Trypanosoma cruzi Prolyl Oligopeptidase Tc80 Is Involved in Nonphagocytic Mammalian Cell Invasion by Trypomastigotes*

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Trypanosoma cruzi, a flagellated protozoan parasite able to invade a wide variety of mammalian cells. To have access to the target organs/cells, the parasite must cross the basal laminae and the extracellular matrix (ECM). We previously characterized an 80-kDa protease (Tc80) secreted by the infective trypomastigotes that hydrolyzes native collagens and might be involved in infection by degrading ECM components. Here, we present evidence indicating a role for Tc80 in the invasion of nonphagocytic cells. Tc80 was classified as a member of the prolyl oligopeptidase (POP) family of serine proteases and was also found to hydrolyze fibronectin. Selective inhibitors for POP Tc80 were synthesized that blocked parasite entry into cells. Blockage occurred when trypomastigotes were preincubated with irreversible inhibitors but not after host cell preincubation, and the blockage correlated with inhibition of POP Tc80 activity in treated parasites. These data and the enzyme location inside a vesicular compartment close to the flagellar pocket, a specialized domain in endocytosis/exocytosis, strongly suggest a role for POP Tc80 in the maturation of parasite protein(s) and/or, after secretion, in a local action on parasite or host cell/ECM components required for invasion.

Trypanosoma cruzi, a flagellated protozoan, is the etiologic agent of Chagas’ disease, a chronic incurable illness prevalent in Latin America (1). In vertebrate hosts, T. cruzi transmission is carried out through reduvid bug feces contaminated with metacyclic trypomastigotes that infect cells via mucosa or skin wounds. The parasite can infect and multiply inside a broad range of mammalian tissues or cells. Trypomastigotes penetrate nonphagocytic cells by a nonconventional mechanism involving host cell lysosome recruitment and their fusion at the parasite attachment site to form a parasitophorous vacuole (2). An active-amine pore-forming protein and a neuraminidase activity synergistically promote the escape from the parasitophorous vacuole to the host cell cytoplasm, where it differentiates into the dividing amastigote form (3). After multiplying, the amastigotes differentiate into trypomastigotes, which are released into the extracellular medium. To have access to different cell types, the trypomastigote must cross the basal laminae (the barrier surrounding organs and blood vessels) and ECM. Specific interactions between trypanomastigote surface molecules and basal laminae/ECM components, such as collagen (4, 5), fibronectin (6), and laminin (7), have been described, which may facilitate the trypomastigote migration through ECM or its interaction with the host cell membrane. T. cruzi proteases play important roles in the host-parasite relationship. They have been involved in nutritional functions, metacyclogenesis, and host cell invasion (8–11). A serine protease, called oligopeptidase B, plays a central role in host cell invasion by generating a Ca$^{2+}$-signaling factor required for the host lysosome recruitment at the invasion site (12–14). Development of specific inhibitors of T. cruzi proteases seems to be a promising approach to novel chemotherapy for Chagas’ disease (15).

We characterized a T. cruzi 80-kDa protease (Tc80), secreted by the infective trypomastigote form, that specifically hydrolyzes purified human collagens types I and IV and native collagen in rat mesentery at neutral pH (16). By its property of cleaving peptide bonds at the carboxyl side of proline residues, Tc80 protease could be classified as prolyl oligopeptidase (POP), a representative of a new serine peptidase family (EC 3.4.21.26) (17). As POPs, Tc80 protease has an unusual inhibitor profile characterized by its inactivation by diisopropyl fluorophosphate but not by the serine protease inhibitor phenylmethylsulfonyl fluoride and its susceptibility to p-chloromercuribenzoate, a cysteine protease inhibitor, indicating the presence of a cysteine residue at or near the active site (18). Tc80 protease secretion by the trypomastigotes suggests that this enzyme could be involved in the host cell infection by facilitating the parasite migration through ECM or the host cell invasion per se (e.g. by cleaving collagens interacting with integrin receptors). Reversible inhibitors of Tc80 protease have been obtained based on 1) the substrate recognition sequence (Leu-

1 The abbreviations used are: ECM, extracellular matrix; AMC, 7-amidino-4-methylcoumarin; Boc, N-tert-butoxycarbonyl; DAPI, 4’,6-diamidino-2-phenylindol; E-64, trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane; IC50, concentration inhibiting 50%; IFA, immunofluorescence assay(s); PBS, phosphate-buffered saline; POP, prolyl oligopeptidase; Suc, succinyl; Z, benzyloxycarbonyl; Tic, tetrahydroisoquinoline carboxylic acid; TLCK, N4-p-tosyl-l-lysine chloromethylketone.
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Gly-Pro, whose C terminus was modified with the functional groups vinyl sulfone, 2-ketobenzothiazole, or nitrile likely to interact with the active site (18); 2) the screening of combinatorial peptide libraries with phenylpropylcarboxyl-
(tetrahydroisoquinoline{(Tic)-pyrrolidine (inhibitor 1; see Table II), which was highly efficient in inhibiting the enzyme with an IC₅₀ of 7 nM (19–20). In this study, we evaluated the specificity of these inhibitors toward Tc80 protease and other known T. cruzi proteases as well as mammalian POP. Furthermore, irreversible inhibitors were synthesized based on the dipeptide I, possessing an electrophilic group on pyrrolidine in the P1 position and a proline or a proline mimic (Tic) in the P2 position. The importance of Tc80 protease in host cell invasion by the trypomastigotes was investigated by using these inhibitors, and its biological role during this essential step of T. cruzi life cycle is discussed.

**EXPERIMENTAL PROCEDURES**

**Materials—**Z-P-prolinal dimethylacetal and Boc-NFP-aldehyde were purchased from Bachem (Voisins-Le-Bretonneux, France). Z-LGP-vinyl sulfone, -ketobenzothiazole, and -nitrile were synthesized as described (18). Inhibitor 1 was synthesized as previously reported (20). The fluorogenic substrates Suc-GPLGP-AMC (AMC), Z-GGR-AMC, Suc-LIVY-AMC, and Z-FR-AMC were from Sigma. Stock solutions of inhibitors and substrates were made in Me₂SO and stored at -20 °C. Purified fibronectin from bovine plasma was purchased from Calbiochem.

**Synthesis of Irreversible Inhibitors—**Inhibitors were prepared in solution phase from two starting materials, Boc-Tic-Pro-OCH₃ and Boc-Pro-OCH₃, obtained by condensing Boc-Tic and Boc-Pro with Pro-OCH₃, respectively, using 2-(1H-benzo[d]imazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/1-hydroxybenzotriazole as coupling reagents and dichloromethane/dimethylformamide as solvents. After trifluoroacetic acid deprotection, an amino-terminal group was substituted by 4-phenylbutyric acid or benzyl isocyanide. The resulting methyl esters were transformed to the chloromethylketones (lithium diisopropylamine/CHCl₃ as reagents), which led to the corresponding acetyloxymethylketones, and then hydrolyzed into hydroxymethylketones. Details will be described elsewhere.²

**Parasites—**T. cruzi epimastigotes of the Tulahuen strain were grown in liver infusion tryptose medium containing 10% fetal calf serum at 28 °C with continuous agitation (21). T. cruzi tissue culture forms of Berenice, Tulahuen, Y, and 22 strains were maintained in monolayers of L-6, HeLa, or NIH-3T3 cells grown in Dulbecco’s minimal essential medium supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 5% (v/v) fetal calf serum at 37 °C in 5% CO₂. Trypomastigotes were obtained and purified as previously described (2). The β-galactosidase-expressing parasite (Tulahuen lacZ clone 4) was kindly provided by W. Van Voorhis (University of Washington, Seattle, Washington) (22).

**Fluorometric Enzyme Assays—**Tc30 protease, POP Tc80, and oligopeptidase B were purified from epimastigotes according to Refs. 23, 16, and 24, respectively. Cruzipain was a generous gift from Dr. J. Scharfstein (Universidade Federal do Rio de Janeiro, Brazil). Purified proteins resolved as one band on SDS-PAGE. Hydrolysis of AMC substrates were recorded up to 30 min at 440 nm upon excitation at 380 nm in a Hitachi 2030 spectrophotometer or at 460 nm upon excitation at 355 nm in 96-well microtiter fluorescence readers (FL600; Bio-Tek Instruments, Inc.; Fluoroskan, Labsystems, Inc.) at 24 °C as previously described (16). The reaction buffers were 25 mM Tris/HCl, pH 7.5, 125 mM NaCl for POP Tc80 and oligopeptidase B, and 0.1 mM sodium phosphate buffer, pH 6.0, for Tc30 protease and the same buffer with 5 mM dithiothreitol for cruzipain.

**Partial Purification of L-6 Cell POP—**Eight 175-cm² flasks of L-6 cells at 70% confluence were rinsed twice with cold phosphate-buffered saline (PBS; 150 mM NaCl, 5 mM sodium phosphate, pH 7.3), and cells were allowed to detach in cold 5 mM EDTA in PBS. After centrifugation (600 × g, 5 min), cells were resuspended in 10 mM HEPES, pH 7.5, and lysed by two cycles of freezing at −80 °C and thawing. POP activity was detected using 100 μM Suc-GPLGP-AMC. After ultrafiltration (100,000 × g, 1 h, 4 °C) more than 90% of the proteolytic activity was found in the supernatant and precipitated with (NH₄)₂SO₄ solution between 30 and 70% saturation. The precipitate was resuspended in 5 ml of 25 mM Tris/HCl, pH 7.5, and dialyzed against 1 liter of the same buffer for 12 h at 4 °C. The solution was then transferred onto a DIA-SEP-Heparin CL-4B column (Sigma), previously equilibrated with 25 mM Tris/HCl, pH 7.5. The proteins were eluted with a linear gradient to 0.5 M NaCl for 40 min and then with 1.0 M NaCl at 0.5 ml/min flow rate. The proteolytic activity eluted as a single peak between 0.20 and 0.25 M NaCl and was then concentrated on Centricon 30 (Millipore Corp., Bedford, MA) at 4 °C. The solution was then submitted to gel filtration on a Superose 12 column (Pharmacia Biotech) and captured with 25 mM Tris/HCl, pH 7.5, 125 mM NaCl at a 0.3 ml/min flow rate. Activity was eluted as a single peak with a molecular mass of 140 kDa according to the column calibration curve. Active fractions were concentrated on Centricon 30 and used for the enzymatic tests. The reaction buffer was 25 mM Tris/HCl, pH 7.5, 125 mM NaCl containing 100 μM leupeptin, trans-epoxysoxycyclohexylamido-(4-guanidino)butane (E-64), Bestatin, and 1 mM EDTA. These protease inhibitors did not affect the L-6 POP activity. Assays with different AMC substrates showed that the partially purified enzyme has a strict postproline activity (data not shown) and a Kᵢ of 243 ± 30 μM for Suc-GPLGP-AMC (25).

**Enzyme Inhibition—**Inhibition constants for reversible inhibitors were determined using the first-order reaction rate (26). After centrifugation, inhibitor was added to a steady-state enzyme-substrate reaction (26). All of the measurements were performed in the corresponding enzyme reaction buffer. Enzyme was mixed with substrate for 5 min before adding inhibitor (<5% of the total assay volume) and incubated at 24 °C up to 30 min. The inhibitor was in at least 20-fold molar excess over enzyme. Reactions were conducted under experimental conditions where less than 5% substrate was hydrolyzed. Under these conditions, the enzyme inhibition rate was calculated from the slope of the double-reciprocal plot and was used to determine the inhibition constant (Ki) and the inhibition type.

**Irreversible inhibitors —**Irreversible inhibitors 4a–4c were derived from the reversible inhibitor 1 (see Table II) were evaluated on the POP Tc80 activity in duplicate with five different concentrations of inhibitor ranging from 1 to 50 μM and final AMC substrate concentration of 3.33 M. For the assay, 30 μl of inhibitor solution were added to 30 μl of Suc-GPLGP-AMC (final concentration 11 μM). The reaction was started by adding 30 μl of the enzyme solution and stopped after 15 min of incubation at room temperature by the addition of 100 μl ethanol, and the fluorescence was measured. Concentration. The active enzyme was needed to reduce activity by 50% (IC₅₀) was determined graphically from the curve of the percentage of inhibition versus the inhibitor concentration.

**Inactivation of POP Tc80 by inhibitors 2–4 was followed by preincubating at 24 °C an aliquot of inhibitor in a buffered enzyme solution in 200 μl. The inhibitor was in at least 20-fold molar excess over enzyme. Inhibitor concentrations (i) varied from 0 to 40 nM, depending on the molecule. Aliquots were removed at regular timed intervals, and residual activity (i) was determined in duplicate by starting the reaction with the substrate Suc-GPLGP-AMC. Pseudo-first-order inhibition rate constants (kₙₐₜ) were obtained from plots of In i/v₀ versus time (t), where v₀ represents the activity prior to the addition of inhibitor. The data were fit to the equation, v = v₀ × e⁻kt, by using a nonlinear regression analysis software (Kaleidagraph, Graphing and Data Analysis Program, Synergy Software). For the determination of k and kᵢ values (limiting rate constant for enzyme inactivation and dissociation constant for the binary enzyme-inactivator complex, respectively), the pseudo-first-order inhibition rate constants (kₙₐₜ) obtained were plotted versus the concentration of inhibitor (i), and the data were fitted to the equation, kₙₜ = k × i/(kᵢ + i), where k represents the rate of inactivation of POP Tc80 by inhibitors 2–4 was evaluated in quadruplicate after dialysis of the complex. Inhibitors 4a, 2a, 2c (15 μM), 2b (30 μM), 3a, 2b, or 3c (75 μM) were added to enzyme assay buffer in the presence of 3.33 M Me₂SO as final concentration (500-μl total volume). The reversible inhibitor 1 (15 μM) was ²S. Vendeville, F. Goossens, M.-A. Debreu-Fontaine, V. Landry, E. Davioud-Charvet, P. Grellier, S. Scharpe, and C. Sergheraert, submitted for publication.

³J. Santana, personal communication.
used as a negative control. 250 μl of each reaction mixture was exten-
sively dialyzed on Centricon 30 at 4 °C for 1 h against the reaction
buffer containing 3.33% Me2SO, with three buffer exchanges. Recovery
of enzyme activity was determined by adding the substrate under
standard assay procedures and compared with the activities from cor-
responding control reaction mixtures in the absence of inhibitor (dialyzed and nondialyzed).

Internal Amino Acid Microsequencing—POP Tc80 was purified by
SDS-PAGE. After Coomassie Blue staining, the band of interest was
excised from the gel and subjected to in-gel proteinolysis (28). Peptides
were purified by reverse phase high pressure liquid chromatography on a
Beckman Ultrasphere ODS column (2.1 mm × 150 mm, 5 μm). Elution was
performed as described above except that experiments were per- 47080
formed in Lab-Tek chambers (Nunc Inc., Naperville, IL). Immediately
after parasite removal, cells were fixed with 3.7% formaldehyde in PBS for 30
min at room temperature, washed twice with PBS, and incubated with
5% nonfat milk in PBS for 30 min. Cells were then incubated for 1 h
at 37 °C with the proteinase inactivated by previous treatment of the
reactant with 2 M Tris–HCl, pH 7.5. Controls consisted of proteinase-in- 20
sensitive parasite cultures and from reaction mixtures of proteinases,
before methanol (20:1 ratio) in the absence of inhibitor. To evaluate the effects of inhibitors on the trypomastigote diferen-
tiation into amastigotes, L-6 cells were maintained with parasites for 1
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NIH-3T3, or HeLa cell cultures plated 24 h before in 24-well plates
(0.5 × 104 cells/well) with a trypomas-
tigotes/cell ratio of 20:1. After a 3-h incubation at 37 °C, cells were
washed three times with culture medium without inhibitor, and cells
were maintained for 72–96 h in the absence of inhibitor before methanol
fixation and Giemsa staining. The percentage of infected cells was
determined by counting 500 host cells under × 50 magnification. The percentage of inhibition of the host cell invasion was calculated by
comparison with the percentage of infected cells in control wells. For the experiments performed with the β-galactosidase-expressing parasite
Trypomastigotes LaCZ clone 4, cells were lysed with 1% Nonidet P-40 after 96 h of
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assay using serum from patients with chronic Chagas’ disease.

Inhibition Assays of Intracellular Amastigote Replication and Host Cell Proliferation—Trypomastigotes were incubated with mammalian
cells (ratio 10:1, respectively) in 24- or 96-well plates for 24 h as
described above. Cells were then washed three times and incubated for
48 h with culture medium containing different concentrations of inhibit-
or before fixation and Giemsa staining. The number of infected cells
was measured using 20 microscopic fields under × 20 magnification.
The number of amastigotes per cell was determined by counting
100 infected cells under × 100 magnification. For host cell prolifer-
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presence of different concentrations of inhibitors for up to 5 days. Cell
proliferation was determined by using the XTT-based colorimetric as-
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cultures received an equivalent amount of Me2SO instead of inhibitor.
Inhibition of proliferation was determined by comparison with control
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ber AW325010). Interestingly, BLAST searches using the 85 residues deduced from this T. cruzi expressed sequenced tag indicated a highly significant similarity to the C-terminal end of various bacterial and mammalian POPs in the data bases (Fig. 1).

Asp and His residues were found in a highly conserved stretch of amino acids between POP sequences that could correspond to the catalytic triad residues of the active site (e.g. Ser164, His680, and Asp644 for porcine muscle POP (30)). No similarity was found in the data bases for the third peptide (EGPPYFVLLR). Such data, in conjunction with the biochemical properties, strongly support the belonging of Tc80 proteinase to the prolyl oligopeptidase family (EC 3.4.21.26). The enzyme was thus named POP Tc80.

To localize POP Tc80, a specific antiserum was produced using the purified proteinase that only reacts by Western blotting with an 80-kDa protein on epimastigote, trypomastigote, and amastigote extracts co-migrating with the purified POP Tc80 (data not shown). A clear difference of cellular localization was observed according to the parasite forms. In tryomastigotes, labeling was mainly associated with vesicles concentrated in proximity to the kinetoplast at the posterior end of the cell (Fig. 2, a, b, and c). Intense fluorescence was concentrated in structures close to the nucleus (Fig. 2, d).

In amastigotes, POP Tc80 is mainly located in vesicles (Ve) concentrated between the kinetoplast (k) and the intracelllar flagellum (Fl) extremity. In amastigotes, POP Tc80 is located in whole cytoplasm and concentrated in structures close to the nucleus (N). a fine labeling of the flagellum was also observed (data not shown).

Table I shows the Ki values of inhibitors of the T. cruzi proteases. All of the inhibitors were inefficient to inhibit the activity of the oligopeptidase B (K<sub>i</sub> values >1 mM, except for Z-LGPG-vinyl sulfone: K<sub>i</sub> = 329 μM), Z-P-prolinal dimethylacetal and Boc-NFPe-aldehyde, were also evaluated. All of these compounds are reversible competitive inhibitors of POP Tc80 (Ref. 18; data not shown). Selected T. cruzi proteases were oligopeptidase B, a serine protease involved in the host cell invasion process (12–14); cruzipain, a cathepsin-L like protease whose inhibition impairs parasite development (15, 35); and Tc30 cysteine-protease, a cathepsin-B like protease (23, 36), since there is evidence that resistance to cruzipain inhibitors is associated with a high level of cathepsin-B expression by the parasite (37). No hydrolysis was observed in controls consisting of TLCK-inactivated POP Tc80.
Ki values were determined by the progress curve method as described under "Experimental Procedures" using the following fluorogenic substrates: Suc-GPLGP-AMC for POP Tc80; Z-GGR-AMC for serine oligopeptidase B; Suc-LLVY-AMC for Tc30 cysteine protease; and Z-FR-AMC for cruzipain. Ki values are the means ± S.D. of several independent experiments.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>POP Tc80</th>
<th>Oligopep. B</th>
<th>Tc30 cysteine protease</th>
<th>Cruzipain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Z-P-prolinal dimethylacetal</td>
<td>1.120 ± 0.120⁰</td>
<td>&gt;10,000⁰</td>
<td>183.5 ± 47.3³</td>
<td>31.3 ± 12.6⁶</td>
</tr>
<tr>
<td>Boc-NFP-aldehyde</td>
<td>0.280 ± 0.110⁰</td>
<td>&gt;10,000⁰</td>
<td>81.5 ± 17.3³</td>
<td>16.5 ± 6.7⁷</td>
</tr>
<tr>
<td>Z-LGP-vinylsulfone</td>
<td>35.5 ± 14.6⁴</td>
<td>&lt;1000⁰</td>
<td>136 ± 39⁶</td>
<td>293 ± 45⁷</td>
</tr>
<tr>
<td>Z-LGP-ketobenzothiazole</td>
<td>0.139 ± 0.064⁴</td>
<td>&gt;1000⁰</td>
<td>132 ± 45⁶</td>
<td>&gt;1000⁶</td>
</tr>
<tr>
<td>Z-LGP-nitrile</td>
<td>0.038 ± 0.014⁴</td>
<td>&gt;5000⁰</td>
<td>132 ± 45⁶</td>
<td>&gt;1000⁶</td>
</tr>
<tr>
<td>1</td>
<td>0.0045 ± 0.0017⁴</td>
<td>&gt;10,000⁰</td>
<td>&gt;10,000⁶</td>
<td>&gt;10,000⁶</td>
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⁰ See Ref. 18.  
⁴ n = 3.  
⁷ n = 7.

The synthesis of irreversible inhibitors based on inhibitor 1, possessing a proline mimic (Tic) (inhibitors of series 2 and 4, Table II) or a proline in the P2 position (inhibitors of series 3, Table II) Several substituents were introduced on the pyrrolidine moiety (P1 position) to act as electrophilic groups toward the active site of the enzyme: the chloromethylketone (inhibitors 2a, 3a, and 4a), acetylomethylketone (inhibitors 2b and 3b), and hydroxymethyl ketone (inhibitors 2c and 3c) groups. These electrophilic groups inhibit serine proteases in an irreversible manner (38).

POP Tc80 was inactivated by inhibitors 2–4 in a time-dependent manner, as illustrated with the chloromethylketone 4a (Fig. 4A). In the same conditions, no time-dependent inhibition of the enzyme was observed in the presence of the reversible inhibitor 1 (Fig. 4B).

The two most potent of these new inhibitors were the chloromethylketone 2a and 4a, with IC₅₀ values below 5 nM and kI/Kᵣ values of 0.38 and 0.30 μM⁻¹ s⁻¹, respectively (Table II). After extensive dialysis, only 0.9% of the enzymatic activity was recovered with inhibitor 4a, indicating that the inhibition was irreversible (Table II). In the same conditions, 83% of the activity was restored with inhibitor 1. For the other inhibitors, 0–12.5% of the enzymatic activity was recovered, suggesting...
that the inhibition was also irreversible or might be due to a very tight binding complex.

Among these different inhibitors, chloromethylketone derivatives were the most efficient at inhibiting the L-6 cell invasion by trypomastigotes, inhibitor 4a being the best with an IC₅₀ value of 15 nM. Furthermore, inhibitor 4a was found to block the invasion by trypomastigotes of the nonphagocytic NIH-3T3 and HeLa cells with similar IC₅₀ values of 11.5 and 14 nM, respectively (Fig. 5).

Inhibition of the Host Cell Invasion Results from the POP Tc80 Inhibition Rather than from the Host Cell POP Inhibition—To discriminate the involvement of POP Tc80 and the host cell POP in the invasion step, trypomastigotes were preincubated for 2 h with inhibitor 4a, washed three times to eliminate the inhibitor, and incubated for 1 h with L-6 cells in the absence of inhibitor. An inhibition of the invasion similar to that measured when inhibitor 4a was maintained during the infection was observed (Fig. 6). In contrast, no significant inhibition of the invasion was recorded when L-6 cells were preincubated for 2 h with inhibitor 4a and incubated with trypomastigotes for 1 h in the absence of inhibitors (Fig. 6).

Similar results were obtained using a direct method to quantify host cell invasion, the immunofluorescence in-out staining of parasites immediately after invasion (2). Trypomastigotes were preincubated for 1 h with the irreversible inhibitor, washed, and incubated for 1 h with L-6 cells in the absence of inhibitors. After washes, cells were fixed with formaldehyde, and IFA associated with parasite DAPI staining allowed us to discriminate the internalized parasites from those attached to the host cells. A typical experiment is depicted in Fig. 7. A decreased number of internalized parasites was observed with increasing concentrations of inhibitors.

To ascertain that the preincubation of trypomastigotes with inhibitor 4a resulted in a POP Tc80 inhibition, parasites were extensively washed after 2 h of incubation with 10 μM inhibitor 4a and then lysed, and POP Tc80 as well as oligopeptidase B and cruzipain activities were measured. More than 80% of inhibition of POP Tc80 activity was observed. In contrast, oligopeptidase B and cruzipain activities were only weakly affected by the treatment (Fig. 8).

Taken together, these data strongly suggest that inhibition of the invasion by the chloromethylketone derivative 4a resulted from an inhibition of POP Tc80 rather than of the host cell POP or other parasite proteases.

To analyze the effects of POP Tc80 inhibitors on the intracellular differentiation of trypomastigotes into amastigotes, cells were maintained with parasites for 1 h in the absence of inhibitor, washed twice and incubated for another 1 h to obtain a complete parasite internalization. After 24 h of exposure to inhibitor 4a, parasite morphology was estimated by IFA, and they all had the typical rounded shape of amastigotes (data not shown), indicating that inhibitor 4a had no effect on the trypomastigote differentiation into amastigotes.

**DISCUSSION**

By its biochemical properties (16, 18) and the high similarity of an internal amino acid sequence to sequences containing the Asp residue of the catalytic site of the POP active site (Fig. 1), the Tc80 proteasine of T. cruzi can be classified as a member of the prolyl oligopeptidase family of serine peptidases. POPs are
ubiquitous proteases found in various organisms including prokaryotes (31), yeast (39), higher eukaryotes (40), and plants (41). Their biological roles are not well known. POPs seem to be unable to hydrolyze peptides longer than 30 residues (33). Access to its catalytic site is impeded by a tunnel of an unusual propeller excluding large structured peptides from the active site (30). POPs were proposed to be involved in the metabolism of proline-containing neuropeptides or hormones (42). How- ever, the substrates of POPs in vivo remain to be identified. In contrast, POP Tc80 specifically hydrolyzes collagens and fibronectin but not other large proteins such as albumin or laminin, or small proteins (insulin or cytochrome c) (Ref. 16; this study). The ability to hydrolyze specific large molecules suggests that POP Tc80 might show divergence with other POPs.

POP Tc80 selectivity for specific ECM components and its secretion by the infective parasite form, the trypomastigote, led us to hypothesize that POP Tc80 might be involved in the host cell invasion either by facilitating the parasite progression through ECM or the invasion per se by modifying interactions of ECM components with the host cells. Indeed, *T. cruzi* interaction with ECM is essential for a successful infection. The parasite must cross the endothelium of blood vessels, the basal laminae, and the extracellular connective medium before invading targeted cells. Receptors for basal laminae or ECM components on the surface of trypomastigotes have been identified and are involved in parasite migration through ECM or parasite attachment to host cells (4–7). Proteases secreted by the infective parasite and active on basal laminae or ECM
components could then be important during this step of the *T. cruzi* life cycle. POP Tc80, by its ability to hydrolyze collagen type IV, a basal lamina component, collagen type I, and fibronectin, ECM components, represents a putative candidate for such a purpose.

To investigate the biological role of POP Tc80, we looked for specific inhibitors (18–20). Their selectivity for POP Tc80 was evaluated by comparison with the inhibition of well-characterized *T. cruzi* serine and cysteine proteases (Table I) and the rodent L-6 cell POP. Surprisingly, a moderate selectivity with *T. cruzi* cysteine proteases was measured for the commercial POP inhibitors, Z-P-proline dimethylacetalate and Boc-NFp-aldehyde (the lowest factors of selectivity were of 28 and 59 for cruzipain, respectively; Table I). The vinyl sulfone derivative based on the POP Tc80 recognition sequence Leu-Gly-Pro was synthesized to react with the cysteine residue in close proximity to the catalytic site as a Michael acceptor (e.g. Cys255 for porcine muscle POP (30)). Covalent attachment to this cysteine residue is predicted to inactivate POPs by steric hindrance (30).

However, it has a weak inhibition activity on POP Tc80, and no selectivity or a weak selectivity was observed between the serine and cysteine proteases tested. It was, however, the only inhibitor to inhibit significantly the serine oligopeptidase B. This suggests the presence of a crucial cysteine residue close to the catalytic site in the folded enzyme, as observed for POPs.

The inhibitor was noted in *T. brucei* POP Tc80 also blocked the invasion, indicating that the inhibitors act on parasite components rather than on host cell components. Furthermore, the selective inhibition of the POP Tc80 activity in trypomastigotes pretreated with the irreversible inhibitor (Fig. 8) strongly suggests that the inhibition of the invasion results from an inhibition of the POP Tc80 activity rather from an interaction with other parasite components. However, this remains to be confirmed. Several attempts to modify inhibitors with specific chemical groups (e.g. biotin) that would allow one to identify parasite proteins interacting with the inhibitor were unsuccessful. Such modifications resulted in a dramatic loss of affinity for purified POP Tc80.

Invasion is an active and complex process whose molecular partners are beginning to be characterized, involving, in particular, heparin-binding protein, fibronectin-binding protein, members of large multigene families including trans-sialidase/sialidases and mucins of the parasite, and ECM components, integrins, and cell surface carbohydrates of the mammalian cells (3). Recent evidence indicates that the invasion process requires early signal transduction events triggered in the host cells as well as in the parasite by parasite-host cell interactions. An increase of intracellular free calcium (2, 48) and cyclic AMP (49), tyrosine phosphorylation (50, 51), mitogen-activated protein kinase activation (50), and transforming growth factor β receptor (52) are all important in invasion. *T. cruzi* oligopeptidase B is a key component in the cascade for the generation of a Ca^2+^-signaling agonist for nonphagocytic mammalian cells required for the recruitment and fusion of host lysosomes that occurs at the parasite attachment site (14). Since POPs are mainly identified as prohormone-processing enzymes, it could be considered that POP Tc80 might be involved in this complex processing cascade. However, trypomastigote extracts treated with 10 μM inhibitor 1 or inhibitor 4A, a concentration that totally inhibits POP Tc80 activity, did not inhibit the generation of the transient increase of intracellular free calcium in NRK fibroblasts. These data rule out POP Tc80 for playing a role in the generation of Ca^2+^-signaling agonist.

**FIG. 8.** Inhibition of POP Tc80 after incubation of trypomastigotes with the irreversible inhibitor 4A. Trypomastigotes (5 × 10^6 parasites, Y strain) were incubated for 2 h at 37 °C with 10 μM inhibitor 4A. After four washes, parasites were lysed in distilled water by freezing/thawing. POP Tc80 (GPLGP), oligopeptidase B (GGPG), and cruzipain (FR) activities were measured as described under “Experimental Procedures.” Percentages of inhibition of activities were determined by comparison with the activities measured in control parasitelmaintained with MeSO instead of inhibitor. Data are the means ± S.D. of three experiments.

**Table I.** Popuiarities of POP Tc80 relative to human POP according to the residue at the subsite P3, supporting divergence between POP Tc80 and POPs.4

Since entry of *T. cruzi* into a wide variety of cell types is facilitated by the parasite’s properties to bind ubiquitous tissue components such as those found in ECM, we looked for the inhibitory effect of POP Tc80 inhibitors on the invasion of adherent nonphagocytic cells. We found that they blocked the invasion process in a dose-dependent manner with an efficiency that reflects their inhibitory effects on the POP Tc80 activity. Preincubation of trypomastigotes with irreversible inhibitors of POP Tc80 also blocked the invasion, indicating that the inhibitors act on parasite components rather than on host cell components.

The discrepancy of inhibition of POP Tc80 relative to human POP according to the residue at the subsite P3, supporting divergence between POP Tc80 and POPs.

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4 S. Vendeville, personal communication.

these ECM components constitute good candidates. A local hydrolysis of these components at the invasion site may be required for the parasite entry. Such hydrolysis could allow a disconnection of the trypomastigote surface proteins from ECM components necessary for penetration. Furthermore, ECM components are connected to the host cell membrane by integrins, and their local hydrolysis by POP Tc80 may trigger a signal that prepares cells for parasite entry (e.g. tyrosine phosphorylation (50) and cytoskeletal rearrangements (2) that are associated with invasion). However, POP Tc80 action on parasite components cannot be excluded, particularly if taking into account its localization in a vesicular compartment that could be involved in protein export. Since POPs are mainly known as prohormone-processing enzymes, localization in such a compartment is the ideal place for a POP Tc80 involvement in maturation of parasite factors essential for invasion. Further work will be required to investigate the biological role of POP Tc80 during trypomastigote invasion. The specific inhibitors we have developed will be useful tools for that purpose.

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