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Oligopeptidase B-dependent signaling mediates host cell invasion by *Trypanosoma cruzi*

Elisabet V.Caler, Silvia Vaena de Avalos[1], Paul A.Haynes[2], Norma W.Andrews[3] and Barbara A.Burleigh

Department of Cell Biology, Yale University School of Medicine, 333 Cedar Street, New Haven, CT 06520, [2]Department of Molecular Biotechnology, University of Washington School of Medicine, Seattle, WA, USA and [1]Departmento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina

[3]Corresponding author
e-mail: norma_andrews@qm.yale.edu

Mammalian cell invasion by the intracellular protozoan parasite *Trypanosoma cruzi* is mediated by recruitment and fusion of host cell lysosomes, an unusual process that has been proposed to be dependent on the ability of parasites to trigger intracellular free calcium concentration ($[Ca^{2+}]_i$) transients in host cells. Previous work implicated the *T.cruzi* serine hydrolase oligopeptidase B in the generation of Ca^{2+}-signaling activity in parasite extracts. Here we show that deletion of the gene encoding oligopeptidase B results in a marked defect in host cell invasion and in the establishment of infections in mice. The invasion defect is associated with the inability of oligopeptidase B null mutant trypomastigotes to mobilize Ca^{2+} from thapsigargin-sensitive stores in mammalian cells. Exogenous recombinant oligopeptidase B reconstitutes the oligopeptidase B-dependent Ca^{2+} signaling activity in null mutant parasite extracts, demonstrating that this enzyme is responsible for the generation of a signaling agonist for mammalian cells.

Keywords: Ca^{2+} signaling/cell invasion/oligopeptidase B/parasite/*Trypanosoma cruzi*

Introduction

Cell invasion is an essential step toward the establishment of infection by intracellular pathogens in susceptible hosts. Recent evidence indicates that in many instances a key feature of the invasion process involves signaling between pathogens and host cells. Insights into the complexity of early signal transduction events triggered in mammalian host cells by pathogen–host cell interactions have come primarily from studies with enteric bacterial pathogens (for reviews see Galan and Bliska, 1996; Falkow, 1997; Finlay and Falkow, 1997). However, mounting evidence indicates that signaling also plays a major role in the interaction of protozoan parasites with their host cells (Moreno *et al*., 1994; Ming *et al*., 1995; Schofield and Tachado, 1996; Gazzinelli *et al*., 1997; Burleigh and Andrews, 1998).

Trypanosoma cruzi, the causative agent of Chagas' disease in humans, is an intracellular protozoan parasite with the ability to invade a wide variety of mammalian cells. Entry of *T.cruzi* into non-phagocytic cells takes place by an unusual mechanism, which involves recruitment and fusion of host lysosomes at the invasion site (Tardieux *et al*., 1992; Rodríguez *et al*., 1996). The highly localized clustering of host cell lysosomes that occurs at the parasite attachment site (Tardieux *et al*., 1992) strongly suggests a role for signal transduction events. Recent evidence indicates that an increase in host cell intracellular free calcium concentration ($[Ca^{2+}]_i$) is required for lysosome-mediated *T.cruzi* entry (Tardieux *et al*., 1994; Rodríguez *et al*., 1995, 1997). Infective *T.cruzi* trypomastigotes trigger transient increases in the $[Ca^{2+}]_i$ of a variety of mammalian cells, an effect not observed with the non-infective epimastigote stages (Tardieux *et al*., 1994; Burleigh and Andrews, 1995; Dorta *et al*., 1995). Buffering host cell cytosolic free Ca^{2+} with MAPTA (Tardieux *et al*., 1994) or depletion of intracellular Ca^{2+} stores with thapsigargin (Rodríguez *et al*., 1995) significantly inhibits trypomastigote invasion of mammalian cells. Furthermore, *T.cruzi*-induced $[Ca^{2+}]_i$ transients in normal rat kidney (NRK) fibroblasts cause transient rearrangement of the cortical actin cytoskeleton (Rodríguez *et al*., 1995), suggesting a role for this signaling event in facilitating access of lysosomes to the plasma membrane. This view is reinforced by the finding that mammalian cell invasion by *T.cruzi* is significantly enhanced by treatment with the microfilament-disrupting agent cytochalasin D (Schenkman *et al*., 1991; Tardieux *et al*., 1992). These observations led to the hypothesis that parasite-induced $[Ca^{2+}]_i$ transients are required for the localized lysosome–plasma membrane fusion events that mediate *T.cruzi* entry.

The Ca^{2+}-signaling activity from *T.cruzi* trypomastigotes is soluble, and linked to the activity of a parasite serine hydrolase, oligopeptidase B (Burleigh and Andrews, 1995; Burleigh *et al*., 1997). Inhibition of oligopeptidase B activity in soluble trypomastigote extracts using protease inhibitors (Burleigh and Andrews, 1995) or specific antibodies to recombinant oligopeptidase B (Burleigh *et al*., 1997) results in inhibition of the Ca^{2+}-signaling activity. Purified oligopeptidase B alone is incapable of triggering $[Ca^{2+}]_i$ transients in mammalian cells, indicating a requirement for additional parasite components to reconstitute the Ca^{2+}-signaling activity. Immunolocalization and subcellular fractionation revealed that *T.cruzi* oligopeptidase B is a cytosolic enzyme. These observations led to the proposal that oligopeptidase B functions as a processing enzyme, generating an active Ca^{2+} agonist from a cytosolic precursor molecule (Burleigh and Andrews, 1995; Burleigh *et al*., 1997). Homology of the *T.cruzi* oligopeptidase B to the prolyl oligopeptidase family of serine peptidases (Burleigh *et al*., 1997), which includes prohormone processing enzymes (Fuller *et al*., 1988), is consistent with this view.

The aim of the present study was to test the hypothesis that oligopeptidase B plays a central role in host cell signaling and invasion by *T.cruzi* trypomastigotes. To this end, we generated oligopeptidase B null mutant *T.cruzi* parasites using a targeted gene replacement approach. We found that *T.cruzi* oligopeptidase B null mutants are unable to elicit the oligopeptidase B-mediated Ca^{2+} response in mammalian cells and are severely impaired with respect to host cell invasion and the establishment of infections in mice. Ca^{2+}-signaling activity was reconstituted in null mutant parasite extracts by addition of exogenous recombinant oligopeptidase B, demonstrating that this enzyme mediates production of a signaling agonist for mammalian cells that is required for efficient invasion and infectivity.

Results

Generation of T.cruzi oligopeptidase B null mutants

To investigate the role of *T.cruzi* oligopeptidase B in host cell Ca^{2+}-signaling and invasion, we have disrupted both alleles of the oligopeptidase B gene (*PEP*). Gene replacement constructs containing the neomycin phosphotransferase gene (p*PEPNEO*) or the phleomycin resistance gene (p*PEPBLE*) (Figure 1A) were targeted to the wild-type *PEP* genomic locus by homologous recombination (Figure 1B). A single *PEP* allele was targeted with the *PEPNEO* construct to generate single knockout (G418-resistant) parasites. One single knockout clone (SKO-1), containing *PEPNEO* integrated at the *PEP* locus, was selected for introduction of the second construct (*PEPBLE*), which resulted in the generation of double knockout (G418/phleomycin-resistant) parasites (Figure 1B). Genomic DNA Southern blots (Figure 1C) of a SKO-1 and a double knockout clone (DKO-1) probed with the 5′ PEP probe (Figure 1A) revealed that the restriction patterns were consistent with integration at the *PEP* locus. A probe specific for a region of the *PEP* gene that was replaced during construction of the knockout cassettes (INT probe; Figure 1A) failed to hybridize with genomic DNA of parasite DKO-1 on Southern blots (Figure 1C, INT, lanes 11–15), but hybridized to the appropriately sized fragments in wild-type (Figure 1C, INT, lanes 1–5) and SKO-1 (Figure 1C, INT, lanes 6–10). PCR analysis using the INT primers did not yield a product when DKO-1 genomic DNA was used as template, but the predicted 700 bp product was observed with both wild-type and SKO-1 genomic DNA (data not shown). Therefore, Southern blot and PCR analysis show that the sequential targeting of drug resistance constructs to the *PEP* locus was successful in generating clones of *T.cruzi* in which one or both *PEP* alleles were replaced.

Disruption of the *PEP* gene in *T.cruzi* was demonstrated further by analyzing oligopeptidase B protein levels and enzymatic activity in single and double knockout parasites as compared with wild-type. Immunoblots probed with specific anti-oligopeptidase B rabbit IgG (Burleigh *et al*., 1997) revealed that the 80 kDa oligopeptidase B polypeptide is not expressed in the null mutants (Figure 2A, DKO-1 and DKO-2). Densitometric analysis of Western blots revealed that SKO (Figure 2A, SKO-1 and SKO-2) contained ~50% less oligopeptidase B than wild-type

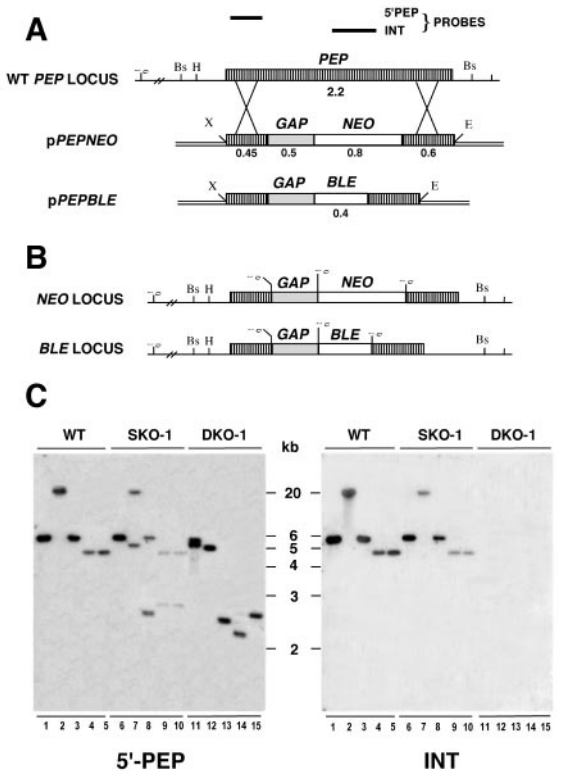

Fig. 1. Generation of *T.cruzi* oligopeptidase B null mutants. (**A**) Wild-type (WT) *PEP* genomic locus and targeted gene replacement constructs p*PEPNEO* and p*PEPBLE* (not to scale). Lined boxes, *PEP* coding region; open boxes, neomycin phosphotransferase gene (*NEO*) and phleomycin resistance gene (*BLE*); solid gray boxes, 500 bp of the *T.cruzi* glyceraldehyde-3-phosphate dehydrogenase intergenic region; double lines, pBluescript-SK vector sequences; solid bars, 5′ PEP and INT probes (450 and 700 bp respectively, generated by PCR as described in Materials and methods) used in Southern hybridizations. Bg, *Bgl*II; Bs, *Bsm*I; E, *Eco*RI; H, *Hin*dIII; X, *Xho*I. (**B**) *Trypanosoma cruzi NEO* and *BLE* genomic loci generated by integration of the constructs excised from p*PEPNEO* and p*PEPBLE* with *Eco*RI and *Xho*I. (**C**) Genomic Southern blot of wild-type *T.cruzi* Y strain (WT), a SKO-1 and DKO-1. Restriction fragments hybridizing with the 5′ PEP probe after digestion with *Hin*dIII (lanes 1, 6 and 11), *Bgl*II (lanes 2, 7 and 12), *Hin*dIII–*Bgl*II (lanes 3, 8 and 13), *Bgl*II–*Bsm*I (lanes 4, 9 and 14) and *Bsm*I (lanes 5, 10 and 15) were of the expected sizes and demonstrated that correct integration had occurred (5′ PEP probe). The INT probe failed to hybridize with DKO-1 DNA, indicating that both alleles of the oligopeptidase B gene were replaced. Migration of molecular size markers (kb) is indicated.

(Figure 2A, WT and WT-1). Quantitation of oligopeptidase B activity in soluble extracts prepared from several wild-type, SKO and DKO clones was achieved by measuring the hydrolysis of the substrate Z-FR-AMC (Burleigh *et al*., 1997). This analysis revealed that the specific activity of oligopeptidase B in SKO clones (Figure 2B, SKO-1, -2, -3 and -4) was 50% lower than the activity measured in the uncloned wild-type population and wild-type clones (Figure 2B, WT, WT-1, -2, -3 and -4). This observation is consistent with previous findings in trypanosomatid parasites, in which gene dosage effects were observed after disruption of one of the alleles of a single-copy gene (Li *et al*., 1996). As expected, no oligopeptidase B activity was detected in any of the null mutant clones (Figure 2B, DKO-1, -2, -3 and -4).

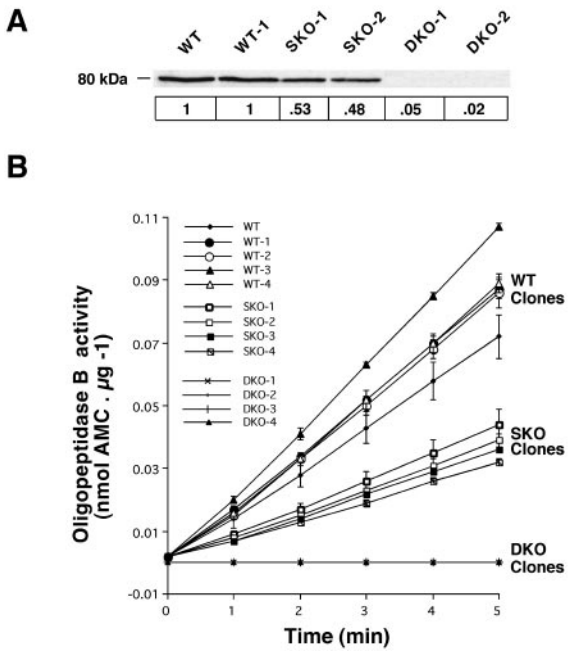

Fig. 2. Disruption of a single *PEP* allele results in a 50% reduction in oligopeptidase B enzyme expression. (**A**) Detergent lysates prepared from wild-type *T.cruzi* Y strain (WT, lane 1), a wild-type clone (WT-1, lane 2), SKO-1, SKO-2 and DKO-1 and DKO-2 were immunoblotted with anti-recombinant oligopeptidase B IgG (αrPEP). The relative intensity of the 80 kDa band determined by densitometric scanning normalized to the density of the wild-type band is indicated below each lane. (**B**) Specific activity of oligopeptidase B in soluble extracts prepared from wild-type, SKO and DKO clones. AMC released from the oligopeptidase B substrate Z-FR-AMC was measured continuously for 5 min in a spectrofluorimeter (excitation 380 nm; emission 440 nm). The data represent the mean of triplicate samples ± SD, of nmol AMC released/microgram of protein at 60 s intervals.

Oligopeptidase B null mutants exhibit reduced invasion capacity for mammalian cells

Since genetic manipulations are not feasible in the non-dividing mammalian-infective trypomastigote life cycle stages of *T.cruzi*, the *PEP* locus was disrupted in epimastigote forms which grow axenically in liquid medium. The successful generation of oligopeptidase B null mutant parasites indicates that oligopeptidase B, which is expressed in all life cycle stages of *T.cruzi* (Burleigh and Andrews, 1995; Burleigh *et al*., 1997), is not essential for the viability of epimastigotes. The rationale behind the generation of oligopeptidase B null mutants was to test whether gene deletion would impair the ability of trypomastigotes, the infective stages of *T.cruzi*, to trigger $[Ca^{2+}]_i$ transients in, and to invade mammalian host cells. Thus, a crucial step toward the further analysis of the null mutant phenotype was the production of metacyclic trypomastigotes (mammalian-infective forms) from stationary phase cultures of epimastigotes. We found that expression of oligopeptidase B is not required for this differentiation event in liquid culture. No morphological differences were noted in the SKO or DKO parasites when compared with wild-type, as judged by light (Figure 3A, a–f) or transmission electron microscopy (data not shown). In addition, oligopeptidase B knockout trypomastigotes did not display a defect in motility (Figure 3B) and incorporated ^{35}S-labeled amino acids into a trichloroacetic acid (TCA)-insoluble fraction at similar rates (Figure 3C). Therefore, the oligopeptidase B null mutants are comparable to wild-type *T.cruzi*, not exhibiting any apparent morphological, motility or metabolic defects.

To examine the effect of oligopeptidase B gene deletion on the ability of *T.cruzi* to invade mammalian cells, purified metacyclic trypomastigotes obtained from stationary phase cultures of epimastigotes (wild-type, SKO-1 and DKO-1) (Figure 3A, insets of a–c) were used to infect NRK fibroblasts and L6E9 myoblasts. A marked difference was observed in the invasive capacity of both SKO-1 and DKO-1 when compared with wild-type (Figure 4A, upper panels). Oligopeptidase B null mutant metacyclics (DKO-1) were 75% less infective for NRK fibroblasts (Figure 4A, upper left panel) and L6E9 myoblasts (Figure 4A, upper right panel) than wild-type metacyclics. In addition, SKO-1 metacyclic trypomastigotes displayed a partial invasion defect, since they were ~40% less infective than wild-type (Figure 4A, upper panels). The few DKO-1 metacyclic trypomastigotes capable of invading host cells, by an oligopeptidase B-independent pathway, exhibited no apparent defect in their ability to progress through the intracellular cycle (Figure 3A, d–f), resulting in the production of tissue culture trypomastigotes. Invasion assays demonstrated that the invasion defect associated with deletion of one or both *PEP* alleles is not exclusive to metacyclic trypomastigotes (Figure 4A, upper panels), since similar results were obtained with tissue culture trypomastigotes (Figure 4A, lower panels). Infection of HeLa cells with wild-type, SKO-1 and DKO-1 tissue culture trypomastigotes resulted in the same relative levels of infection observed in NRK and L6E9 cells (Figure 4A, lower right panel). These results reveal a strong correlation between oligopeptidase B expression levels and the capacity of *T.cruzi* to invade cultured mammalian cells. The *PEP* SKO parasites express 50% less oligopeptidase B enzyme than wild-type (Figure 2), and exhibit intermediate levels of host cell invasion when compared with wild-type and DKO mutants (Figure 4A).

Residual invasion of L6E9 myoblasts by oligopeptidase B null mutants is independent of a signaling pathway involving Ca^{2+} release from intracellular stores

The marked reduction in host cell invasion by oligopeptidase B null mutants is predicted to be a consequence of the inability of these parasites to generate a Ca^{2+} agonist and trigger inositol 1,4,5-trisphosphate (IP_3)-mediated mobilization of Ca^{2+} from host cell intracellular stores (Rodríguez *et al*., 1995; Burleigh *et al*., 1997). To test this prediction, we examined the effect of depletion of host cell intracellular Ca^{2+} stores on the residual invasion by oligopeptidase B null mutants. Pre-treatment of L6E9 myoblasts with thapsigargin, which inhibits the endoplasmic reticulum (ER) Ca^{2+}-ATPase leading to intracellular Ca^{2+} store depletion (Lytton *et al*., 1991), failed to reduce the level of myoblast invasion by null mutant trypomastigotes (Figure 4B, DKO-1). In contrast, infection of thapsigargin-treated myoblasts by SKO-1 trypomastigotes was reduced by ~50%, to a level comparable to that of the residual DKO-1 invasion (Figure 4B). A similar reduction in invasion levels is observed in thapsigargin-treated NRK fibroblasts infected with wild-type trypomastigotes (Rodríguez *et al*., 1995). These

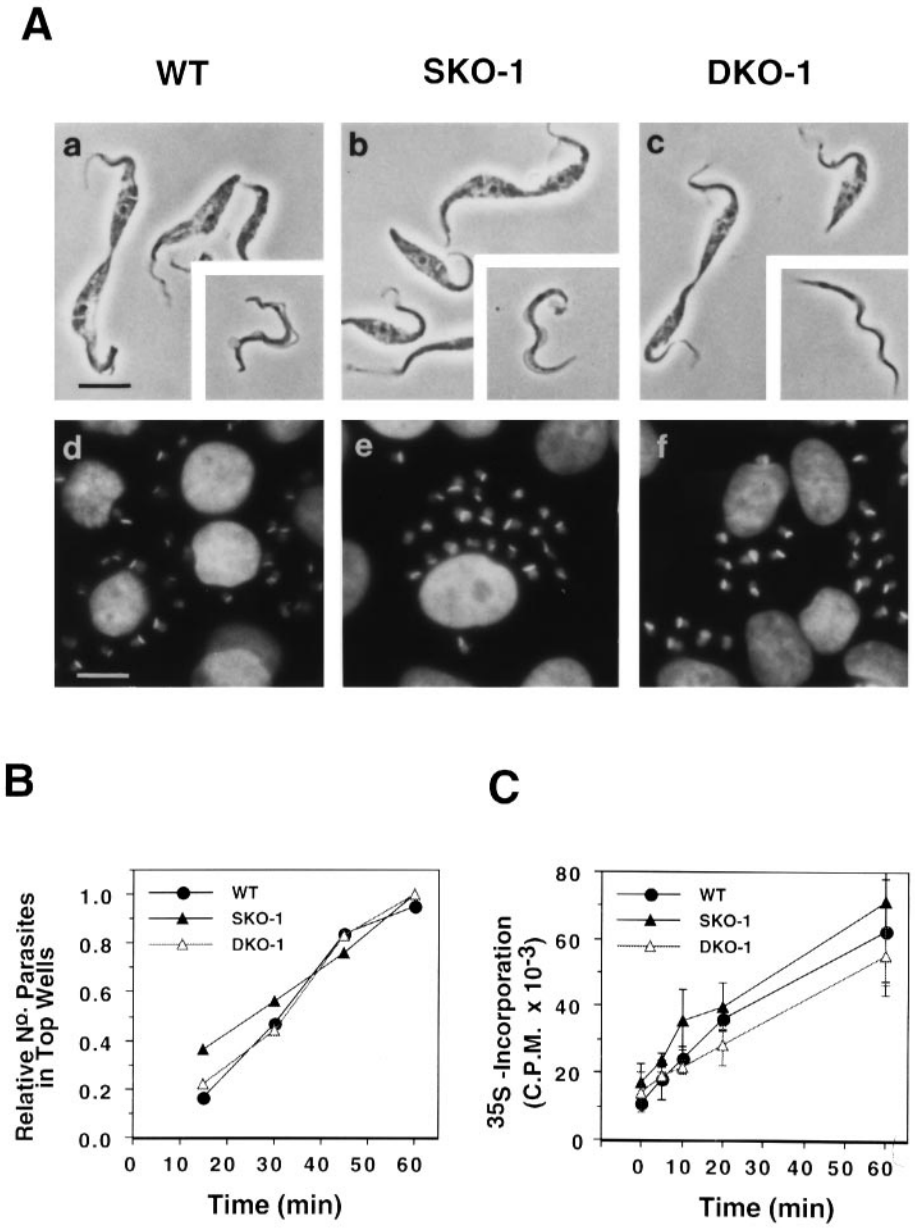

Fig. 3. Oligopeptidase B gene deletion does not affect *T.cruzi* morphology, motility or differentiation. (**A**) Phase-contrast micrographs of wild-type, SKO-1 and DKO-1 epimastigotes (panels a, b and c), metacyclic trypomastigotes (a, b and c insets), and fluorescence micrographs of DAPI-stained intracellular amastigotes in NRK cells (panels d, e and f). Scale bars = 5 μm. (**B**) Motility of wild-type, SKO-1 and DKO-1 trypomastigotes was determined by their ability to pass through a 5 μm pore membrane in a Transwell plate. Parasites were added to the bottom; equilibration between the top and bottom chambers was reached at 60 min. The data represent numbers of trypomastigotes counted in the top chamber relative to the final equilibration concentration of 10^6 parasites/ml at the time points indicated. (**C**) Metabolic incorporation of ^{35}S-TransLabel by trypomastigotes. Results are plotted as the mean ± SD of triplicate samples, of counts per minute (c.p.m.) ^{35}S incorporated into a TCA-insoluble fraction as a function of time (minutes).

results demonstrate that the residual invasion by oligopeptidase B null mutant trypomastigotes is mediated by a mechanism that is independent of the ability of these parasites to mobilize Ca^{2+} from thapsigargin-sensitive host cell intracellular stores. To determine whether this residual invasion mechanism still required $[Ca^{2+}]_i$ transients, L6E9 myoblasts were pre-treated with the membrane-permeant Ca^{2+}-chelating agent, MAPTA-AM, to buffer intracellular free Ca^{2+} prior to infection. MAPTA loading significantly inhibited invasion of L6E9 myoblasts by DKO-1 trypomastigotes, as well as by SKO-1 and wild-type parasites (Figure 4C). This suggests that the residual host cell invasion by oligopeptidase B null mutants is also dependent on $[Ca^{2+}]_i$ transients. Cytochalasin D pre-treatment of L6E9 myoblasts enhanced the level of invasion by oligopeptidase B SKO and DKO mutants (data not shown) to an extent that is similar to the previously reported enhancement of wild-type *T.cruzi* invasion (Tardieux *et al.*, 1992). Taken together, our results indicate that *T.cruzi* utilizes two independent pathways for host cell invasion. The major, oligopeptidase B-dependent pathway involves mobilization of host cell intracellular Ca^{2+} stores. A minor, oligopeptidase B-independent pathway does not require thapsigargin-sensitive intracellular Ca^{2+} stores,

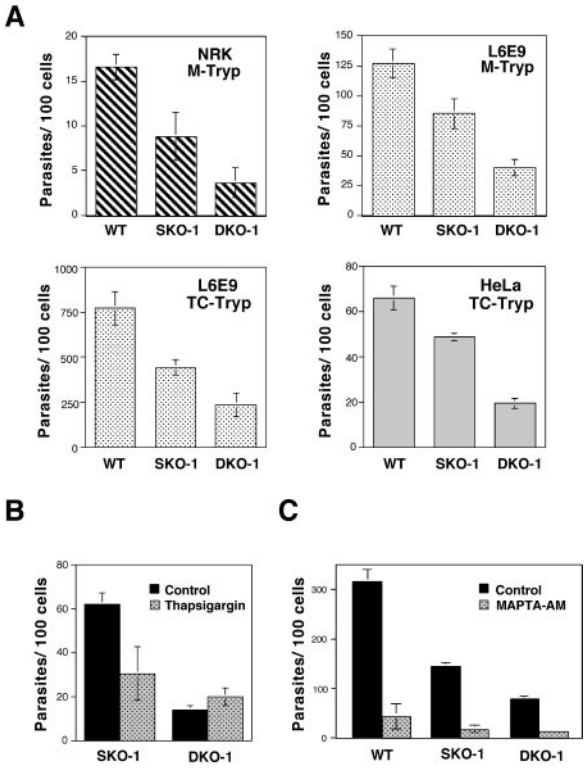

Fig. 4. Oligopeptidase B null mutants exhibit an impaired invasion phenotype that is insensitive to thapsigargin and sensitive to MAPTA-AM pre-treatment of host cells. (**A**) Mammalian cells were infected with 5×10^7/ml wild-type (WT), SKO-1 or DKO-1 *T.cruzi* metacyclic trypomastigotes (M-Tryp) or tissue culture trypomastigotes (T-Tryp) for 1 h. (**B**) L6E9 myoblasts mock-treated (solid bars) or pre-treated with thapsigargin (stippled bars) were washed and infected with 2×10^7/ml SKO-1 or DKO-1 tissue culture trypomastigotes for 30 min. (**C**) L6E9 myoblasts pre-treated with MAPTA-AM (stippled bars) or mock-treated (solid bars) were washed and infected with 5×10^7 parasites/ml of wild-type, SKO-1 and DKO-1 tissue culture trypomastigotes for 1 h. The number of parasites/100 cells was determined as described in Materials and methods. Values represent the mean ± SD of triplicate samples.

but still appears to be dependent on $[Ca^{2+}]_i$ transients. The effect of cytochalasin D indicates that both pathways are facilitated by disruption of host cell actin microfilaments.

Reconstitution of the soluble Ca^{2+}-signaling activity of oligopeptidase B null mutants with recombinant oligopeptidase B

To demonstrate directly a role for oligopeptidase B in the generation of a soluble Ca^{2+}-signaling agonist, our aim was to carry out reconstitution experiments in null mutant extracts by addition of recombinant oligopeptidase B. Unexpectedly, we found that DKO-1 soluble extracts retained the ability to trigger $[Ca^{2+}]_i$ transients in NRK fibroblasts in the absence of oligopeptidase B (Figure 5A). This activity was abolished by several protease inhibitors which inhibit both oligopeptidase B and the wild-type soluble Ca^{2+}-signaling activity (Burleigh and Andrews, 1995; Burleigh *et al*., 1997), and by an additional inhibitor, Z-FA-FMK, which does not affect either oligopeptidase B or the wild-type Ca^{2+}-signaling activities (Table I). These results suggested that an additional peptidase activity, sensitive to Z-FA-FMK, was present in trypomastigote extracts and was also able to generate a Ca^{2+} agonist for NRK cells. As shown in Figure 5B, the Ca^{2+}-signaling activity in DKO-1 extracts is abolished by the protease inhibitor Z-FA-FMK, while the wild-type activity is not. Therefore, reconstitution experiments were carried out in the presence of Z-FA-FMK to inhibit the residual Ca^{2+}-signaling activity in null mutant extracts. Figure 5C shows that exogenous recombinant oligopeptidase B, when added to Z-FA-FMK-treated DKO-1 trypomastigote soluble extracts, restores Ca^{2+}-signaling activity. Reconstitution is only possible with functional oligopeptidase B, since the catalytically inactive enzyme (S→T565 rPEP) failed to restore the Ca^{2+}-signaling activity (Figure 5C). Thus, the ability of exogenous recombinant oligopeptidase B to reconstitute the Ca^{2+}-signaling activity in null mutant soluble extracts directly demonstrates that this enzyme is involved in the generation of a soluble Ca^{2+}-signaling agonist for mammalian cells.

To examine the significance of the Z-FA-FMK-sensitive activity in host cell signaling and invasion by *T.cruzi*, live trypomastigotes were pre-treated with the membrane-permeant protease inhibitors, Z-FA-FMK and Z-FR-FMK. These inhibitors previously were demonstrated to enter all life cycle stages of *T.cruzi* efficiently, as judged by inhibition of the lysosomal cysteine protease cruzipain (Harth *et al*., 1993). Both of these inhibitors abolished the residual Ca^{2+}-signaling activity in null mutant soluble extracts (Table I, DKO-1), but failed to reduce the residual invasion of host cells by oligopeptidase B null mutants (Figure 6, DKO-1). In contrast, Z-FR-FMK, which inhibits oligopeptidase B activity (Burleigh and Andrews, 1995; Table I), reduced the level of L6E9 myoblast invasion by wild-type and SKO-1 trypomastigotes (Figure 6). These results indicate that inhibition of cytosolic oligopeptidase B in *T.cruzi* trypomastigotes leads to a decrease in invasive capacity, while inhibition of the additional Z-FA-FMK-sensitive activity has no consequence. The failure of both Z-FA-FMK and Z-FR-FMK to reduce the residual invasive capacity of DKO-1 trypomastigotes further suggests that generation of the soluble Ca^{2+} agonist by the additional protease/peptidase activity in soluble parasite extracts (Figure 5A) is not physiologically relevant to the mechanism of *T.cruzi* invasion. It is conceivable that the substrate of oligopeptidase B, postulated to be the Ca^{2+} agonist precursor, is not in the same subcellular compartment as the Z-FA-FMK-sensitive activity, and that both are brought into contact only as a consequence of cell disruption.

Deletion of the oligopeptidase B gene causes marked reduction in T.cruzi infectivity for mice

To determine if the invasion phenotype observed with single and double oligopeptidase B null mutants for cultured mammalian cells (Figure 4) would be reflected *in vivo*, mice were infected with purified tissue culture trypomastigotes and the resulting parasitemia was recorded for 8–10 days post-infection. Initial experiments compared infectivity of the *T.cruzi* wild-type Y strain with SKO and DKO trypomastigotes (Figure 7A). This experiment revealed that mice infected with either SKO-1 or DKO-1 displayed markedly reduced parasitemias, as well as a delay in the onset of the peak when compared with wild-type (Figure 7A). To rule out the possibility that the lower infectivity of SKO-1 and DKO-1 in mice was due to

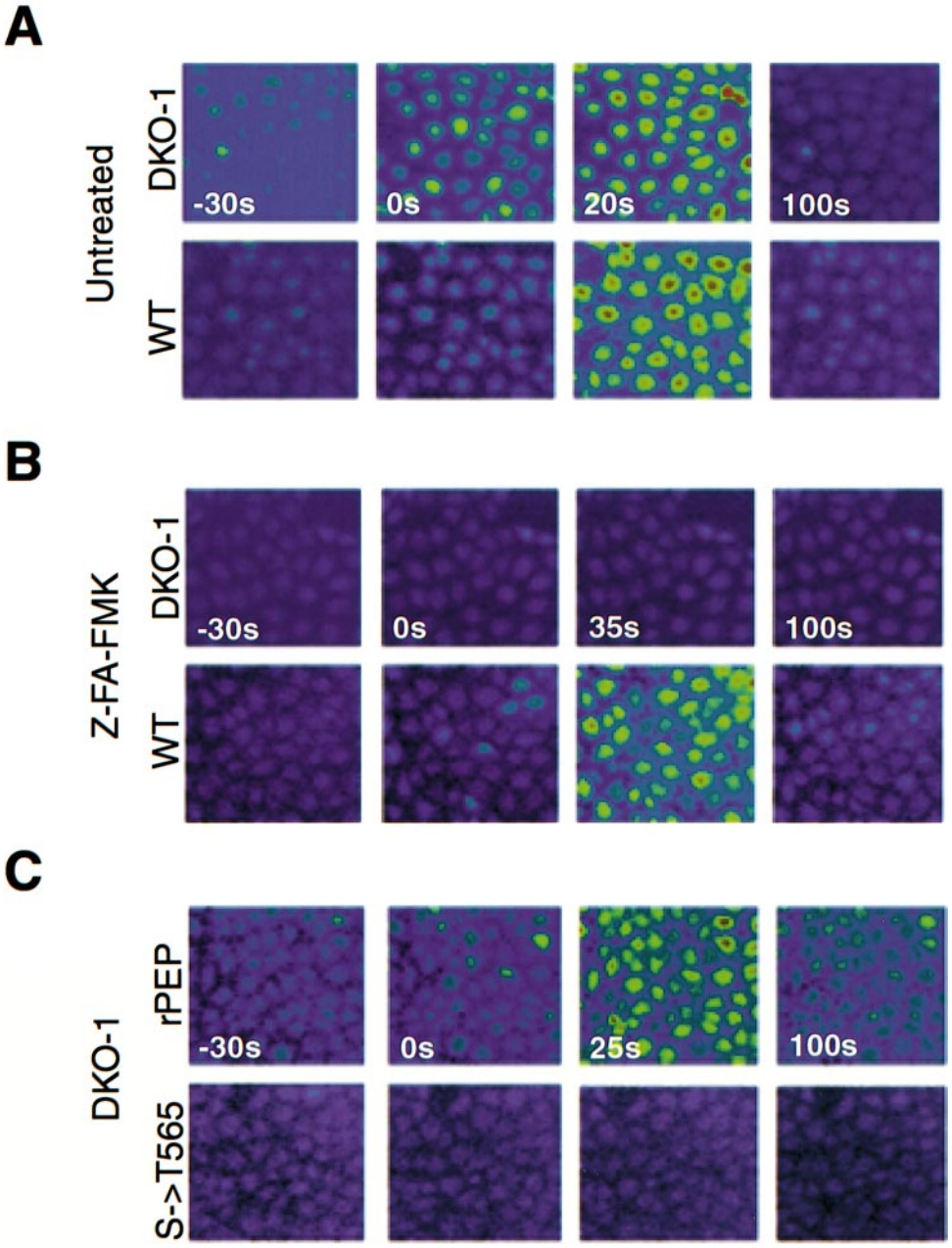

Fig. 5. Reconstitution of Ca^{2+}-signaling activity in *T.cruzi* oligopeptidase B null mutant extracts with recombinant oligopeptidase B. Time-lapse fluorescence microscopy of fluo-3/AM-loaded NRK cells before and after addition of: (**A**) DKO-1 or wild-type trypomastigote soluble extracts pre-incubated for 5 min at 37°C; (**B**) DKO-1 or wild-type soluble extracts pre-treated for 5 min at 37°C with 100 μM Z-FA-FMK; (**C**) DKO-1 soluble extract pre-treated for 5 min at 37°C with 100 μM Z-FA-FMK and 40 μg/ml of either wild-type recombinant oligopeptidase B (rPEP) or catalytically inactive recombinant oligopeptidase B rPEP (S→T565 rPEP). Images were acquired at 1 s intervals beginning 30 s prior to addition of extracts (–30 s). Images were pseudocolored with purple indicating the lowest $[Ca^{2+}]_i$ and red the highest.

random clonal variation, mice were infected with several independent clones of SKO, DKO and wild-type parasites. All of the wild-type clones gave rise to consistently higher parasitemias than SKO-1 (Figure 7B), and all SKO (Figure 7C) and DKO (Figure 7D) clones produced significantly lower levels of parasitemia than the wild-type population. The lack of a consistently discernible difference in the infectivity of SKOs and null mutant trypomastigotes is probably due to the reduced sensitivity of the assay at very low levels of parasitemia. Our results thus demonstrate that the defect in mammalian cell invasion associated with replacement of one or both *PEP* alleles in *T.cruzi* (Figure 4) is exacerbated in *in vivo* infections of mice (Figure 7). This suggests that a 50% reduction in oligopeptidase B expression levels already has important consequences for parasite proliferation in mice.

Discussion

In this study, we demonstrate that the *T.cruzi* serine hydrolase oligopeptidase B is involved in the activation of a host cell signaling pathway, which is a key event in the infection process. The *T.cruzi* oligopeptidase B null mutant trypomastigotes generated through targeted gene replacement are 75% less infective than wild-type parasites for fibroblasts, myoblasts and HeLa cells. This indicates

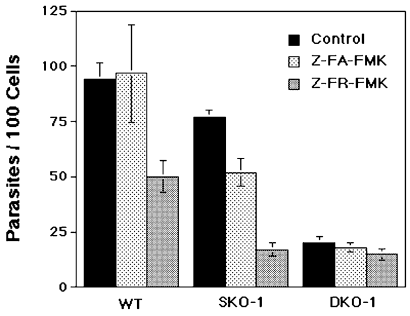

Fig. 6. Membrane-permeant protease inhibitors Z-FA-FMK and Z-FR-FMK fail to reduce the residual invasion of oligopeptidase B null mutant trypomastigotes. Trypomastigotes at 5×10^7/ml were pre-treated for 15 min at 37°C with 50 μM Z-FA-FMK (stippled bars), 50 μM Z-FR-FMK (gray bars) or mock treated (black bars), prior to incubation with L6E9 myoblasts for 1 h. The number of parasites/100 cells was determined as described in Materials and methods. Values represent the mean ± SD of triplicate samples.

Table I. Oligopeptidase B and Ca^{2+}-signaling activities in soluble *T.cruzi* extracts in the presence of protease inhibitors

Inhibitor	Oligopeptidase B activity	Ca^{2+}-signaling activity	
		Wild-type	DKO-1
No inhibitor	+	+	+
Leupeptin	–	–	–
TLCK	–	–	–
E-64	+	+	+
Z-FR-FMK	–	–	–
Z-FA-FMK	+	+	–

Oligopeptidase B activity in soluble wild-type trypomastigote extracts was assayed with 20 μM Z-FR-AMC in the presence of 100 μM protease inhibitors (TLCK was 10 μM) as previously described (Burleigh *et al*., 1997).
Soluble extracts prepared from wild-type and DKO-1 metacyclic trypomastigotes were pre-treated with protease inhibitors (100 μM; TLCK was 10 μM) for 5 min at 37°C prior to adding to NRK fibroblasts loaded with fluo3-AM.
+, activity detected; –, no activity detected.

that oligopeptidase B plays a major role in *T.cruzi* invasion of mammalian cells, a conclusion that is further supported by the phenotype associated with trypomastigotes containing only one functional *PEP* allele. These single knockout parasites express 50% less oligopeptidase B enzyme than wild-type, and exhibit intermediate levels of host cell invasion when compared with wild-type and DKO mutants.

A role for oligopeptidase B in the generation of a *T.cruzi* Ca^{2+}-signaling agonist for mammalian cells was demonstrated directly by reconstitution of the Ca^{2+}-signaling activity in null mutant parasite extracts with recombinant oligopeptidase B (Figure 5). Addition of the protease inhibitor Z-FA-FMK to reconstitution assays was necessary to inhibit a previously undetected activity capable of generating a Ca^{2+} agonist in null mutant trypomastigote extracts. Since the residual invasive capacity of null mutant trypomastigotes is not inhibited by the membrane-permeant inhibitor Z-FA-FMK (Harth *et al*., 1993), we conclude that while a Z-FA-FMK-sensitive peptidase/protease appears to substitute for oligopeptidase B in soluble trypomastigote extracts, it is unlikely to function in the generation of a signaling factor in live, intact trypomastigotes. Consistent with our previous results showing that the oligopeptidase B inhibitor Z-FR-FMK reduces trypomastigote invasion in a dose-dependent manner (Burleigh and Andrews, 1995), we found that host cell invasion by oligopeptidase B-expressing trypomastigotes, both wild-type and SKO, is inhibited by Z-FR-FMK. On the other hand, oligopeptidase B null mutants are refractory to pre-treatment with Z-FR-FMK. These results provide further evidence in support of a central role for oligopeptidase B in mammalian cell invasion by *T.cruzi*. The ability of recombinant oligopeptidase B to reconstitute Ca^{2+}-signaling activity in null mutant soluble extracts provides proof that this enzyme is responsible for the generation of a mammalian cell signaling agonist. The *T.cruzi* oligopeptidase B null mutants are incapable of Ca^{2+} agonist generation, and consequently are impaired in host cell signaling and invasion. In the oligopeptidase B SKO trypomastigotes, the intermediate levels of host cell invasion relative to wild-type and the null mutant may be explained by a decrease in Ca^{2+} agonist production and a concomitant reduction in host cell signaling.

Thapsigargin pre-treatment of mammalian cells inhibits entry of wild-type trypomastigotes (Rodríguez *et al*., 1995), indicating that the *T.cruzi*-induced mobilization of Ca^{2+} from intracellular stores (Tardieux *et al*., 1994; Rodríguez *et al*., 1995) plays an important role in the parasite entry process. The finding that the residual invasion capacity of null mutants is not affected by thapsigargin treatment of host cells demonstrates that this pathway was effectively abolished by deletion of the oligopeptidase B gene. This result is fully consistent with the predicted function for oligopeptidase B in a pathway that leads to mobilization of Ca^{2+} stores in mammalian cells (Burleigh *et al*., 1997).

Our findings demonstrate that the oligopeptidase B-dependent signaling of host cells by infective forms of *T.cruzi* is a major signaling pathway leading to parasite entry. However, neutralization of the oligopeptidase B-dependent pathway via (i) *PEP* gene deletion, (ii) inhibition of cytosolic oligopeptidase B with the membrane-permeant inhibitor Z-FR-FMK or (iii) depletion of host cell intracellular Ca^{2+} stores with thapsigargin does not completely abolish *T.cruzi* entry. The residual invasion mechanism of the oligopeptidase B null mutants appears to be also dependent on intracellular free Ca^{2+} levels, since parasite entry is inhibited when host cells are pre-loaded with the Ca^{2+}-chelator MAPTA. Therefore, *T.cruzi* may be capable of triggering $[Ca^{2+}]_i$ transients in mammalian cells using an additional mechanism, distinct from the oligopeptidase B-dependent pathway. This could occur by involvement of thapsigargin-insensitive intracellular Ca^{2+} stores, or by Ca^{2+} influx from the extracellular medium. Thapsigargin-insensitive Ca^{2+} pools have been described in mammalian cells (Fasolato *et al*., 1991; Rizzuto *et al*., 1994; Pizzo *et al*., 1997), where they account for approximately half of all sequestered Ca^{2+}. However, these stores are not likely to mediate signal transduction events, since they are either non-inducible (Fasolato *et al*., 1991; Rizzuto *et al*., 1994) or not released by physiological stimulation (Pizzo *et al*., 1997). Ca^{2+} influx from the extracellular medium is thus more likely to contribute to the mechanism of residual invasion by *T.cruzi* null mutants. Trypomastigotes of *T.cruzi* are highly

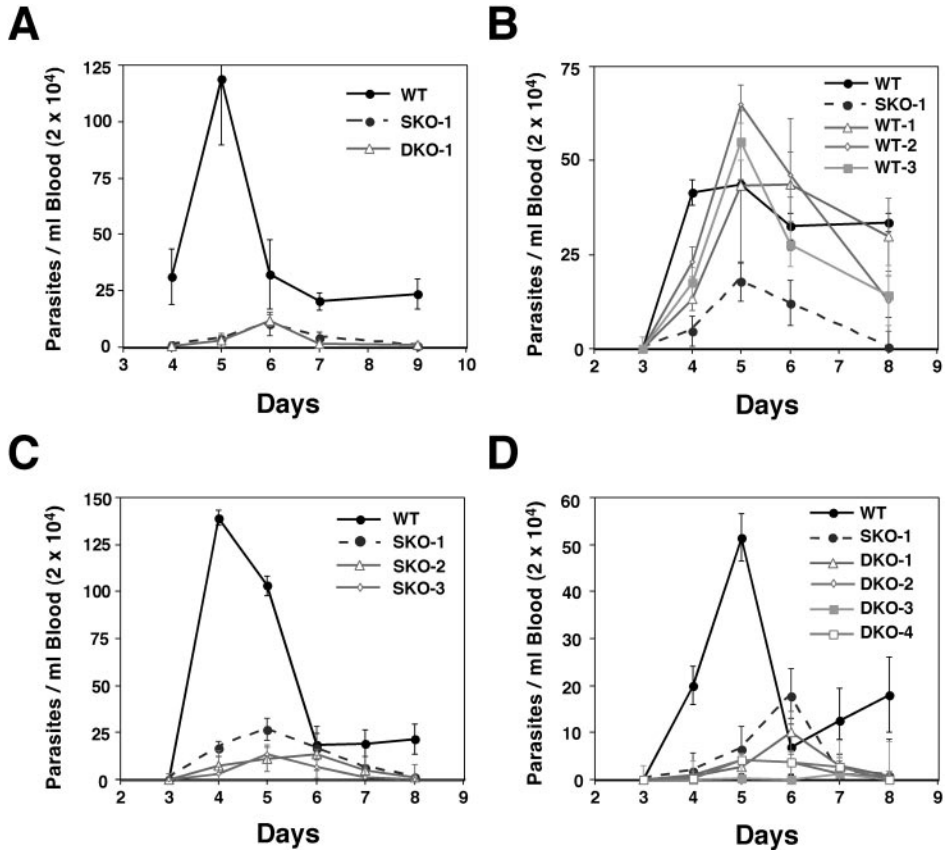

Fig. 7. Single and double *PEP* allele replacement decreases the infectivity of *T.cruzi* in mice. Female C_3H mice were injected with 2×10^6 [(**A**) and (**C**)] and 1×10^6 [(**B**) and (**D**)] tissue culture-derived trypomastigotes. The number of parasites/ml of blood was counted beginning at day 3 post-infection and followed until the first peak declined (8–9 days). Data points represent the mean parasitemia ± SD of five (A) or three mice (B–D). WT-1, WT-2 and WT-3 represent clones of the wild-type Y strain population (WT). SKO-1, SKO-2 and SKO-3 are three independent single knockout clones in which one allele of oligopeptidase B was replaced with the *PEPNEO* construct. DKO-1, DKO-2, DKO-3 and DKO-4 are double knockout clones, generated by integration of the *PEPBLE* construct into SKO-1.

motile organisms which attach to the surface of mammalian cells prior to invasion, a process which requires 8–10 min for completion (Rodríguez *et al*., 1996). It is conceivable that mechanical stimulation provided by the rapidly beating flagellum of attached parasites (Rodríguez *et al*., 1996) may promote Ca^{2+} influx through the opening of Ca^{2+} channels on the plasma membrane (Sanderson *et al*., 1990). Alternatively, *T.cruzi* trypomastigotes may produce an additional, oligopeptidase B-independent agonist which may activate plasma membrane Ca^{2+} channels directly. Although such an agonist should have been detected in previous studies (Burleigh and Andrews, 1995; Rodríguez *et al*., 1995), it may be unstable or not released in soluble form in *T.cruzi* extracts. High resolution Ca^{2+} imaging at the single cell level may clarify this issue, since it may allow comparisons between the frequency and spatial patterns of the mammalian cell $[Ca^{2+}]_i$ responses to wild-type and oligopeptidase B null mutant trypomastigotes.

Transforming growth factor β (TGFβ)-receptor signaling pathways were reported to be important for *T.cruzi* invasion of mink lung epithelial cells, observations that led to the proposal that trypomastigotes may produce a TGFβ-like molecule (Ming *et al*., 1995). Since there is no evidence that activation of the TGFβ receptor leads to IP_3 generation and mobilization of Ca^{2+} from intracellular stores in mammalian cells (Heldin *et al*., 1997), the relationship of that pathway to the oligopeptidase B-mediated signaling described here is unclear. Although *T.cruzi* trypomastigotes may have evolved different strategies to promote their entry into different cell types, the results obtained in our study demonstrate that the oligopeptidase B-dependent mobilization of host cell intracellular Ca^{2+} is the dominant pathway regulating *T.cruzi* entry into NRK fibroblasts, L6E9 myoblasts and HeLa cells. Furthermore, the marked defect of single and double oligopeptidase B SKO and DKO mutants in mouse infections significantly reinforces the general importance of this pathway in mammalian tissues.

The *T.cruzi* invasion mechanism is known to involve the movement and localized fusion of host cell lysosomes with the plasma membrane (Tardieux *et al*., 1992; Rodríguez *et al*., 1996). In addition to lysosomes, microtubules and the microtubule-dependent plus-end-directed motor, kinesin, are required for the invasion process (Rodríguez *et al*., 1996). It is tempting to speculate that lysosome-mediated entry is possible because *T.cruzi* utilizes an existing pathway in mammalian cells regulating lysosome recruitment. Recent studies on the mechanism of membrane repair in mammalian cells have revealed that in response to plasma membrane injury, the rapid influx of extracellular Ca^{2+} leads to exocytosis of vesicles at the site of damage (McNeil and Steinhardt, 1997). The delivery of vesicles to the site of wound repair at the plasma membrane involves kinesin- and myosin-dependent

pathways (Bi *et al*., 1997). Although the origin of these exocytosed vesicles has not been identified, lysosomes are likely candidates, in light of recent findings that mammalian cell lysosomes can behave as Ca^{2+}-regulated exocytic organelles (Rodríguez *et al*., 1997). Thus, the $[Ca^{2+}]_i$ transients induced in host cells by *T.cruzi* may contribute to the lysosome–plasma membrane recruitment and fusion events that occur during host cell invasion. Since these events are very localized (Tardieux *et al*., 1992), it is conceivable that they are mediated by delivery of oligopeptidase B-generated Ca^{2+} agonist directly to the parasite attachment site at the plasma membrane.

Oligopeptidase B is an 80 kDa cytosolic polypeptide that lacks a distinct signal sequence (Burleigh *et al*., 1997). The enzyme is not constitutively released from trypomastigotes (B.Burleigh, unpublished observations) nor does it interact directly with mammalian cell surface receptors to trigger $[Ca^{2+}]_i$ transients (Burleigh and Andrews, 1995). Studies with soluble trypomastigote extracts have provided experimental evidence to suggest that in addition to oligopeptidase B, a trypomastigote factor with affinity for heparin is also required for Ca^{2+}-signaling activity (Burleigh and Andrews, 1995). Based on these observations, it was proposed that oligopeptidase B participates in the generation of an active Ca^{2+} agonist for mammalian cells through the processing of a trypomastigote-specific precursor. Significant homology of *T.cruzi* oligopeptidase B with the prolyl oligopeptidase family of serine peptidases (Burleigh *et al*., 1997), of which some members are pro-hormone-processing enzymes (Fuller *et al*., 1988), suggests that the *T.cruzi* enzyme may have a similar function. The *T.cruzi* Ca^{2+}-signaling agonist is predicted to be a peptide, since other members of the prolyl oligopeptidase family were shown to have a strong preference for peptide substrates of $<$30 amino acids (Barrett and Rawlings, 1992). The oligopeptidase B-generated Ca^{2+} agonist is postulated to interact with a receptor on the surface of host cells (Leite *et al*., 1998) and trigger the intracellular cascade leading to the mobilization of Ca^{2+} stores, as illustrated in Figure 8.

A central question in the model proposed for oligopeptidase B-mediated entry of *T.cruzi* into mammalian cells (Figure 8) relates to the mechanism of Ca^{2+} agonist delivery to host cells. The cytosolic localization of oligopeptidase B suggests that following generation of the Ca^{2+} agonist within the trypomastigotes, it must be exported from the parasites to interact with a host cell surface receptor. Two possibilities for the mechanism of Ca^{2+} agonist delivery to host cells can be envisaged. The first is that the Ca^{2+} agonist, once generated in the cytosol, is translocated into the classical secretory pathway and transported out of the parasite by vesicular transport steps, in a manner similar to the ER TAP transporters which carry processed class I peptide antigens from the cytosol to the secretory pathway in mammalian cells (Kleijmeer *et al*., 1992; Kvist and Levy, 1993). If a vesicular transport pathway through the ER–Golgi is utilized in *T.cruzi*, then Ca^{2+} agonist secretion may occur from a specialized region of the plasma membrane referred to as the flagellar pocket, from which endocytosis/exocytosis is presumed to occur in kinetoplastid parasites (Webster and Russell, 1993; Clayton *et al*., 1995). Noteworthy in this respect is that trypomastigote invasion is an oriented process, with

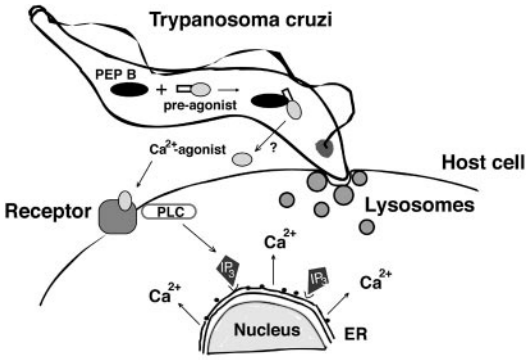

Fig. 8. A model for *T.cruzi* oligopeptidase B-mediated signaling in mammalian cells. The *T.cruzi* oligopeptidase B (PEP B) is postulated to process a precursor molecule in the cytoplasm of trypomastigotes (pre-agonist) generating an active Ca^{2+} agonist for mammalian cells. Following export from the parasite, the Ca^{2+} agonist binds to a receptor on the surface of the mammalian host cell, activating phospholipase C (PLC) and generating inositol 1,4,5-triphosphate (IP_3). IP_3 binds to its receptor on the membrane of the endoplasmic reticulum (ER) promoting Ca^{2+} efflux. Host cell lysosome fusion with the plasma membrane at the parasite attachment site may be regulated by $[Ca^{2+}]_i$ transients triggered in host cells by the oligopeptidase B-generated Ca^{2+} agonist.

the posterior end of the parasite where the flagellar pocket is located always entering the mammalian cell first (Rodríguez *et al*., 1996). Another possibility for Ca^{2+} agonist export from *T.cruzi* would be the involvement of non-classical secretion mechanisms, independent of the ER–Golgi pathway, which have been detected in yeast and mammalian cells (Kuchler *et al*., 1989; Florkiewicz *et al*., 1995; Siders and Mizel, 1995; Cleves *et al*., 1996; Chen *et al*., 1997).

Disruption of a single *PEP* allele, resulting in a 50% reduction in the level of oligopeptidase B expression and a similar reduction in mammalian cell invasion, causes a significantly more pronounced defect in mouse infections. We found that wild-type *T.cruzi* populations give rise to consistently higher parasitemias than SKO or DKO oligopeptidase B trypomastigotes. This was observed with several independent clones of wild-type, SKO or DKO parasites, indicating that the differences were not generated by clonal variations, which have been reported to arise in populations of *T.cruzi* (Doyle *et al*., 1984). It is conceivable that, because SKO and DKO clones invade mammalian cells less efficiently, they are more susceptible to immune clearance mechanisms, which would explain why the impaired invasion phenotype observed in cultured mammalian cells is exacerbated *in vivo*.

In this study, we directly demonstrate, using reconstitution experiments, that the cytosolic *T.cruzi* enzyme oligopeptidase B is responsible for the generation of a Ca^{2+}-signaling agonist for mammalian cells. Analysis of the phenotype of *T.cruzi* oligopeptidase B mutant parasites revealed that this enzyme plays a central role in the ability of *T.cruzi* trypomastigotes to invade mammalian cells, and to establish infections in mice. The residual cell invasion capacity of the null mutant parasites is, as predicted, insensitive to inhibitors which affect the oligopeptidase B-mediated pathway. We have thus provided genetic evidence that is fully consistent with a major role for the oligopeptidase B-mediated host cell signaling pathway,

which involves mobilization of host cell intracellular Ca^{2+} stores in promoting *T.cruzi* invasion.

Materials and methods

Cells and parasites

$LLcMK_2$ monkey kidney epithelial cells, NRK fibroblasts, L6E9 rat myoblasts and HeLa cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2. Epimastigotes from the *T.cruzi* Y strain were maintained in liver infusion tryptose medium containing 10% FBS (LIT) at 28°C (Nogueira and Cohn, 1976). Clonal populations were obtained by limiting dilution of Y strain epimastigotes in LIT medium in 96-well plates. Metacyclic trypomastigotes were obtained from stationary phase cultures of epimastigotes by complement lysis selection (Nogueira *et al*., 1975). Tissue culture trypomastigotes were generated by infection of confluent monolayers of $LLcMK_2$ cells with metacyclic trypomastigotes to establish the intracellular cycle and were maintained by weekly passages (Andrews *et al*., 1985). Soluble parasite extracts were prepared as described previously (Burleigh and Andrews, 1995). Micrographs of formaldehyde-fixed [2% (w/v) paraformaldehyde/phosphate-buffered saline (PBS)] epimastigotes, metacyclics or *T.cruzi*-infected NRK cells were taken with a Zeiss Axiovert 135 fluorescence microscope.

Plasmid constructs and transfection

To replace the endogenous oligopeptidase B gene by homologous recombination, two gene replacement cassettes were constructed with different resistance markers (Figure 1A). Homologous targeting sequences were generated by PCR amplification of ~500 bp from the extreme 5′ and 3′ ends of the oligopeptidase-coding region using a full-length cDNA encoding the *PEP* gene in pBluescript-SK (Burleigh *et al*., 1997) as template. The PCR primers (1, 5′-CG**GATATC**CAGTTTGTTCTCGTCCAG-3′; and 2, 5′-CC**AGATCT**GTGTGCCGTTTGTAGATG-3′) were designed to anneal at the +480 bp and +1860 bp positions respectively of the *PEP* gene [where +1 represents the A in the initiation codon ATG (see Burleigh *et al*., 1997)], and to introduce an *Eco*RV or *Bgl*II restriction site (indicated in bold on the primer sequence). PCR amplification was carried out as follows: initial denaturation at 94°C for 4 min followed by 35 cycles of: 98°C, 20 s; 56°C, 1 min; and 68°C, 10 min followed by 72°C, 10 min using the Takara LA PCR Kit (Panvera Corporation). The amplification product consisted of the entire pBluescript-SK plasmid flanked by the 5′ and 3′ ends of the *PEP* gene (pPEPENDS vector; not shown). The neomycin phosphotransferase gene (*NEO*) was PCR-amplified from the construct (p72*neo*72; Cooper *et al*., 1993) using the primers (1, 5′-GTG**GATATC**ATGATTGAACAAGATGGAA-3′; and 2, 5′-GGAC**AGATCT**GATCAGAAGAACTCGTCAA-3′) containing *Eco*RV (primer 1) and *Bgl*II (primer 2) restrictions sites (bold) under the following conditions: initial denaturation at 94°C for 5 min followed by 25 cycles of: 94°C, 1 min; 56°C, 1 min; 72°C, 1 min; followed by 72°C for 10 min. The phleomycin resistance gene (*BLE*) was excised from pUT56 (Cayla, France) using *Eco*RV and *Bgl*II. *NEO* and *BLE* were cloned into the *Eco*RV–*Bgl*II-digested pPEPENDS vector (not shown). Introduction of a splice acceptor site upstream of the drug resistance markers was accomplished by cloning the glyceraldehyde-3-phosphate dehydrogenase intergenic region (*GAP*) (Kendall *et al*., 1990) into the *Eco*RV site of pPEPENDS-NEO/BLE. *GAP* was PCR amplified from pTEX-Ble (Nozaki and Cross, 1994) using primers (1, 5′-GG**AGATCT**GCGTGGCGATGACT-3′; and 2, 5′-GG**AGATCT**TTGGTAGATGGCTTTC-3′) containing *Bgl*II sites (bold) using the conditions outlined for amplification of *NEO* except that primer annealing was carried out at 50°C. To ensure that the final constructs (pPEPNEO and pPEPBLE; Figure 1A) were correct, restriction analysis was performed and the junctions between cloned fragments were sequenced (data not shown). *NEO*, *BLE* and *GAP* were amplified using AmpliTaq DNA polymerase (Perkin Elmer) according to the manufacturer's instructions. PCRs were performed in a GeneAmp PCR System 9600 (Perkin Elmer) thermal cycler.

Parasite transfections and selection

Oligopeptidase B gene replacement cassettes excised from plasmid constructs with *Eco*RI and *Xho*I were purified from 1% (w/v) agarose/TAE gels using a Geneclean II kit (Bio 101). Mid-log phase epimastigotes were washed and resuspended at 6×10^7/ml in Zimmerman fusion media (Bellofatto and Cross, 1989). A 10 μg aliquot of the purified 2.5 kb *Eco*RI–*Xho*I fragment containing the *PEPNEO* cassette was added to 0.5 ml of epimastigotes in a 0.4 cm electrode Gene-Pulser® Cuvette (Bio-Rad) and pulsed twice at room temperature using a Gene Pulser® electroporator (Bio-Rad) set at 1.5 K_v, 25 μF and resistance (Ω) set to infinity. Immediately following electroporation, parasites were diluted in 5 ml of LIT and incubated for 48 h at 28°C prior to the addition of 500 μg/ml geneticin (G418; Sigma). G418-resistant parasites were cloned by limiting dilution in LIT prior to characterization. Several clones were obtained which contained the correctly integrated *PEPNEO* replacement cassette as determined by analysis of restriction fragment size on genomic DNA Southern blots (described below). One clone (SKO-1) was selected for introduction of the *PEPBLE* cassette using the same strategy outlined for *PEPNEO*, except that drug selection was carried out with 500 μg/ml each of G418 and phleomycin (Cayla, France).

Genomic DNA isolation and analysis

Trypanosoma cruzi genomic DNA was isolated as described (Medina-Acosta and Cross, 1993). Restriction digests of genomic DNA from individual neomycin or neomycin/phleomycin-resistant clones were analyzed by Southern blot hybridization. [^{32}P]dCTP-labeled DNA probes were generated by PCR using the oligopeptidase B cDNA as template (Burleigh *et al*., 1997). The primers used for amplification of probes are as follows: 5′ PEP probe (1, 5′-ATGAAGTGTGGTCCCATTGCCACG-3′; and 2, 5′-CAGTTTGTTCTCGTCCAGTATCACG-3′); NEO probe (1, 5′-GTGGATATCATGATTGAACAAGATGAA-3′; and 2, 5′-GGACAGATCTGATCAGAAGAACTCGTCAA-3′); and INT probe (1, 5′-GAGATCGGGTCGCATGTTTAACAC-3′; and 2, 5′-CTGCGCGTCAGCTACACTTCCATG-3′). The plasmid probe was obtained by random priming labeling of pUC19 with [^{32}P]dCTP (*Redi* prime, Amersham).

Western blotting

Detergent lysates of wild-type, SKO and DKO *T.cruzi* parasites were prepared by lysing a pellet of 5×10^7 parasites in 20 mM Tris, pH 7.4, 150 mM NaCl (TBS) containing 1% (v/v) NP-40 and 10 μg/ml each of pepstatin A, leupeptin, aprotinin, antipain, soybean trypsin inhibitor and 1 mM iodoacetamide. Parasite soluble fractions were resolved under reducing conditions on SDS–PAGE prior to transfer of proteins to Immobilon™ membranes (Millipore). Oligopeptidase B was detected on Western blots using 2 μg/ml rabbit anti-peptidase IgG (Burleigh *et al*., 1997) diluted in TBS containing 5% (w/v) non-fat milk, 1% (w/v) bovine serum albumin followed by peroxidase-labeled goat anti-rabbit IgG (Kirkegaard & Perry). Immunoreactive bands were detected by ECL (Amersham). Blots were exposed to Hyperfilm™ (Amersham) for different periods and analyzed after scanning the films using a GS-700 Imaging Densitometer (Bio-Rad).

Parasite motility and metabolic incorporation of ^{35}S-TransLabel™

The ability of trypomastigotes to traverse a 5.0 μm pore membrane filter was used as a measure of parasite motility. A 600 μl aliquot of a 10^6 parasites/ml trypomastigote suspension in DMEM/2% FBS was placed in the bottom chamber of a Transwell® plate (Costar) and 100 μl of DMEM/2% FBS was added to the top chamber and incubated at 37°C. Samples (10 μl) were removed from the top chamber at the indicated time points and parasites counted in a hemocytometer. For metabolic studies, trypomastigotes were resuspended at 5×10^7 parasites/ml for 20 min in methionine- and cysteine-free DMEM supplemented with 2% dialyzed FBS, prior to addition of 100 μCi of ^{35}S-TransLabel™ (ICN Biomedicals). At the times indicated, triplicate samples (10 μl) were spotted onto 0.45 μm filters (Millipore), precipitated with 10% (v/v) TCA and the amount of ^{35}S incorporation was determined.

Oligopeptidase B activity assay

Oligopeptidase activity in soluble *T.cruzi* extracts was determined by measuring the release of the fluorescent group 7-amino-4-methyl-coumarin (AMC) from the synthetic substrate Z-FR-AMC. A 100 μl aliquot of parasite extracts (~10 μg protein) was added at t_0 to 1.9 ml of 50 mM Tris pH 8.0, 150 mM NaCl containing 20 μM Z-FR-AMC, and the fluorescence emission at 440 nm was recorded continuously for 5 min following excitation at 380 nm.

Generation of catalytically inactive recombinant oligopeptidase B by site-directed mutagenesis

Based on the sequence homology of the *T.cruzi* oligopeptidase B with the prolyl oligopeptidase family, and the presence of a consensus sequence which demarcates the active site serine residue in these enzymes (Barrett and Rawlings, 1992), Ser565 was predicted to be the active

site serine in the *T.cruzi* enzyme. Catalytically inactive recombinant oligopeptidase B was generated by a single amino acid substitution of Ser565 by Thr565 using the QuickChange™ Site-Directed Mutagenesis Kit (Stratagene). The primer sequences used for the codon substitution were 1, 5′-GCATGTGAAGGGCGTACGGCGGGAGGATTGTTA-3′; and 2, 5′-TAACAATCCTCCCGCAGTACGCCCTTCACATGC-3′. Wild-type (rPEP) and mutant (S→T565 rPEP) were cloned into the pET19b expression vector (Novagen) and expressed in *Escherichia coli* as N-terminal polyhistidine-tagged recombinant proteins as previously described (Burleigh *et al*., 1997). Peptidase activity was assayed using Z-FR-AMC as described above; no hydrolysis of Z-FR-AMC was detected with S→T565 rPEP.

Cell invasion assays

Mammalian cells were plated at a density of 2.5×10^4 cells/cm^2 in DMEM/10% FBS on 12 mm round coverslips placed in 6 cm plastic tissue culture dishes and grown for 48 h at 37°C in a humidified atmosphere containing 5% CO_2. Coverslips with attached cells were washed briefly with DMEM/2% FBS immediately prior to incubation for 1 h at 37°C with purified trypomastigotes at $1–5\times10^7$ parasites/ml. For cell pre-treatments, drugs were added at the following final concentrations: 0.5 μM thapsigargin (Sigma) from a 10 mM stock solution in dimethylsulfoxide (DMSO) for 30 min at 37°C; 80 μM MAPTA-AM (Molecular Probes) from a 10 mM stock in DMSO, with the addition of 0.05% (w/v) pluronic acid (Molecular Probes) for 1 h at 37°C. Drug-treated cells were rinsed three times with fresh DMEM/2% FBS before incubation with parasites. In other experiments, *T.cruzi* trypomastigotes were pre-treated with the peptidyl fluoromethyl ketones Z-Phe-Arg-FMK (Z-FR-FMK) or Z-Phe-Ala-FMK (Z-FA-FMK) at a final concentration of 50 μM for 15 min at 37°C before incubation with mammalian cells. Infected cells were washed, fixed in 2% (w/v) paraformaldehyde/PBS and the number of intracellular parasites was determined by immunofluorescence as described previously (Tardieux *et al*., 1992).

Trypanosoma cruzi infections of mice

Groups of three or five 8-week-old female C_3H mice were infected by intraperitoneal injection with 1×10^6 or 2×10^6 purified tissue culture trypomastigotes in 100 μl of PBS. Parasitemia was monitored on a daily basis beginning at day 3 post-infection. A 5 μl aliquot of blood obtained from the tail vein was placed under a 22×22 cm glass coverslip (Fisher) and the number of trypomastigotes in 50 fields was counted upon examination by phase-contrast microscopy under 400× magnification (Nikon Labophot). The number of parasites/ml of blood was calculated as described previously (Andrews *et al*., 1985).

Time-lapse fluorescence video microscopy

The Ca^{2+}-signaling activity associated with soluble parasite extracts was detected at the single cell level, using time-lapse fluorescence video microscopy, as previously described (Burleigh *et al*., 1997). Briefly, 80 μl of parasite soluble extracts was added to NRK cells (pre-loaded for 1 h with 5 μM fluo 3-AM, 0.05% pluronic acid, 0.5 mM probenecid) 30 s after initiation of the time-lapse recording. In some experiments, parasite soluble extracts were pre-incubated with protease inhibitors and/or recombinant oligopeptidase B at a final concentration of 40 μg/ml for 5 min at 37°C, prior to addition to NRK cells. Fluorescent images were collected with a video camera (CCD-C72; Dage/MTI) through a 25× objective and recorded on an optical laser memory disk (TQ-3031F; Panasonic), at time-lapse intervals of 1 s using a computer-controlled shutter system (MetaMorph, Universal Imaging). Pseudocolor images were transferred to Adobe Photoshop (Adobe Systems), composed and printed in a high resolution color printer (XLS 8600PS; Eastman Kodak Co.).

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