyclic trypomastigotes of these strains [57]. We had previously observed that extracellular amastigotes from the T. cruzi I G strain, which is poorly virulent and only causes subpatent parasitemia in outbred Swiss mice, are much more infective to HeLa cells than forms of the more virulent T. cruzi II isolates like Y and CL strains [48]. The results obtained here with MDCK transfecteds have provided, for the first time, a comprehensive comparison between infective forms and parasite strains. Thus far, we have confirmed the poor infectivity of intracellular amastigotes when compared with the extracellular forms, and the notion is strengthened that the infectivity of metacyclic trypomastigotes and extracellular amastigotes differs: T. cruzi II metacyclic trypomastigotes are more infective than T. cruzi I, whereas the opposite is true for extracellular amastigotes. Whether or not the differences in signaling pathways described for T. cruzi I and II metacyclic trypomastigotes upon invasion of cultured cells [4] are retained in their extracellular amastigote counterparts, is a subject of our current research.

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