Trypanosoma cruzi: Phosphatidylinositol 3-Kinase and Protein Kinase B Activation Is Associated with Parasite Invasion

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Multiple signal transduction events are triggered in the host cell during invasion by the protozoan parasite Trypanosoma cruzi. Here, we report the regulation of host cell phosphatidylinositol 3-kinase (PI3K) and protein kinase B (PKB/Akt) activities by T. cruzi during parasite-host cell interaction. Treatment of nonphagocytic cells (Vero, L6E9, and NIH 3T3) and phagocytic cells (human and J774 murine macrophages) with the selective PI3K inhibitors Wortmannin and LY294002 significantly impaired parasite invasion in a dose-dependent fashion. A strong activation of PI3K and PKB/Akt activities in Vero cells was detected when these cells were incubated with trypomastigotes or their isolated membranes. Consistently, we were unable to detect activation of PI3K or PKB/Akt activities in host cells during epimastigote (noninfective) membrane-host cell interaction. Infection of transiently transfected cells containing an inactive mutant PKB showed a significant inhibition of invasion compared with the active mutant-transfected cells. T. cruzi PI3K-like activity was also required in host cell invasion since treatment of trypomastigotes with PI3K inhibitors prior to infection reduced parasite entry. Taken together, these results indicate that PI3K and PKB/Akt activation in parasites, as in host cells induced by T. cruzi, is an early invasion signal required for successful trypomastigote internalization.

Key Words: Trypanosoma cruzi; cell invasion; signal transduction; PI3K; PKB/Akt; Wortmannin; LY294002

INTRODUCTION

The intracellular protozoan parasite Trypanosoma cruzi (T. cruzi) is the causative agent of Chagas’ disease or American trypanosomiasis. In the mammalian host, trypomastigotes are the infective stage that invade host cells through the formation of a membrane-bound vacuole. Soon after invasion, disruption of this vacuolar membrane allows parasites to reach the cytoplasm, where they differentiate into amastigotes and begin intracellular replication. Several lines of evidence have suggested that parasite attachment and invasion is a complex process in which multiple host and parasite molecules are required [1]. Moreover, T. cruzi internalization has been associated with recruitment and fusion of host cell lysosomes at the invasion site [2], suggesting the activation of a signal transduction pathway in the host cell. In addition to these findings, transient Ca2+ increases in the host cell have been detected when trypomastigotes or their isolated membranes were added to these cells [3–5]. These transient signals were associated with a yet undescribed trypomastigote membrane factor [6]. The Ca2+ signals were pertussis toxin-sensitive and mediated by phospholipase C, with concomitant inositol triphosphate (IP3) formation [7].

Besides the phosphoinositide pathway involving IP3 (which in turn releases Ca2+ from intracellular stores) and diacylglycerol, other phosphoinositides, such as phosphatidylinositol (PI) 3-phosphate (PIP3), PI 3,4-bisphosphate (PIP2), and PI 3,4,5-triphosphate (PIP3), have also been implicated in signaling roles [8]. Upon binding of hormones or growth factors to plasma membrane receptors, the levels of these lipids are rapidly increased in eukaryotic cells through the activity of phosphoinositide kinases. One of these kinases, PI3K, is a ubiquitously expressed enzyme that catalyses the phosphorylation of phosphoinositides at the D-3 hydroxyl of the myoinositol ring generating PIP2, PIP3, and PIP4 [9]. The form of PI3K involved in tyrosine kinase receptor signal transduction is composed of a regulatory 85-kDa subunit and a catalytic 110-kDa subunit [9]. On the other hand, several studies have indicated that the serine threonine kinase B (PKB), also known as c-Akt, is one of the major targets of PI3K [10, 11]. Activation of PKB/Akt occurs primarily by phosphorylation of Thr and Ser residues [12] and/or binding of PIP3 [13] and involves translocation of the...
enzyme to the plasma membrane. Both PI3K and PKB/Akt have been implicated in mitogenesis, vesicular trafficking, and cell survival through signal transduction mechanisms [14]. Recent reports on the activation of the host cell PI3K or PKB/Akt pathway by pathogenic bacteria [15, 16], lipopolysaccharide [16, 17], or certain viruses [18, 19] have shown that these pathogens or their isolated molecules can subvert host cell signal transduction pathways and modify the course of parasite survival. In the present work, we have studied the involvement of host PI3K and PKB/Akt in the process of T. cruzi invasion. Our findings show that parasitic molecules present in the plasma membrane of trypomastigotes trigger activation signals for host PI3K and PKB/Akt. These signals are undoubtedly associated with infectivity since membranes from the noninfective epimastigote stage were unable to induce any effect. The activation of host PI3K and PKB/Akt is a requisite for successful infection by T. cruzi, since parasite invasion is strongly reduced when this pathway is blocked by selective inhibitors or inactive mutant cells. Besides, a participation of a T. cruzi’s PI3K-like in host cell invasion indicates that common pathways in the parasite and the host cell are mutually involved.

MATERIALS AND METHODS

Chemicals. Polyclonal antibodies to PI3K-γ-p85 and to PKB/Akt were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The lipid substrate phosphatidylinositol was obtained from Avanti Polar Lipids (Alabaster, GA). Wortmannin and LY290042 were purchased from Biomol (Plymouth Meeting, PA) and dissolved in dimethyl sulfoxide. All working solutions were prepared immediately before use and sterilized through a 0.22-μm-pore-size filter. Protein A-Sepharose beads, protease inhibitors, and all other reagents were from Sigma (St. Louis, MO).

Mammalian cells and parasites. Infection experiments were performed using the nonphagocytic cell line Vero (African green monkey kidney cells) L-1210, myeloblasts, and NIH3T3 fibroblasts. Human and murine J774 macrophages were grown in Dulbecco’s minimal essential medium (DMEM) containing 10% fetal bovine serum (FBS), at 37°C with 5% CO2.

NIH3T3 cells transiently expressing wild-type, active, and inactive PKB/Akt-hemagglutinin (HA) constructs were generated by using the pcDNA3 vector as described previously [20]. Human peripheral blood monocyte-derived macrophages were prepared as described [21]. J774 macrophages were grown in alpha-minimal essential medium (MEM) containing 10% FBS at 37°C with 5% CO2.

T. cruzi epimastigotes of the acute RA lethal strain [22] were cultured in biphasic medium at 28°C as previously described [23]. Trypomastigotes from the same strain were obtained from the culture medium of irradiated Vero cells following the method described by Moreno et al. [4]. This method yields 95% pure trypomastigotes with minor contamination of amastigotes. Final concentrations of parasites were determined using a Neubauer chamber.

T. cruzi membrane fraction. T. cruzi trypomastigote and epimas- tigote membranes were prepared as previously described [5]. Briefly, parasites at a concentration of 1×108/ml were washed twice and resuspended in phosphate buffer solution plus 1% bovine serum albumin (PBS–BSA) containing the following protease inhibitors: 0.01% leupeptin; 2 mM phenylmethylsulfon fluoride; 25 U/ml aprotinin; 1 mM benzamidine; 0.2 mg/ml soybean trypsin inhibitor, and 0.5 mM tosyl-L-lysine chloromethyl ketone. Parasites were disrupted by four freeze-thaw cycles and centrifuged at 14,000g for 10 min at 4°C. The supernatant was further centrifuged at 105,000g for 1 h at 4°C and the pellet containing the microsomal fraction was resuspended in PBS–BSA, aliquoted at 1×108 parasite equivalents/ml, and stored at -70°C. This fraction was further used as the parasite membrane fraction in the PI3K and PKB/Akt assays and contained 15 μg of protein per 1×108 trypomastigotes.

PI3K assay. To determine PI3K activity, 3×105 Vero cells were washed twice with ice-cold PBS and lysed in modified RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM Na3VO4, 1% NP-40, 1 mM EDTA, and protease inhibitors (0.01% leupeptin; 2 mM phenylmethylsulfon fluoride; 25 U/ml aprotinin, 1 mM benzamidine; 0.2 mg/ml soybean trypsin inhibitor), 1 mM NaF, 10 mM β-glycerophosphate) by incubation in a rocker shaker for 15 min at 4°C. Cells were removed from the plate with a cell scraper and were centrifuged at 14,000g for 15 min at 4°C. The supernatants were incubated overnight at 4°C with γ-p85 PI3K antibody. Protein A-Sepharose beads (40 μl) were added to each tube and incubated for 2 h at 4°C with constant agitation. The immunoprecipitates were washed once by centrifugation with RIPA buffer, twice with 100 mM Tris, pH 7.5, 0.1 mM LiCl, 1 mM Na3VO4, and twice with TNE (10 mM Tris, 150 mM NaCl, 5 mM EDTA, 0.1 mM Na3VO4) and finally resuspended in 50 μl of TNE buffer. Sonicated PI (1 μl of 2 mg/ml in 10 mM Tris–HCl, pH 7.4, 1 mM EDTA) was added to 1 μl of [γ-32P]ATP, 1 μl of [γ-32P]ATP, 10 μl of 100 mM MgCl2, and 10 μl of 100 mM MnCl2 were added and the samples were incubated for 15 min at 37°C. The reaction was stopped by the addition of 20 μl of 6 N HCl and 160 μl of chloroform:methanol (1:1). After the tubes were vortexed, the organic phase was extracted and centrifuged at 2000 rpm for 5 min at room temperature. Samples were applied to potassium oxalate-treated thin-layer chromatography (TLC) plates and developed using a solvent system of CHCl3:CH3OH:H2O:NH4OH (60:47:11:3). Dried TLC plates were exposed to an autoradiography film and the PIP spots were identified with authentic standards and quantitated by densitometry using the NIH Image Program or excising the spot and scintillation counting [24]. This assay was performed with unstimulated Vero cells (control), Vero cells stimulated with 100 nM EGF (epidermal growth factor) (positive control), parasite membrane-stimulated cells, and cells previously treated with LY294002 and stimulated with a trypomastigote membrane fraction. Parasite membranes (from 1×108 para-sites) used in each point were equivalent to 15 μg of protein. In all cases, parallel samples were processed for protein immunoblot using the same γ-p85 PI3K antibody to verify that equivalent amounts of enzyme were present in each experimental point.

PKB/Akt assay. For the determination of PKB activity, the method of Kohn et al. was used [20]. For these immune complex kinase assays, Vero cells were cultured, stimulated, washed, and lysed exactly as described for PI3K assays. After a 3-h incubation at 4°C with 5 μl of the anti-PKB antibody followed by an overnight incubation with 20 μl of protein A–agarose, the immunoprecipitates were washed twice with lysis buffer (25 mM HEPES, pH 7.9, protease inhibitors, 1% Triton X-100, 150 mM NaCl, 10 mM β-glycerophosphate, and 1 mg/ml soybean trypsin inhibitor) and twice with kinase buffer (25 mM HEPES, pH 7.9, 10 mM MgCl2, 10 mM MnCl2). Reactions were done in 25 μl of kinase buffer containing 1 μl of histone H2A (1 mg/ml) and 1 μl of [γ-32P]ATP (10 μCi/ml) and incubated at 30°C for 20 min. Reactions were terminated by addition of 3× Laemmli buffer. Samples were boiled and proteins were separated by SDS–polyacrylamide gel electrophoresis (PAGE) (12% gel). Phosphorylated PKB was visualized by autoradiography and quantified by densitometry as in the PI3K assays.
equivalent amounts of enzyme were present in each experimental point.

Cell invasion assay. Cell culture trypomastigotes were washed twice in DMEM + 10% FBS and resuspended in the same medium at 1 × 10⁷ parasites/ml. Mammalian cells were seeded on coverslips in 24-well plates and grown for 24 h. The cells (1 × 10⁴/well) were treated for 1 h at 37°C with LY 294002 (50 and 100 μM) or Wortmannin (0, 10, 100, and 500 nM). Nontreated cells as well as cells incubated with DMSO in the assay concentrations were used as controls. After extensive washing, cells were infected with trypomastigotes (1 × 10⁴/well) for 2 h at 37°C. Coverslips were washed twice with the same medium to remove unbound parasites and cells were incubated for 24 h at 37°C with 5% CO₂. To determine the percentage of infected cells, four coverslips of each point were washed twice with PBS, fixed with absolute methanol, and stained with Giemsa. Infection was determined microscopically, evaluating the percentage of infected cells containing intracellular amastigotes and counting no fewer than 500 cells at random [5].

Immunofluorescence confocal microscopy. For morphological analysis by immunofluorescence confocal microscopy, cells overexpressing either the wild type or the active or inactive mutant of PKB/Akt were grown on 12-mm round coverslips in 12-well microtest plates and transfected as described earlier [20]. After transfection the cells were fixed in 80 mM Pipes (Sigma), pH 6.8, 1 mM MgCl₂, 0.25 M sucrose, 5 mM EGTA, and 3% (w/v) paraformaldehyde. After 20-min fixation at room temperature, the cells were washed twice with PBS and then incubated for 20 min with 50 mM NH₄Cl in PBS. The coverslips were then placed with the cells facing down on Parafilm containing 30-μl droplets of 20% goat serum in PBS and incubated in a humidified chamber at room temperature for 30 min. The overexpressed tagged HA-PKB/Akt proteins were visualized with an affinity-purified mouse anti-HA monoclonal antibody, whereas T. cruzi was visualized with an anti-T. cruzi rabbit polyclonal serum. After the incubation with the first antibody, the coverslips were washed three times for 15 min with 500 μl PBS and then incubated for 30 min at room temperature with rhodamine-labeled goat anti-rabbit IgG antibodies and fluorescein isothiocyanate-labeled goat anti-rabbit IgG antibodies (both from Sigma). After the incubation with the second antibody, the coverslips were washed three times for 15 min with 500 μl PBS and mounted on 4 μl of mounting medium on a microscopy slide. Control experiments were carried out to ensure that the antibodies did not cross-react. The cells were viewed in a confocal scanning beam fluorescence microscope at excitation wavelengths of 476 and 529 nm, respectively.

Statistical analysis. Statistical analysis was determined by Student's t test and significance was considered P < 0.05.

RESULTS

PI3K Inhibitors Block T. cruzi Invasion in Host Cells

To determine the effect of the PI3K inhibitors in the invasion process of T. cruzi, macrophages and nonphagocytic cells were incubated with the PI3K inhibitors Wortmannin and LY 294002 prior to infection, as described under Materials and Methods. As shown in Fig. 1A, infection of Vero, L₆E₉, and NIH3T3 cells by trypomastigotes was greatly inhibited by pretreatment of host cells with different concentrations of LY 294002. The results showed a dose-dependent pattern of inhibition, with maximal percentages calculated between 75.5 and 96% for the three cell lines (P < 0.05). Vero and NIH 3T3 cells were the most sensitive to the effect of this inhibitor since approximately 90% inhibition was obtained with the lowest concentration tested (50 μM) (Fig. 1A). Similar results were obtained with the three cell lines when Wortmannin, the structurally unrelated PI3K inhibitor, was used (Fig. 1B). We also determined the effect of both PI3K inhibitors in the invasion process of T. cruzi in human macrophages and the murine phagocytic cell line J774. Furthermore, Wortmannin was a potent inhibitor of the invasion of human and murine macrophages by T. cruzi (Fig. 2A) and this inhibitory effect was dose dependent. Similar results were also obtained with LY 294002 in both macrophage cell types (Fig. 2B).
Activation of Host PI3K and PKB/Akt Activities by Trypomastigote Membranes

Based on the results that PI3K inhibitors blocked T. cruzi entry in host cells, we queried whether activation of this kinase could be triggered by trypomastigote membranes in contact with mammalian cells. Vero cells were stimulated with trypomastigote membranes and PI3K activity was evaluated by immunoprecipitation of cell lysates and PIP product quantitation as described under Materials and Methods. As shown in Fig. 3, contact of Vero cells with trypomastigote membranes for 10 min induced a 10-fold increase in enzyme activity compared with unstimulated cells (Fig. 3, lane A vs lane B). Treatment of cells for 1 h with 50 μM LY294002 before the addition of parasitic membranes abolished the stimulatory effect of this fraction (Fig. 3, lane C). Similar results were obtained when cells were pretreated for the same period with 10 nM Wortmannin (data not shown). Concentrations of both inhibitors, which gave the maximal inhibition of invasion (Figs. 1A and 1B), were used. In order to determine if the activation of host cell PI3K by trypomastigote membranes was specific for this infective stage, parallel experiments were performed using membranes obtained from the noninfective epimastigote stage. The results showed that these membranes were unable to induce any PI3K activation in host cells (Fig. 3, lane D), suggesting that only infective trypomastigote membranes are able to activate host cell PI3K. Similar results to these described in Fig. 3 were also obtained

**FIG. 2.** Inhibition of T. cruzi invasion in phagocytic cells by PI3K selective inhibitors. (A) J774 (■) and human (□) macrophages were incubated in DMEM alone (control) or plus 1, 10, 100, or 500 nM Wortmannin for 1 h. After incubation, cells were washed and infected with cell-cultured trypomastigotes (parasite cell ratio 10:1) for 2 h. Infection percentages were determined 24 h postinfection, as described under Materials and Methods. Values shown are the mean ± SD of three independent experiments. (B) J774 (■) and human (□) macrophages were incubated in DMEM alone (control) or plus 50 or 100 μM LY294002 for 1 h. After incubation, cells were washed and infected with cell-cultured trypomastigotes (parasite cell ratio 10:1) for 2 h. Infection percentages were determined 24 h postinfection, as described under Materials and Methods. Values shown are the mean ± SD of three independent experiments.

**FIG. 3.** T. cruzi activates PI3K in Vero cells. Cells were starved of FBS for 18 h prior to PI3K stimulation. PI3K activity was measured in control cells (lane A) or in cells (in the absence of serum) incubated for 10 min with T. cruzi membranes prepared from either trypomastigotes (lane B) or epimastigotes (lane C). In lane D, 50 μM LY294002 was added to the cells for 1 h before stimulation with T. cruzi trypomastigote membranes. After all treatments, cell lysates were prepared and were immunoprecipitated with an anti-p85 PI3K antibody. PI3K activity in the immunoprecipitate was then measured. The experiment was repeated three times with similar results. An anti-PI3K blot is shown as a loading control.
when intact parasites were incubated with host cells (data not shown).

On the other hand, to evaluate the kinetics of activation of PI3K by trypomastigote membranes compared with a normal growth factor and to determine the magnitude of the response to both stimuli, we performed a time course experiment with parasite membranes and EGF (as a PI3K known activator). The results shown in Fig. 4 indicate that parasite activation followed a time course pattern with maximal activity at 10 min; meanwhile, EGF showed a more rapid response (5 min) but of a less magnitude (100% vs 80%, respectively). The activation was time dependent and the level of PI3K at 60 min was very similar to the basal levels (data not shown).

PKB/Akt is widely accepted as a PI3K downstream target [11]. To confirm this hypothesis, the kinase activity of Vero cell PKB/Akt after stimulation with T. cruzi membranes was analyzed by immunoprecipitation with anti-PKB/Akt antibodies. The results shown in Fig. 5 indicate that, as observed with PI3K, only trypomastigote but not epimastigote membranes induced PKB/Akt activation.

In order to examine if T. cruzi invasion was also regulated by the overexpression of PKB/Akt active or inactive kinase mutants, infection experiments with further antibody labeling and confocal microscopy analysis were performed with PKB-transfected cells. The results shown in Fig. 6 indicate that the overexpression of wild-type or constitutively activated PKB/Akt increases T. cruzi invasion (P < 0.05). In contrast, the overexpression of the inactive mutant PKB/Akt significantly blocked T. cruzi invasion (P < 0.05) (Fig. 6).
DISCUSSION

Numerous studies using genetics, PI3K inhibitors, and PI3K overexpression have implicated this enzyme in the regulation of cell proliferation, survival, metabolism, cytoskeletal reorganization, and membrane trafficking [25]. One well-established target of PI3K activation is PKB/Akt, also called PKB [26, 27]. PKB/Akt is thought to play an important role in protecting cells from apoptosis and in promoting cell survival [28]. The present report provides substantive evidence for the involvement of host signal transducing molecules like PI3K and PKB/Akt during T. cruzi invasion of phagocytic and nonphagocytic cells. Our results show that the PI3K inhibitors Wortmannin and LY294002 significantly reduced the extent of T. cruzi infection in macrophagic and nonmacrophagic cells (Figs. 1 and 2). Simultaneously with the revision of this paper, Todorov et al. [29] have reported similar results, but using peritoneal macrophages. Vero cells were highly sensitive to the effects of both compounds, although the concentration necessary to inhibit at least 50% invasion was shown to be higher for T. cruzi than for Listeria monocytogenes [30]. Both PI3K inhibitors significantly reduced T. cruzi invasion in a dose-dependent fashion, as reported for L. monocytogenes [30]. In agreement with our results, Wortmannin has been shown to inhibit infection by another parasitic protozoan, Cryptosporidium parvum, in epithelial cells [31]. The observation that both compounds were strong inhibitors of parasite invasion at concentrations that are known to inhibit only PI3K [32, 33] indicates that this host enzyme could be part of a signaling pathway required for successful infection. As seen with other signal transduction molecules like Ca\(^{2+}\), the PI3K/PKB cascade could be a common pathway used by several pathogens to invade and survive within host cells. During T. cruzi infection, parasite contact with nonphagocytic cells is associated with changes in the actin cytoskeleton [7] and the migration of host cell lysosomes to the invasion site [2]. PI3K activity may be involved in the regulation of actin microfilament formation and a role of this enzyme in actin cytoskeleton reorganization cannot be excluded [9]. Activation of host PI3K leads to the production of phosphorylated PI, which can activate several downstream targets, including PKB/Akt [12, 24, 25]. Our findings showing that the activation of host PI3K and PKB/Akt by a membrane fraction isolated from the infective trypomastigotes, coupled with the absence of enzyme activation by the noninfective epimastigote stage (Figs. 3–5), suggest that the membrane fraction contains the factor(s) that facilitates T. cruzi invasion. The kinetics of the PI3K activation induced by trypomastigote membranes resulted in a pattern similar to that of a well-known activator of this enzyme as EGF, although the response to the parasitic membranes was higher and sustained during a longer period (Fig. 4). Parasitic membranes constituted a good approach to studying the biochemical changes in host cells associated with parasite invasion since activating molecules are probably located on the parasite surface. To date the trypomastigote molecules that cause the signaling processes described above are still largely unknown. We are currently investigating the nature of this factor(s).

Many signaling molecules that activate host cell intracellular cascades have been described from various pathogenic microorganisms [15–19]. Only one molecule, trans-sialidase (or gp83), has been reported to have a role in upregulation of invasion through host MAP kinases during T. cruzi infection [34].

On the other hand, parasite attachment is a complex process that leads to activation of parasitic signaling pathways as well, even within the host cell. Ca\(^{2+}\) signals have been detected in trypomastigotes when they became associated with mammalian cells [5] and these increments in Ca\(^{2+}\) flux were related to an increased infective capacity [35]. Since molecules involved in signal transduction are common in the parasite and in the host cell, we investigated T. cruzi PI3K participation in host cell invasion. Here, we show for the first time that T. cruzi PI3K-like activity is required in trypomastigotes for their invasion into host cells, since treatment of parasites with Wortmannin resulted in dose-dependent inhibition of invasion in Vero cells (Fig. 6).

Finally, in this work we have shown that T. cruzi trypomastigotes are able to activate the host PI3K/
PKB/Akt cascade. There could be two possible hypotheses for the parasite activation of these host cell signal transduction pathways. First, PI3K and PKB/Akt are known to couple the initiation of endocytic membrane trafficking via Rab 5 function, the rate-limiting endosomal GTPase [36–38]. This would explain the higher infection percentages obtained with the PKB/Akt-positive cells in which endocytic trafficking is increased. In this sense, an enhanced T. cruzi invasion has been detected in Rab-5-transfected cells (Wilkowsky et al., personal communication). Second, PKB/Akt has been shown to phosphorylate BAD [39, 40], which represents a key anti-apoptotic signal in the host cell. The upregulation of these enzymes could be considered a parasitic strategy for preserving host cell life and allowing parasites to establish a secure environment for their survival and propagation.

REFERENCES


