Novel PI 3-kinase-dependent mechanisms of trypanosome invasion and vacuole maturation

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Summary

Mammalian cell invasion by the protozoan parasite, Trypanosoma cruzi, is facilitated by the activation of host cell phosphatidylinositol 3 (PI 3)-kinases. We demonstrate that the well-characterized Ca²⁺-regulated lysosome-mediated parasite entry pathway is abolished by wortmannin pretreatment. In addition, we have characterized a novel route of T. cruzi invasion unexpectedly revealed in the course of this study. For over a decade, targeted exocytosis of lysosomes at the host cell plasma membrane was considered as the primary mechanism for T. cruzi entry into non-professional phagocytic cells. We now provide evidence that a significant fraction (50% or greater) of invading T. cruzi trypomastigotes exploit an alternate actin-independent entry pathway that involves formation of a tightly associated host cell plasma membrane-derived vacuole enriched in the lipid products of class I PI 3-kinases, PtdInsP₃/PtdIns(3,4)P₂. Initially devoid of lysosomal markers, the resultant parasite-containing vacuoles gradually acquire lysosome associated membrane protein 1 (lamp-1) and fluid phase endocytic tracer from the lysosomal compartment. In striking contrast to latex bead phagosomes, few T. cruzi vacuoles associate with the early endosomal marker, EEA1 and the ‘maturation’ process becomes refractory to PI 3-kinase inhibition immediately following parasite internalization. Jointly, these data provide a new paradigm for T. cruzi invasion of non-professional phagocytic cells and reveal a novel vacuole maturation process that appears to bypass the requirement for EEA1.

Movie available online

Key words: Phosphatidylinositol, Phagocytosis, Invasion, Trypanosoma cruzi, Maturation

Introduction

Phosphatidylinositol 3 (PI 3)-kinases are activated by a wide variety of extracellular stimuli to generate specific lipid products that provide docking/activation sites for downstream effectors (Toker and Cantley, 1997). Distinct cellular processes including membrane trafficking along the endocytic pathway (Li et al., 1995; Kjeken et al., 2001), insulin-stimulated exocytosis (Watson and Pessin, 2001), phagocytic events (Araki et al., 1996; Cox et al., 1999; Marshall et al., 2001; Vieira et al., 2001; Fratti et al., 2001) and actin remodeling (Greenwood et al., 1998; Insall and Weiner, 2001) are regulated by phosphoinositol phosphates (PtdInsP) generated by three distinct classes of PI 3-kinases. Modulation of host cell PI 3-kinase-dependent signaling by microbial pathogens is becoming recognized as an important strategy for establishment of infection and intracellular survival of these organisms (Duclos and Desjardins, 2000; Fratti et al., 2001; Coombes and Mahony, 2002). For instance, PI 3-kinase activation promotes the actin-dependent uptake of Yersinia (Schulte et al., 1998) and Listeria (Ireton et al., 1996) while enteropathogenic E. coli prevents its own phagocytic uptake by thwarting PI 3-kinase signaling in macrophages (Celli et al., 2001). Moreover, the ability of some pathogens to survive within an intracellular vacuolar compartment involves pathogen-specific strategies for manipulation of the phagosome maturation process at different stages (Xu et al., 1994; Sinai and Joiner, 1997; Scianimanico et al., 1999; Steele-Mortimer et al., 1999; Hackstadt, 2000; Fratti et al., 2001).

The protozoan parasite, Trypanosoma cruzi, invades and replicates within a wide variety of nucleated cell types, exhibiting tropism for cardiomyocytes and smooth muscle in vertebrate hosts. More than a decade ago, it was demonstrated that infective T. cruzi trypomastigotes exploit a unique actin-independent mechanism to enter non-professional phagocytes, which involves targeted exocytosis of host cell lysosomes at the site of parasite attachment (Tardieux et al., 1992). Prior to invasion, lysosomes are recruited to the plasma membrane along microtubules in a kinesin-dependent manner (Rodriguez et al., 1996), where they undergo Ca²⁺-dependent fusion (Rodriguez et al., 1997), delivering membrane required to form the nascent parasitophorous vacuole (Tardieux et al., 1992). In striking contrast to phagocytosis or other actin-driven uptake mechanisms frequently exploited by intracellular pathogens (Steele-Mortimer et al., 2000;
Materials and Methods

Antibodies

*T. cruzi* trypomastigotes were stained with a rabbit anti-*T. cruzi* antibody (Tardieux et al., 1992) or mouse monoclonal antibody (mAb) 2A1 (Andrews et al., 1987). Mouse anti-rat lamp-1 mAb LY1C6 was a generous gift from I. Mellman, Yale University. Rat anti-mouse lamp-2 (lysosome associated membrane protein 2; mAb GL2A7) and mouse anti-hamster (mAb UH1) were obtained from the Developmental Studies Hybridoma Bank, University of Iowa. Early endosomes were detected using a mouse monoclonal antibody against early endosome antigen-1 (EEA1) (BD Transduction labs). Mouse anti-fibronectin antibodies were obtained from (BD Transduction Laboratories). Texas Red (TR)-phalloidin and TR-dextran 10,000 *M* were obtained from Molecular Probes. Secondary antibody conjugates used include: Alexafluor-350, -488 or TR-conjugated anti-mouse, anti-rabbit or anti-rat IgG were obtained from Molecular Probes. Cy5-labelled goat anti-rabbit IgG was purchased from Amersham-Pharmacia.

Tissue culture and parasite maintenance

LLC-MK2, L6E9 rat myoblasts and mouse embryonic fibroblasts (MEF) were maintained in DMEM (Gibco) with 10% FBS, 1% penicillin-streptomycin, and 2 mM glutamine (DMEM-10). CHO and FcRyIII-CHO cells were maintained in α-MEM (Gibco) supplemented with 10% FBS and 1% penicillin-streptomycin. Mouse embryonic fibroblasts (MEF) were derived from embryos of *p85α−/− p85β−/−* (KO) and *p85α−/− p85β−/−* (ghet) mice (129 × C57BL/6). The generation of the *p85β−/−* mice and establishment of the embryonic fibroblasts from crosses with *p85α−/−* mice will be described in detail elsewhere (S.M.B. and L.C.C., unpublished).

Tissue culture-derived *T. cruzi* trypomastigotes (Y strain) were generated by weekly passage in confluent monolayers of LLC-MK2 cells in DMEM containing 2% FBS as described previously (Caler et al., 1998). Trypomastigotes harvested from culture supernatants were washed three times in Ringers/BSA prior to use in invasion assays as described (Tardieux et al., 1992).

Isolation of primary cardiomyocytes

Primary cultures of ventricular cardiomyocytes from 1-day-old Sprague-Dawley rats (Charles River Laboratories) were prepared as described previously (Baliga et al., 1999). Briefly, neonatal rat pups were humanely sacrificed, their hearts removed and placed into ice-cold Hanks buffered salt solution (HBSS) (Worthington Biochemical). Ventricular tissue was digested overnight in a 1% trypsin/HBSS (Sigma), washed with ice-cold HBSS and transferred into HBSS containing a 1% collagenase solution (Worthington Biochemical). Isolated cells were subjected to two rounds of pre-plating onto tissue culture-treated plastic to enrich for cardiomyocytes. Cell mixtures containing 90-95% cardiomyocytes were adjusted to the appropriate concentration in DMEM-10 containing 1% sodium pyruvate and plated on laminin-coated tissue culture plates or coverslips.

Mammalian cell transfection

Cells were seeded onto coverslips in 3.5 cm dishes at 5x10^4/mL, cultivated overnight, and transfected with 1 μg DNA by lipofection (FuGENE 6, Roche) for 3-4 hours in complete medium. Cells were washed and incubated in complete medium for an additional 8-12 hours. Constructs used in transfection include: Akt-PH-GFP and Akt-PH-GFPB25C (provided by J. Downard, ICRF, UK), PLC6-PH-GFP (provided by T. Meyer, Stanford University), 2xFYVE-GFP (provided by H. Stenmark, Norwegian Radiant Hospital, Oslo) and Rab5-GFP (from M. Desjardins, U. Montreal). Myristylated (myr)-GFP was constructed as follows: the myristylation sequence and the extreme N-terminal end (114 nucleotides) of Akt was amplified from the myr-Akt-pcDNA3 (provided by N. Hay, U. Chicago) by PCR using the following primers: forward: 5’-TTATGGGGAGCAGC- AAGAGCAAG-3’, reverse: 5’-TGTAGCCAATAAAGGTGCCAT- CGTTC-3’. PCR was performed using pfu polymerase (Strategene) with the accompanying reaction buffer under the following conditions: denaturation at 94°C, 1 minute; annealing at 50°C, 1 minute; elongation at 72°C, 2 minute; 40 cycles. The resulting PCR product was ligated into the CT-GFP Fusion TOPO cloning vector (Invitrogen) and clones were sequenced to verify orientation and continuity of the open reading frame.

Immunofluorescence

Coverslips with adherent cells were fixed in 2% paraformaldehyde (PFA)/PBS, washed with PBS and incubated for 10 minutes in 50 mM NH4Cl/PBS. Antibody dilutions, incubations and washes were carried out in TBS/BSA (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% BSA) for staining of external antigens or permeabilized with TBS/BSA/0.1% saponin to detect intracellular antigens. Antibody incubations were carried out for 40 minutes with extensive washes for
25 minutes following primary and secondary incubation steps. Parasite and mammalian DNA was stained with 1.25 μg/ml DAPI (Pierce) for 3 minutes. Coverslips were washed with PBS and mounted in 10% Mowiol (Calbiochem) containing 2.5% DABCO (1,4-diazobicyclo-[2.2.2]-octane). Fluorescence images were obtained with a Nikon TE-300 inverted epifluorescence microscope equipped with an Orca-100 CCD camera (Hamamatsu) and analyzed using MetaMorph imaging software (Universal Imaging Corporation).

Quantitative parasite invasion assay

Mammalian cells were plated onto glass coverslips in 3.5 cm² dishes at a density of 5×10⁴/ml and grown for 48 hours (~80% confluence). Freshly harvested *T. cruzi* trypomastigotes were incubated with cells in Ringers/BSA for 15-60 minutes at 37°C. Following incubation, the remaining extracellular parasites were removed by washing monolayers 3 times with ice-cold PBS and cells were fixed in 2% PFA/PBS. Immunostaining was carried out in TBS/BSA using a rabbit anti-*T. cruzi* antibody to detect extracellular parasites. Parasite and mammalian cell nuclei were visualized with 1.25 μg/ml DAPI (Pierce). The number of intracellular parasites per 200-400 mammalian cells was determined by counting cells on triplicate coverslips. Drug pretreatments of mammalian cells were as follows: 20-40 nM wortmannin for 30 minutes; 2 μM cytochalasin D for 10 minutes.

Endocytic tracer internalization assays

Chinese hamster ovary (CHO) cells (for infection and fibronectin-coated inert particle assays) or FcRγIII-CHO cells (for infection and IgG-coated inert particle assays) were grown on glass coverslips to 80% confluence (48 hours). Latex beads (3 μm; Sigma) were coated with mouse fibronectin (0.2 mg/ml; Invitrogen) in PBS at room temperature overnight or with rabbit IgG (1 mg/ml) in Ringers/BSA for 2 hours at room temperature. Beads were washed and resuspended in Ringers/BSA at a concentration of 1×10⁷ beads/ml. Latex beads or *T. cruzi* trypomastigotes were incubated with cells (under various conditions of drug pre-treatment) for 10-15 minutes at 37°C, washed extensively with Ringers/BSA to remove uninternalized beads or parasites and further incubated at 37°C for the indicated times. Immunofluorescence staining was carried out on fixed cells to distinguish the remaining extracellular latex beads (anti-fibronectin or anti-rabbit IgG) from internalized beads detected by DIC microscopy.

Endocytic tracer internalization assays

L6E9 myoblasts or CHO cells grown on glass coverslips were incubated with TR-conjugated 10,000 kDa dextran (TR-dextran) for 2 hours at room temperature overnight or with rabbit IgG (1 mg/ml) in Ringers/BSA for 2 hours at room temperature. Beads were washed and resuspended in Ringers/BSA at a concentration of 1×10⁸ beads/ml. Latex beads or *T. cruzi* trypomastigotes were incubated with cells (under various conditions of drug pre-treatment) for 10-15 minutes at 37°C, washed extensively with Ringers/BSA to remove uninternalized beads or parasites and further incubated at 37°C for the indicated times. Immunofluorescence staining was carried out on fixed cells to distinguish the remaining extracellular latex beads (anti-fibronectin or anti-rabbit IgG) from internalized beads detected by DIC microscopy.

Endocytic tracer internalization assays

L6E9 myoblasts or CHO cells grown on glass coverslips were incubated with TR-conjugated 10,000 kDa dextran (TR-dextran) (Molecular Probes) at 0.5 mg/ml in DMEM with 10% FBS for 3 hours at 37°C, followed by washing and a 3-hour chase in DMEM-10 prior to infection with *T. cruzi* trypomastigotes. TR-dextran-loaded cells were infected with *T. cruzi* and processed as described above. For some experiments, cells were washed extensively following parasite internalization and fresh medium added with or without 40 nM wortmannin. Coverslips were removed at 10 minute intervals and fixed with 2% PFA/PBS and immunofluorescence staining carried out as described above.

Supplemental movie

*T. cruzi* infection of Akt-PH-GFP-expressing L6E9 myoblasts

Cells seeded onto 25 mm² glass coverslips at a density of 5×10⁴/ml were transfected by lipofection with an Akt-PH-GFP construct as described above. Coverslips were washed with warm Ringers/BSA and placed in a temperature-controlled chamber held at 37°C (Nikon). Transfected cells were located in the field using 100× objective and a narrow band pass GFP filter (ex: 480 nm; em: 510 nm, Chroma Technologies) and fluorescent images were collected with a Nikon TE-300 inverted fluorescence microscope equipped with an Orca-100 CCD camera (Hamamatsu) at time-lapse intervals of 5 seconds using a computer-controlled shutter system (Sutter) controlled by MetaMorph imaging software (Universal Imaging). Several images were taken prior to the addition of 5×10⁷ *T. cruzi* trypomastigotes in Ringers/BSA. Time-lapse image files were pseudocolored and converted to a QuickTime movie (1 frame/3 second). Selected frames were saved separately, transferred to Adobe Photoshop 6.0 (Adobe Systems) to generate the composite Fig. 2.

Results

Rapid accumulation of host cell PtdIns(3,4,5)P₃/PtdIns(3,4)P₂ on the nascent *T. cruzi* vacuole during cell entry.

To gain insight into host cell PI 3-kinase signaling events triggered early in the *T. cruzi* entry process, we examined the distribution of the lipid products of class I PI 3-kinases, PIP₂/PtdIns(3,4)P₂, in CHO cells expressing the pleckstrin homology (PH) domain of Akt fused to GFP (Akt-PH-GFP). In response to infective *T. cruzi* trypomastigotes, cytosolic Akt-PH-GFP is recruited to the site of trypomastigote entry where intimate association with invading (Fig. 1A) and recently internalized parasites (Fig. 1B) is observed. Localized membrane targeting of Akt-PH-GFP in response to *T. cruzi* required a functional PH domain (Fig. 1C) and was sensitive to wortmannin pretreatment (Fig. 1D) as expected (Watton and Downward, 1999). Changes in PtdInsP₃/PtdIns(3,4)P₂

![Fig. 1. Rapid accumulation of PtdIns(3,4,5)P₃/PtdIns(3,4)P₂ at the *T. cruzi* invasion site. Fluorescence images of (A) CHO cells or (B) primary rat cardiomyocytes transiently expressing Akt-PH-GFP following incubation with infective *T. cruzi* trypomastigotes for 15 minutes. (C) *T. cruzi* invasion of CHO cells expressing Akt-PH-GFPR25C which fails to bind to PtdIns(3,4,5)P₃/PtdIns(3,4)P₂. Extracellular parasites were stained with *T. cruzi*-specific antibodies in non-permeabilized cells. Host cell and parasite DNA were visualized with DAPI (A-C, blue). (D) Recruitment of Akt-PH-GFP to membranes surrounding invading *T. cruzi* trypomastigotes is sensitive to wortmannin. The relative number of Akt-PH-GFP-associated parasites was determined in CHO cells infected for 15 minutes following treatment with 20 nM wortmannin or vehicle control for 30 minutes. Data are represented as means ± s.d. n=3.](image-url)
distribution in response to *T. cruzi* were monitored using time-lapse fluorescence microscopy. Images were acquired prior to, and following, addition of infective trypomastigotes to myoblasts transiently expressing Akt-PH-GFP (Fig. 2; see movie: http://jcs.biologist.org/supplemental/). It is important to note that *T. cruzi* trypomastigotes are highly motile organisms that initiate host cell attachment and invasion by gliding over the cell surface to promote interaction of parasite ligands with host receptors (reviewed by Burleigh and Woolsey, 2002). While the abundance of motile parasites precluded the simultaneous acquisition of informative DIC images, the movement of trypomastigotes along the top of GFP-expressing cells, in a similar focal plane, produced a ‘shadow’ and permitted detection of cell-proximal parasites; two such parasites are highlighted in this time-lapse series (Fig. 2; see movie: http://jcs.biologist.org/supplemental/). Recruitment of Akt-PH-GFP to host cell membranes at the site of parasite contact is a gradual process with appreciable accumulation beginning ~2 minutes after interaction is initiated (Fig. 2, arrowheads). Within 5 minutes, the trypomastigote becomes completely enveloped in host cell membrane that is highly enriched in PtdIns(3,4)P_2 (Fig. 2, 13.6-16.3 minutes). Although not obvious from the static images, one of the cell-associated parasites (Fig. 2, arrow) initiates PI 3-kinase signaling then dissociates from the cell, moving out of the field of view (no parasite is present in the 8.3 minute frame). By 9.4 minutes another, or possibly the same, parasite associates specifically with this activated region of the host cell membrane, promoting additional Akt-PH-GFP recruitment. While the fate of this parasite was not determined, it clearly fails to become engulfed in PtdInsP_3/PtdIns(3,4)P_2-enriched membranes and Akt-PH-GFP dissociates from the membrane. These observations highlight the heterogeneity inherent in the early interactions of live motile *T. cruzi* trypomastigotes with mammalian cells and indicate that a series of reversible binding events take place which can trigger activation of class I PI 3-kinases and localized accumulation of PtdInsP_3/PtdIns(3,4)P_2 at the parasite attachment site.

**Plasma membrane invagination defines a novel actin-independent pathway for *T. cruzi* entry.**

Given earlier observations that *T. cruzi* invasion of non-professional phagocytic cells involves targeted fusion of host cell lysosomes with the plasma membrane (Tardieux et al., 1992), we predicted that Akt-PH-GFP, which is rapidly recruited to nascent *T. cruzi* vacuoles, would colocalize with host cell lysosomal markers on the parasite vacuole. Despite our ability to detect lysosome association with *T. cruzi* early in the infection process (Fig. 3A,B), overlap of lamp-1 staining with Akt-PH-GFP on the *T. cruzi* vacuole was not observed (Fig. 3C,D). While several instances of lysosome clustering near Akt-PH-GFP-positive *T. cruzi* vacuoles was observed at 15 minutes post-infection, (Fig. 3C,D), the distribution of these two markers remained distinct in control (not shown) and infected cells. Similarly, delivery of endocytosed fluid phase markers from Texas Red (TR)-dextran-loaded lysosomes to Akt-PH-GFP-positive *T. cruzi* vacuoles was not detected (not shown). By 60 minutes post-infection, the majority of parasite-containing vacuoles were lamp-1-positive (Fig. 3E,F), and minimal residual Akt-PH-GFP, which is rapidly recruited to nascent membrane-parasite interface prior to and during trypomastigote invasion suggests that invading this time-lapse series (Fig. 2; see movie: http://jcs.biologist.org/supplemental/).
parasites associate with host-cell plasma membrane as a first step in an alternate mode of entry, prior to interaction with the lysosomal compartment.

To examine this possibility, short-term invasion experiments (15 minutes) were carried out using CHO cells transiently expressing myristoylated GFP (myr-GFP) or the chimeric PtdIns(4,5)P_2-binding protein, PLCδ-PH-GFP, both of which associate predominantly with the plasma membrane (Stauffer et al., 1998; McCabe and Berthiaume, 2001). Similar to the interaction of trypomastigotes with Akt-PH-GFP-enriched membranes, intimate association of invading (Fig. 4A,B) and recently internalized parasites (Fig. 4C) with the plasma membrane markers is observed. Intimate association of invading trypomastigotes with the host cell plasma membrane is suggestive of molecular interactions between T. cruzi surface ligands and host cell receptors and is supported by the exclusion of membrane-impermeant fluorescent dextran from the nascent T. cruzi vacuole (data not shown). Although these events resemble actin-dependent ‘zippering’ processes that occur during receptor-mediated phagocytosis of inert particles (Swanson and Baer, 1995) or uptake of some bacterial pathogens (Mengaud et al., 1996; Cossart and Lecuit, 1998; Isberg et al., 2000; Kwok et al., 2002), several observations indicate that host cell actin polymerization is not required for this novel route of T. cruzi entry. First, examples of F-actin co-localization with plasma membrane-associated T. cruzi trypomastigotes were rarely observed (Fig. 4D-F), contrasting markedly with the intense actin staining around latex beads during uptake into cells (Fig. 4G). In addition, enhancement of parasite internalization following disruption of the host cell actin cytoskeleton (Fig. 4H) is a well-documented feature of the T. cruzi invasion process (Tardieux et al., 1992; Rodríguez et al., 1995; Kima et al., 2000). Furthermore, we find that cytochalasin D pretreatment failed to inhibit the early association of invading parasites with plasma membrane markers (Fig. 4I). Together, these findings provide the first demonstration that T. cruzi trypomastigotes enter non-professional phagocytic cells by two distinct actin-independent routes: the classic lysosome-mediated entry pathway and a novel pathway that involves intimate association with host cell plasma membrane markers as an initial step in cell invasion. Moreover, as ~50% of invading or recently internalized parasites were in plasma membrane-derived vacuoles (Fig. 1D; Fig. 4I) this novel pathway constitutes a major route of T. cruzi entry into non-professional phagocytic cells.

**Fig. 3.** Lamp-1 association with the Akt-PH-GFP-enriched T. cruzi vacuole occurs after parasite entry. (A,B) Immunofluorescence staining of lamp-1 (red) in T. cruzi-infected CHO cells at 15 minutes post-infection showing diffuse (arrows) and punctuate (arrowheads) staining patterns outlining intracellular parasites. External parasites stained with T. cruzi-specific antibodies (green); parasite and mammalian DNA stained with DAPI (blue). (C-F) L6E9 myoblasts expressing Akt-PH-GFP (green), were infected with T. cruzi for 15 minutes (C,D) or 60 minutes (E,F) and stained with anti-lamp-1 (red) and DAPI (blue). No overlap of Akt-PH-GFP and lamp-1 staining is observed (C-F; merge).

**Transient interaction of T. cruzi vacuoles with the early endocytic pathway is minimal**

To determine if trypomastigotes that first enter cells in a plasma membrane-derived vacuole interact with components of the endocytic pathway, fluorescence microscopy was carried out to examine T. cruzi co-localization with early endosomal markers (Fig. 5). Rab5-GFP transiently expressed in CHO cells was found to associate with T. cruzi trypomastigotes during the entry process (Fig. 5A) and immediately following internalization (Fig. 5B,C). These observations, which support recent findings (Wilkowski et al., 2002), suggest that rapid recruitment of Rab5 to the site of parasite entry may direct downstream signaling/trafficking events required for T. cruzi invasion. However, a small fraction of invading parasites were found to associate with Rab5 during entry (<10%). Association of the PI3P-binding 2xFYVE-domain-GFP chimera (Fig. 5D,E) or endogenous EEA1 (Fig. 5F) with T. cruzi was only detected following complete internalization of the parasite (Fig. 5E,F). These observations suggest that EEA1/2xFYVE-domain recruitment to the T. cruzi vacuole occurs at a step subsequent to Rab5 association and formation of the vacuole as shown previously (Lawe et al., 2002).

Quantitation of the association of intracellular parasites with EEA1 or lamp-1 revealed that within the first 10 minutes of infection, ~20% of the T. cruzi-containing vacuoles were EEA1-positive and a similar number were associated with lamp-1 (Fig. 5G). As intracellular infection progressed, under...
Fig. 4. T. cruzi associates with host cell plasma membrane markers in an actin-independent entry process. (A,B) CHO cells transfected with myr-GFP were infected with T. cruzi trypomastigotes for 15 minutes. The extracellular region of partially internalized parasites was stained with a T. cruzi-specific antibody followed by TR-conjugated secondary antibody (arrowheads) highlighting the ability of T. cruzi to enter cells either (A) posterior end (DAPI-stained, blue) or (B) anterior end first. (C) Fully internalized trypomastigote in CHO cells expressing PLC8-PH-GFP 15 minutes after infection. (D) Akt-PH-GFP-expressing L6E9 infected with T. cruzi for 15 minutes were stained with TR-phalloidin (E; arrowhead). (F) Partial co-localization of T. cruzi-associated Akt-PH-GFP and F-actin is observed (merged image). (G) Phagocytic uptake of IgG-coated 3 μm latex beads by FCRγIII-CHO cells stained with TR-phalloidin (arrowheads). (HI) CHO cells transfected with PLC8-PH-GFP were pre-treated with 10 μM cytochalasin D or vehicle control for 10 minutes, infected with trypomastigotes for 15 minutes and the number of intracellular parasites (H) and the percentage of intracellular parasites associating with PLC8-PH-GFP (I) was determined. Data are represented as means ± s.d. for 3 coverslips. Scale bars: 5 μm.

conditions where no additional invasion occurred, a decrease in EEA1 association coincided with increased lamp-1 acquisition by the T. cruzi vacuole (Fig. 5G). To compare these events to phagosome maturation in the same cell type, we examined the kinetics of EEA1 and lamp-1 association with phagosomes formed following internalization of fibronectin (FN)-coated 3 μm latex beads (LB) (Fig. 5H). While generally similar, important differences are noted when comparing these two processes. Most striking is the relative ability of newly formed vacuoles to associate with EEA1. Only ~20% of T. cruzi vacuoles are EEA1-positive at early time points of infection (10-20 minutes) (Fig. 5F), whereas ~70% of LB phagosomes associate with this early endosomal marker shortly after internalization (Fig. 5H). Association with EEA1 is transient and lamp-1 is gradually acquired by both T. cruzi vacuoles and LB phagosomes (Fig. 5G,H). Few LB phagosomes are lamp-1-positive at 10 minutes whereas ~20% of internalized parasites associate with lamp-1 at this early time point, probably representing the fraction of parasites that have gained access to the host cell via the lysosome recruitment/fusion pathway. These data indicate that a minor fraction of the total invading parasites exploit the lysosome-mediated entry pathway for invasion. This finding is consistent for several different cell types examined. In CHO, L6E9 myoblasts, mouse embryonic fibroblasts, as well as normal rat kidney fibroblasts (NRK), the cell line in which the lysosome recruitment/fusion mechanism of T. cruzi entry was originally described (Tardieux et al., 1992), the relative number of lamp-positive T. cruzi vacuoles ranged from ~15-25% at 15 minutes post-infection. Thus, utilization of a novel alternative route of entry appears to be a common mechanism of T. cruzi invasion of a variety of non-professional phagocytic cells.

PLC8 3-kinase inhibition blocks lysosome association with invading T. cruzi trypomastigotes

Inhibition of host cell PLC 3-kinases significantly impairs T. cruzi invasion (Fig. 6A) as previously described (Wilkowsky et al., 2001). Since our data clearly demonstrate that this pathogen exploits two distinct routes to enter non-professional phagocytic cells, we examined the effect of wortmannin pretreatment on the relative ability of T. cruzi to immediately associate with lysosomal (lamp-1) or plasma membrane markers. Strikingly, early lamp-1 association with invading or intracellular parasites was completely abolished by this treatment, strongly suggesting that host cell PLC 3-kinase activity is required for the lysosome-mediated entry pathway. In contrast, the relative ability of trypomastigotes to associate with the plasma membrane marker PLC8-PH-GFP was unchanged (~50% of total cell-associated parasites) in wortmannin pretreated cells (Fig. 6B). A fraction (~22%) of intracellular parasites is not associated with either marker in untreated cells, which may reflect the population associated with early endosomes (Fig. 5G). Since this fraction increases upon inhibition of PLC 3-kinase activity, it appears as though the dynamics of the T. cruzi interaction with this compartment are significantly altered by wortmannin pretreatment, possibly impacting parasite progression into, or out of, this compartment.

To specifically address the role of class I PLC 3-kinases in the lysosome-mediated entry pathway, we examined the relative ability of T. cruzi to associate with lamp-2 in mouse embryonic fibroblasts (MEF) lacking α and β isoforms of p85 (p85αβ−β−; ‘KO’) as compared to MEF heterozygous for p85α (p85αβ−β−; ‘αHET’) (S.M.B. and L.C.C., unpublished). Although a dramatic reduction (70%) in infectivity of p85-deficient fibroblasts was observed (Fig. 6C), the immediate association of T. cruzi with host cell lysosomes was not abrogated in these fibroblasts (Fig. 6D). In addition, the kinetics of subsequent lamp-2 acquisition by the T. cruzi...
PI 3-kinase-dependent trypanosome invasion

vacuole was not altered in cells lacking p85 expression (Fig. 6D). Overall, these data demonstrate that disruption of PI 3-kinase signaling in host cells, either using membrane-permeant drugs or abrogation of signaling through class I PI 3-kinas, leads to a dramatic reduction (>60%) in the ability of *T. cruzi* to infect host cells and indicates that both routes of *T. cruzi* entry are affected by PI 3-kinase inhibition. While the lysosome-mediated entry pathway is exquisitely sensitive to wortmannin-pretreatment, signaling through class I PI 3-kinase is not required for this route of entry, and suggests that other wortmannin-sensitive PI 3-kinas are involved in this process. However, the potential contribution of an alternatively spliced form of p85 (p55γ) which is still expressed in p85-deficient fibroblasts (S.M.B. and L.C.C., unpublished) cannot be excluded.

*Fig. 5*. Early association of *T. cruzi* with early endosomes is minimal and precedes lamp-1 acquisition. Immunofluorescence staining of extracellular *T. cruzi* (arrowheads) following infection of CHO cells transiently expressing (A–C) Rab5-GFP or (D,E) 2×FYVE-GFP for 7.5 minutes. Host cell and parasite DNA is visualized with DAPI (blue). (F) Immunostaining of endogenous EEA1 in *T. cruzi*-infected CHO cells (15 minutes post-infection). (G) *T. cruzi* trypomastigotes or (H) fibronectin-coated 3 µm latex beads, incubated with L6E9 myoblasts for 10 minutes were washed extensively to remove uninternalized beads or parasites and further incubated for a total of 60 minutes. Three samples from each condition were fixed at 10, 20, 30 45 and 60 minutes post-incubation and immunostained for EEA1 or lamp-1. Data are represented as means ± s.d.; n=3. Scale bars: 3 µm.

*Fig. 6*. Inhibition of host cell PI 3-kinas during *T. cruzi* invasion blocks the lysosome-mediated entry pathway. (A,B) CHO cells expressing PLCδ-PH-GFP were treated with 40 nM wortmannin or vehicle control for 30 minutes prior to incubation with trypomastigotes for 15 minutes. Fixed permeabilized cells were stained with antibodies to lamp-1. The relative number of (A) intracellular parasites and (B) lamp-1 or PLCδ-PH-GFP-positive intracellular parasites was determined. (C) The relative number of intracellular parasites determined following *T. cruzi* infection of p85<sup>α</sup>β<sup>+</sup> (αHET) and p85<sup>α</sup>β<sup>–</sup> (KO) MEFs 15 minutes. (D) Kinetics of lamp-2 association with intracellular *T. cruzi* in αHET and KO cells at 10, 20, 30, 45, and 60 minutes post-infection. Data are represented as means ± s.d.; n=3.

*T. cruzi* vacuole maturation is distinct from phagosome maturation

*T. cruzi*-containing vacuoles formed by plasma membrane invagination transiently associate with early endosomal markers and gradually acquire lysosomal markers in a process that is reminiscent of phagosome maturation (Desjardins et al., 1994). A critical step in the phagosome maturation process is the generation of PtdInsP<sub>3</sub> at the phagosome membrane following recruitment of the class III PI 3-kinase, VPS34 (Vieira et al., 2001), such that acquisition of lysosomal markers by the phagosome can be efficiently blocked by the addition of PI 3-kinase inhibitors immediately following particle uptake (Fratti et al., 2001). To examine whether *T. cruzi* vacuole maturation is distinct from phagosome maturation.
*T. cruzi* vacuole maturation is sensitive to PI 3-kinase inhibition, wortmannin was added to cells at 10 minutes post-infection. To our surprise, wortmannin failed to alter the kinetics of either lamp-1 (Fig. 7A) or endocytosed TR-dextran (Fig. 7B) acquisition by the *T. cruzi* vacuole, despite the ability of this treatment to block EEA1 association with the *T. cruzi* vacuole (not shown). Control experiments clearly demonstrate that wortmannin effectively arrests maturation of phagosomes formed following uptake of IgG-coated latex beads by FcRIII-CHO cells (Fig. 7C). Similar results were obtained with LY492002 (data not shown). These findings highlight fundamental differences in the maturation of *T. cruzi* vacuoles compared to latex bead phagosomes and suggest that acquisition of lysosomal markers by the *T. cruzi* vacuole may occur by an unprecedented PI 3-kinase-independent pathway.

To explore this possibility, we exploited the ability of wortmannin to block the lysosome-mediated entry pathway (Fig. 6B), which provided a useful approach to specifically examine the maturation of *T. cruzi* vacuoles compared to latex bead phagosomes formed following internalization at the plasma membrane. In cells pretreated with wortmannin, the kinetics of lamp-1 acquisition mimics that observed for untreated control cells, except that the ‘set point’ is lower because of the absence of lysosome-mediated entry (Fig. 7D; pre). This result can be interpreted in two ways: either lamp-1 acquisition by the *T. cruzi* vacuole is completely refractory to PI 3-kinase inhibition or cells are recovering from drug pretreatment. To distinguish between these possibilities experiments were carried out in which wortmannin was added back to pre-treated cells following parasite internalization for 10 minutes to prevent recovery (Fig. 7D; pre + post). Under conditions of continued PI 3-kinase inhibition, lamp-1 acquisition by the *T. cruzi* vacuole was completely abolished, indicating that an early PI 3-kinase-dependent step is clearly required for *T. cruzi* vacuole maturation. However, this step becomes refractory to inhibition within 10 minutes of parasite entry in marked contrast to phagosome maturation as shown here and previously (Fratti et al., 2001).

**Discussion**

Targeted lysosome exocytosis at the site of *T. cruzi* trypomastigote attachment to host cells has long been considered to be the predominant mechanism by which this intracellular pathogen gains access to non-professional phagocytic cells (Tardieux et al., 1992). In the present study, we have made the surprising discovery that the majority of invading trypomastigotes do not rely on lysosome recruitment to deliver the membrane necessary for vacuole formation. Instead, these highly motile parasites penetrate cells by inducing plasma membrane invagination in a process that is facilitated by disruption of host cell actin microfilaments. The resulting *T. cruzi*-containing vacuole is enriched in the lipid products of class I PI 3-kinases, PtdInsP3/PtdIns(3,4)P2, and devoid of lysosomal markers. Exploitation of this lysosome-independent entry pathway by *T. cruzi* was evident in several different non-professional phagocytic cell types including primary cardiomyocytes, a highly relevant cell type for *T. cruzi* infection and pathology in the host.

A critical question raised by our findings is how such a prominent pathway for *T. cruzi* invasion escaped earlier detection. First, novel use of plasma membrane-GFP markers coupled with time-lapse fluorescence imaging in the present study afforded a definitive view of the early interactions of *T. cruzi* trypomastigotes with the host cell plasma membrane. This methodology presented a clear advantage over previous attempts to localize surface-targeted membrane proteins in the nascent *T. cruzi* vacuole by immunofluorescence staining (Procipio et al., 1999; Kima et al., 2000). Secondly, the original paper describing the unusual lysosome-mediated entry pathway failed to report the frequency of this event (Tardieux et al., 1992). On the contrary, quantitation is emphasized in our study where we consistently find that the fraction of intracellular trypomastigotes that immediately associates with host cell lysosomes is minimal (~20%). While we cannot absolutely rule out the possibility that the fluorescence visualization techniques used to detect lamp-1 and TR-dextran

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**Fig. 7.** *T. cruzi* vacuole maturation involves an early PI 3-kinase-dependent step. CHO cells were pulsed with (A,B) *T. cruzi* or (C) IgG-coated latex beads for 10 minutes, washed extensively and incubated in the absence or presence of 40 nM wortmannin for a total of 10, 20, 30, 45 and 60 minutes. (D) Kinetics of lamp-1 association with the *T. cruzi* vacuole in untreated CHO cells (control), cells pretreated with 40 nM wortmannin for 30 minutes (wortmannin-pre) or in wortmannin pretreated cells to which drug was added after 10 minutes of infection (wortmannin pre+post). Data are represented as means ± s.d.; n=3.
association with the *T. cruzi* vacuole are inefficient and result in an underestimation of the number of parasites, we are able to account for ~90% of the intracellular parasites: (50% plasma membrane; 20% lysosome; 20% EEA1) shortly after *T. cruzi* internalization. Thus, our data do not support gross under-representation of lysosome-associated parasites. Moreover, the existence of a significant, lysosome-independent route for *T. cruzi* entry may offer an explanation for previous observations showing that interference with the function of synaptotagmin VII, an important component of Ca\(^{2+}\)-regulated lysosome exocytosis machinery (Reddy et al., 2001), resulted in an ~90% inhibition of Ca\(^{2+}\)-dependent lysosome exocytosis (Martinez et al., 2000), while the effect on *T. cruzi* invasion was considerably less profound (Caler et al., 2000).

A consistent feature of *T. cruzi* invasion of non-professional phagocytic cells is facilitation of the process by disruption of the host cell actin cytoskeleton (Tardieux et al., 1992; Rodriguez et al., 1995; Kima et al., 2000). While the existence of an actin-associated pathway for *T. cruzi* invasion of non-professional phagocytic cells has also been suggested (Procopio et al., 1999; Rosestolato et al., 2002), we find that F-actin co-localization with invading trypomastigotes is minimal and that envelopment of infective *T. cruzi* trypomastigotes in a confined plasma membrane-derived vacuole does not require host cell actin polymerization. On the contrary, the overall capacity for cell invasion by *T. cruzi* is enhanced by cytochalasin D pretreatment, in support of previous reports (Tardieux et al., 1992; Rodriguez et al., 1995; Kima et al., 2000). Time-lapse fluorescence imaging showed motile trypomastigotes penetrating the cell at an activated region of the plasma membrane where the lipid products of class I PI 3-kinases accumulate. As signaling through class I PI 3-kinases has been implicated in the reorganization of actin microfilaments (Greenwood et al., 1998; Hooshmand-Rad et al., 2000) it is possible that this early parasite-induced signaling pathway may participate in actin remodeling events during the cell invasion process. It has already been established that intracellular Ca\(^{2+}\) transients triggered in host cells by infective *T. cruzi* trypomastigotes results in a rapid, transient disruption of the cortical actin cytoskeleton (Rodriguez et al., 1995) while the role of PI 3-kinases in this process remain to be determined. Given the dramatic reduction of *T. cruzi* invasion of fibroblasts and myoblasts following inhibition of host cell Ca\(^{2+}\) signaling (Tardieux et al., 1994; Rodriguez et al., 1995) and class I PI 3-kinase-dependent signaling, it is likely that both of these early signaling pathways function as global regulators of the *T. cruzi* invasion process. However, as class I PI 3-kinases are not strictly required for lysosome-mediated entry, despite the exquisite sensitivity of this process to wortmannin pretreatment, our findings suggest that preferential activation of class I PI 3-kinase signaling may favor entry by the plasma membrane invagination route and activation of a distinct wortmannin-sensitive pathway may direct entry through the targeted lysosome exocytosis.

The molecular events regulating the *T. cruzi*-dependent activation of class I PI 3-kinases have yet to be established. However, the intimate association of invading trypomastigotes with PI(3,4)P\(_2\)/PI(3,4,5)P\(_3\)-enriched host cell plasma membrane is suggestive of specific ligand-receptor interactions which directly or indirectly trigger PI 3-kinase activation. Obvious candidates for such signaling ligands are members of the *T. cruzi* trans-sialidase/gp85 family of surface glycoproteins (Pereira-Chioccola and Schenkmam, 1999).

These abundant parasite proteins have been implicated in mediating attachment of *T. cruzi* to mammalian cells (Magdesian et al., 2001; Ming et al., 1993; Pereira et al., 1996) and stimulation of PI 3-kinase/Akt-dependent pro-survival pathways in neuronal cells was shown to be mediated by trans-sialidase (Chuenkova et al., 2001; Chuenkova and Pereira, 2000). Although the complexities of the early interactions of *T. cruzi* trypomastigotes with mammalian host cell interactions are still emerging, the extent to which specific early signaling pathways are engaged by this parasite during the infective process are likely to have important downstream consequences with respect to regulation of host cell gene expression (Vaena de Avalos et al., 2002) and intracellular survival (Chuenkova et al., 2001).

Transient residence within an acidic, lysosomal compartment is a prerequisite for *T. cruzi* progression into the host cell cytosol where replication takes place (Andrews et al., 1990; Ley et al., 1990). This requirement is fulfilled directly when parasites enter host cells using the lysosome recruitment pathway (Tardieux et al., 1992) and indirectly following the alternate route of entry described here. Parasites that first enter cells in a plasma membrane-derived vacuole gradually accumulate the lysosomal markers, lamp-1 and endocytosed TR-dextran, with kinetics similar to those observed for the maturation of latex bead phagosomes. These results predict that acquisition of lysosomal markers by the *T. cruzi* vacuole would follow the ordered process reported for the maturation of latex bead phagosomes (Desjardins et al., 1994). Nascent phagosomes undergo a series of transient fusion and fission events with early and late endosomes, progressively assuming the properties of a degradative lysosome (Desjardins et al., 1994; Duclos et al., 2003). Recent studies have identified the generation of PI(3)P on the phagosome membrane, through the activity of the class III PI 3-kinase, VPS34 (Vieira et al., 2001) and the subsequent recruitment of the Rab5 effector protein, EEA1, via its PI3P-binding FYVE domain (Stenmark et al., 1996; Lawe et al., 2000), as critical steps in the maturation process (Fratti et al., 2001; Vieira et al., 2001). Phagosome maturation can be effectively blocked by microinjection of antibodies to EEA1 or VPS34 or by the addition of PI 3-kinase inhibitors to cells shortly after phagocytic uptake (Fratti et al., 2001). The importance of EEA1 in phagosome maturation is underscored in studies of the mycobacterial vacuole. This intracellular pathogen survives in cells by avoiding fusion with host lysosomes (Sturgill-Koszycki et al., 1994; Clemens and Horwitz, 1995), exploiting a strategy whereby EEA1 is excluded from the mycobacterial vacuole (Fratti et al., 2001).

While *T. cruzi*-containing vacuoles associate with the early endosomal markers, Rab5 and EEA1, early in the infective process, our data strongly suggest that EEA1 recruitment to the parasite vacuole is not a strict requirement for progression to a lysosomal compartment. Relatively few *T. cruzi* vacuoles become EEA1 positive (~20%) following infection of myoblasts as compared to latex bead phagosomes (~70%) in the same cell type. Furthermore, the kinetics of lamp-1 and lysosomal TR-dextran acquisition by the *T. cruzi* vacuole was found to be insensitive to PI 3-kinase inhibition by
concentrations of wortmannin that disrupt EEA1 localization with the parasite vacuole. In contrast, maturation of latex bead phagosomes in CHO cells was significantly attenuated by the addition of wortmannin, similar to that shown previously for macrophages (Fratti et al., 2001). Additional investigation of the T. cruzi vacuole maturation process exposed the requirement for a very early wortmannin-sensitive step that was only effectively blocked with a combination of wortmannin pretreatment of cells and post-treatment following parasitae internalization. Further studies are required to delineate the early molecular events that regulate T. cruzi vacuole maturation; our data strongly suggest that this process is clearly distinct from classical phagosome maturation with respect to interactions with the early endosomal compartment as well as susceptibility to PI 3-kinase inhibition. Since immediate association of invading trypomastigotes with host cell lysosomes (i.e. the lysosome-mediated entry pathway) is also blocked by wortmannin pretreatment, PI 3-kinases are implicated in this entry pathway as well, although neither process appears to rely on class I PI 3-kinases. These findings suggest that lysosome-dependent entry and T. cruzi vacuole maturation may be mechanistically related, temporally distinct processes.

In this study we have defined a novel pathway for T. cruzi trypomastigotes entry and a unique vacuole maturation process that appears to bypass the requirement for association with EEA1. While some pathogens skillfully avoid fusion with lysosomes, entry into and escape from this acidic compartment is a prerequisite for T. cruzi intracellular survival (Ley et al., 1990). Thus, it is tempting to speculate that T. cruzi-induced signaling pathways may accelerate or even bypass the early maturation steps to ensure its successful delivery to lysosomes. With an increased understanding of the complexities of pathogen-specific strategies for intracellular survival (Fratti et al., 2001; Coppens et al., 2000; Ghigo et al., 2002), it is becoming difficult to retain the classical concept of phagosome maturation (Desjardins et al., 1994). Instead, the mechanisms by which pathogen-containing vacuoles interact with intracellular compartments (or markers for these compartments) suggest that intracellular pathogens and the maturation process as a network. The challenge and excitement will be to elucidate the molecular mechanisms underlying this subversive activity that holds great potential to reveal novel regulators of the endocytic trafficking in mammalian cells.

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