Proteasome Activity Is Required for the Stage-specific Transformation of a Protozoan Parasite

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Summary

A prominent feature of the life cycle of intracellular parasites is the profound morphological changes they undergo during development in the vertebrate and invertebrate hosts. In eukaryotic cells, most cytoplasmic proteins are degraded in proteasomes. Here, we show that the transformation in axenic medium of trypomastigotes of *Trypanosoma cruzi* into amastigote-like organisms, and the intracellular development of the parasite from amastigotes into trypomastigotes, are prevented by lactacystin, or by a peptide aldehyde that inhibits proteasome function. Clasto-lactacystin, an inactive analogue of lactacystin, and cell-permeant peptide aldehyde inhibitors of *T. cruzi* cysteine proteinases have no effect. We have also identified the 20S proteasomes from *T. cruzi* as a target of lactacystin in vivo. Our results document the essential role of proteasomes in the stage-specific transformation of a protozoan.

Infection by *Trypanosoma cruzi*, the causative agent of Chagas' disease, is initiated by metacyclic trypomastigotes present in the feces of triatomine bugs. The trypomastigotes invade host cells and enter the cytoplasm, where they transform into amastigotes. The amastigotes replicate and, a few days later, transform back into trypomastigotes, rupture the host cells, and invade the bloodstream (1). Thus, on two occasions during its intracellular stage, *T. cruzi* undergoes shape and volume changes, restructures its flagellum and kinetoplast, and synthesizes new sets of surface molecules. These striking modifications are precisely timed, take place in an orderly fashion, and must involve selective degradation of cytoplasmic proteins.

In eukaryotic cells, most proteins in the cytoplasm and nucleus are degraded not in lysosomes, but within proteasomes, after they are marked for destruction by covalent attachment of ubiquitin (Ub)[1] molecules (2–5). In addition to their role in nonlysosomal protein turnover, proteasomes are involved in specific cellular functions, including the following: the programmed inactivation of mitotic cyclins, transcription factors, and transcriptional regulators; the elimination of mutated or damaged proteins; and antigen presentation. The function of the proteasomes is also tightly regulated, and their structure may vary to match function (6–7).

The experiments described below were designed to document the participation of proteasomes in the developmental pathways of protozoan parasites. *T. cruzi* has an advantage as an experimental model because its trypomastigote form can be induced to change rapidly into amastigotes in axenic medium. The resulting amastigote-like parasites cannot be distinguished from intracellular amastigotes by light or electron microscopy, or by stage-specific surface markers. Thus, in this model, the effects of protease inhibitors on transformation can be studied independently from their effect on the cells of the host.

[1]*Abbreviations used in this paper:* BSA, bovine serum albumin; CAPS, (3-[Cyclohexylamino]-1-Propanesulfonic acid), Ch-L, Chymotrypsin-like; EDTA, ethylene di-amino tetra acetic acid; E-64, trans-epoxysuccinyl-L-leucylamido-3-methyl-butane ethyl ester; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; MES, (2-[N-morpholino]-ethanesulfonic acid); MG-132, carboxybenzoxyl-leucinyl-leucinyl-leucinal-H; PGPH, peptidylglutamyl peptide hydrolase; T-L, Trypsin-like; Ub, ubiquitin.

Materials and Methods

Cell Lines. LLC-MK_2 fibroblasts were obtained from American Type Culture Collection, Rockville, MD (ATCC CCL-7). L6E9 myoblasts were a gift of Dr. R. Docampo (University of Il-

linois, Urbana-Champaign, IL.). Cells were grown in RPMI 1640 medium supplemented with 10% FCS, 100 μg/ml penicillin, and streptomycin.

Reagents. Protease inhibitors E-64, E-64d, Cbz–Phe–Ala–FMK, Cbz–(S-BZ)–Cys–Phe–CHN2, and fluorogenic substrates were purchased from Sigma Chemical Co. (St. Louis, MO). Lactacystin and clasto-lactacystin were synthesized as previously described (8, 9). MG-132 was from Proscript, Inc. (Cambridge, MA). Chromatography columns and resins were from Pharmacia Biotech AB (Uppsala, Sweden).

Inhibition of Trypomastigote Transformation into Amastigotes. LLC-MK_2 cells were infected with *T. cruzi* trypomastigotes, Y strain (10). 4 d later, the supernatants contained more than 95% trypomastigotes and small number of amastigotes or intermediate forms. Parasite transformation into amastigotes was induced by lowering the pH of the incubation medium (11, 12). To assay for the effect of inhibitors in the transformation, twofold dilutions of each inhibitor were distributed in 96-microwell plates. Dilutions were made with DMEM buffered with 20 mM MES (pH 5.0) containing 0.4% BSA. Lactacystin or clasto-lactacystin, MG-132, E-64, Cbz–(S-BZ)–Cys–Phe–CHN2 and Cbz–Phe–Ala–FMK were prepared at 200 μM, and 50 μl were added to wells to final dilutions of 100–0.78 μM. Depending on the inhibitors used, DMSO dilutions or medium were used as controls. Trypomastigotes were centrifuged (3,000 *g* × 15 min) and resuspended at 2×10^7/ml in DMEM (pH 5.0). 50 μl of this suspension was added to each well, mixed, and incubated for 4 h at 37°C in a 5% CO_2 atmosphere. The plate was centrifuged and the supernatants were removed and replaced by DMEM (pH 7) containing 10% FCS. The plates were reincubated overnight at 37°C in a CO_2 incubator. The percentage of transformed parasites was determined by microscopically scoring 200 cells in each well in a blinded fashion. All experiments were carried out in duplicate.

FACS® Analysis. Parasites (2.5×10^7) were transformed in the presence or absence of proteinase inhibitors as described. At the end of the incubation, parasites were resuspended in 250 μl of DMEM at 4°C, and an equal volume of monoclonal antibodies 2C2 anti-Ssp-4 or 3C9 anti-Ssp-3 (13) was added. The incubation proceeded for 30 min on ice. The suspension was then centrifuged for 7 min at 3,500 rpm in a refrigerated centrifuge (Sorvall RT6000B), using a horizontal rotor. The supernatant was removed, and the parasites were fixed with 4% paraformaldehyde in PBS. After 30 min at 4°C, the fixative was removed and the parasites were washed with 1 ml of cold 0.4% BSA–DMEM. The parasites were then incubated for 30 min with anti-mouse IgG conjugated with FITC. The suspensions were centrifuged, washed with 0.4% BSA–DMEM, resuspended in 50 μl of PBS, and postfixed with 4% paraformaldehyde. The cell suspensions were analyzed in a Becton Dickinson FACScan®.

Inhibition of Development of Intracellular Parasites. L6E9 myoblasts were irradiated with 2,000 rads (14) and plated in 4-well Lab-Tek microchamber slides (NUNC, Naperville, IL). Trypomastigotes were pretreated for 1 h with 10 μM lactacystin or clasto-lactacystin at 37°C. Parasites were washed twice, resuspended in DMEM, and used to infect myoblasts at a parasite to L6E9 cells ratio of 5:1. After 2 h incubation at 37°C, trypomastigotes were removed, and the L6E9 cells were washed with DMEM. To study the effect of inhibitors on invasion, one set of cells was fixed with 4% paraformaldehyde in PBS for 30 min. Extracellular trypomastigotes were detected by immunofluorescence with a polyclonal antibody to *T. cruzi*, and the total number of parasites was determined by staining with Hoechst dye (Sigma) after permeabilization of the L6E9 cells with cold methanol for 10 min. The number of intracellular parasites was calculated by subtracting the extracellular from the total parasites (15). To determine the fate of lactacystin-treated parasites, the remaining infected cell cultures were reincubated at 37°C. At 24, 48, and 72 h, triplicate wells were washed and stained with May–Grunwald–Giemsa. The slides were examined under light microscopy and the number of intracellular amastigotes in 100 cells was counted. Results are expressed as means ± SD.

In another set of experiments, we studied the effect of inhibitors on the transformation of intracellular amastigotes into trypomastigotes. Cell cultures were infected with *T. cruzi* trypomastigotes. 48 h after infection, the cultures were treated for 2 h with 0.75, 1.5, and 3 μM of lactacystin or clasto-lactacystin. The cultures were washed and reincubated at 37°C for an additional 2 d, when the first parasite burst occurred. The culture supernatants were collected and the numbers of exiting trypomastigotes were determined in a Neubauer chamber. To document further the inhibitory effect of lactacystin in the amastigote/trypomastigote transformation, infected cultures were lysed 72, 80, 88, and 96 h after infection with a buffer containing 3% *n*-octylglucopiranoside, 50 mM Tris–HCl (pH 7.4), 0.1 mM EDTA, 20 μM E-64 and 5 μg/ml leupeptin, antipain, and pepstatin. The extracts were analyzed for levels of transialidase, an enzyme expressed in trypomastigotes, but not in amastigotes (16). Measurements were made in triplicate samples, and transialidase activity was expressed as cpm ± SD.

Enzymatic Assays. Proteolytic activity was assayed using as substrate 100 μM fluorogenic peptides diluted in 50 mM Tris–HCl (pH 7.8). 10 μl of chromatographic fractions was added to 90 μl of the fluorogenic peptide, and the mixtures incubated at 37°C for 30 min before quenching with 200 μl of cold ethanol. Fluorescence was measured on a Fluoroskan II (Labsystems, Helsinki, Finland) using an excitation wavelength of 380 nm and an emission wavelength of 440 nm. Fluorescence values were compared with a standard curve prepared with 7-amino-4-methylcoumarin or 2 naphthylamide, as described by Rivett et al. (17). The following fluorogenic peptides were used: Suc–Leu–Leu–Val–Tyr–MCA and Suc–Ala–Ala–Phe–MCA to measure chymotrypsin-like (Ch-L) activity, Cbz–Leu–Leu–Glu–2–naphthylamide to measure peptidylglutamyl peptide hydrolyzing activity (PGPH), and Boc–Leu–Arg–Arg–MCA to measure trypsin-like activity (T-L). Cruzipain activity was measured using Cbz–Phe–Arg–AMC as a substrate.

Purification of T. cruzi Proteasomes. For purification of proteasomes, *T. cruzi* epimastigotes (Y strain) were used. Parasites were harvested from 3 l of 6-day cultures by centrifugation at 2,000 *g* for 20 min and washed three times with PBS. Parasites were suspended in 5 v of 20 mM Tris–HCl, 1 mM EDTA, sonicated, and the homogenate clarified by centrifugation. The pellet was discarded and the supernatant was centrifuged at 100,000 *g* for 1 h. The 100,000 *g* supernatant was concentrated by filtration in a Centricon 10 unit (Amicon, Beverly, MA), and fractionated by fast perfomance liquid chromatography (FPLC) using a Superose 6 HR 16/50 column equilibrated with 25 mM Tris–HCl, 1 mM EDTA (pH 7.5). Fractions of 1.2 ml were collected and assayed for Ch-L activity. The active fractions were again assayed in the presence of 50 μM of either lactacystin or E-64. Those that were inhibited by lactacystin but not by E-64 were pooled and loaded onto a Mono-Q 5/5 column equilibrated with 20 mM Tris–HCl (pH 8.0). Bound proteins were eluted using a 0–1M KCl linear gradient in 20 mM Tris–HCl (pH 8.0). Fractions of 0.5 ml were collected and assayed for proteolytic activity as above. The active fractions eluted at aproximately 400–500 mM KCl. They were pooled and concentrated in a Centricon 10 unit. The concen-

trated sample was loaded onto a Superose 6 HR 16/30 equilibrated with 25 mM Tris–HCl, 1 mM EDTA, (pH 7.5). Fractions of 0.6 ml were collected and assayed for Ch-L, T-L and PGPH activities (17).

Protein Determination. Protein concentration was determined by the Bradford method (18), using BSA as a standard.

Electrophoretic Techniques. Samples were analyzed by SDS–PAGE electrophoresis according to Laemmli (19) in a 12% separating gel and 3% stacking gel. Two-dimensional gel SDS-PAGE electrophoresis was performed as in O'Farrell (20).

Antibodies and Immunoprecipitation Studies. Anti-*T. cruzi* proteasome antibodies were obtained by injecting rabbits with three doses of 50 μg of purified proteasomes using Titer Max (CytRx Corp, Norcross, GA) as adjuvant. The antiserum strongly reacted with the 25–35 kD proteasome subunits by Western blotting. Two weaker unidentified bands of about 70 kD were also seen on the blots (data not shown). For immunoprecipitation studies, aliquots of 3×10^7 trypomastigotes were incubated for 3 h in transformation medium alone, or in the presence of lactacystin or clasto-lactacystin. The parasites were washed, resuspended in 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, and sonicated. Sonicates were centrifuged for 5 min at 10,000 *g*. The supernatants were pretreated with preimmune rabbit serum and protein A–Sepharose (Pharmacia Biotech, Uppsala, Sweden), and then incubated overnight with anti-*T. cruzi* proteasome antisera diluted 1:250. The immunocomplexes were collected by incubation with 100 μl of a 50% suspension of protein A–Sepharose. The immunoprecipitates were washed and Ch-L activity measured in the presence or absence of protease inhibitors, as explained in the text and figure legends. Experiments were performed in triplicate and expressed as fluorescence units ± SD.

Electron Microscopy. Purified proteasomes (50 μg/ml) were attached to carbon-coated and glow-discharged formvar film for 1 min, and subjected to negative staining with 1% uranyl acetate as described (21). Electron micrographs were recorded with magnification of 80,000 in a Zeiss EM 910 electron microscope.

NH_2-terminal Sequences. Samples were separated on SDS-PAGE, transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore; Milford, MA) using CAPS (Sigma) pH 11, containing 10% (v/v) methanol, stained with Coomassie blue, and the protein bands were excised and sequenced. Automatic Edman degradation analysis was carried out on a 477A protein sequencer, and the resulting phenylthiohydantoin derivatives identified using an online 120A phenylthioidantoin analyser (Applied Biosystems, Foster City, CA).

Results

Effect of Protease Inhibitors on the Transformation of T. cruzi in Axenic Medium. Figs. 1 *A* and 1 *B* show that proteasome inhibitors prevented the transformation of *T. cruzi* trypomastigotes into amastigote-like parasites. 50% inhibition of transformation was achieved at 1–2 μM concentrations of lactacystin and MG132, a peptide aldehyde (22) (Fig. 1 *A*). Clasto-lactacystin dihydroxy acid, an inactive analogue of lactacystin (Figs. 2 *A* and 2 *B*) (23), did not prevent transformation. Lactacystin has no effect on cysteine proteinases (24), including cruzain (or cruzipain), the major lysosomal cathepsin L–like enzyme of *T. cruzi* (25–27) that has been implicated in the growth and differentiation of the parasite (28–30). The hydrolysis of Cbz–Phe–Arg–AMC by recombinant cruzain (a gift from Dr. J. McKerrow, University of California, San Francisco, CA), or by cruzain purified from parasite extracts, was not affected by high concentrations (100 μM) of lactacystin (data not shown). Conversely, parasite remodeling was not affected by Cbz–Phe–Ala–FMK or Cbz–(S-Bz)Cys–Phe–CHN2, cell-permeant inhibitors of cysteine proteases, or by E-64 at concentrations as high as 50 μM (Fig. 1 *A* and 1 *B*).

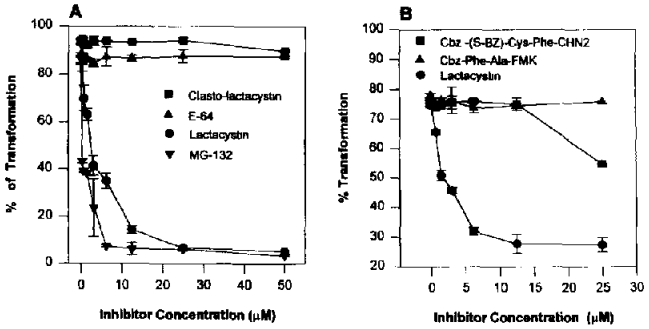


Figure 1. (*A* and *B*) Effect of protease inhibitors on the transformation of *T. cruzi* trypomastigotes into amastigotes. Parasites were incubated for 4 h at 37°C in transformation medium with the protease inhibitors, and then reincubated overnight in DMEM 10% FCS. Transformation was scored in a double-blind fashion by light microscopy, and results expressed as mean ± SD.

The trypomastigotes treated with 10 μM lactacystin for 18 h appeared normal on the basis of motility and morphology, when examined by light microscopy (Fig. 2 *C*) and electron microscopy (data not shown). Nevertheless, higher concentrations of lactacystin were toxic for the parasite, similar to what has been described for other eukary-

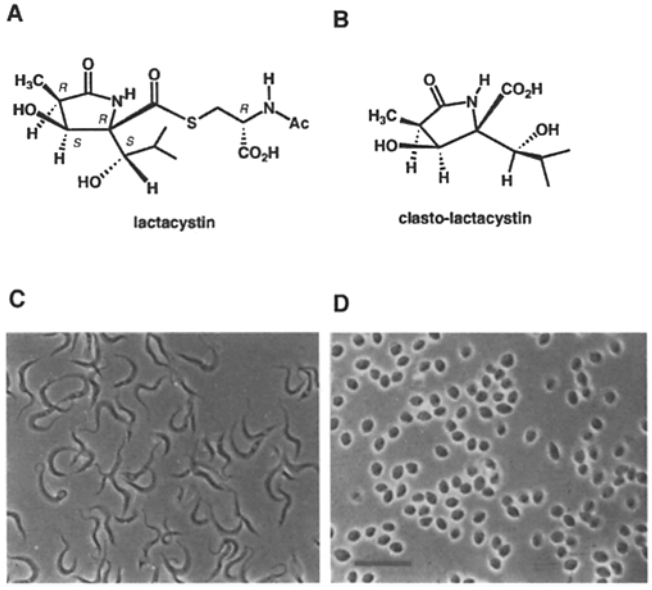


Figure 2. Effect of lactacystin and clasto-lactacystin on *T. cruzi*. (*A*) Lactacystin. (*B*) Clasto-lactacystin dihydroxy acid. (*C* and *D*) Morphology of *T.cruzi* trypomastigotes that were incubated in DMEM (pH 5.0) in the presence of lactacystin or clasto-lactacystin, respectively.

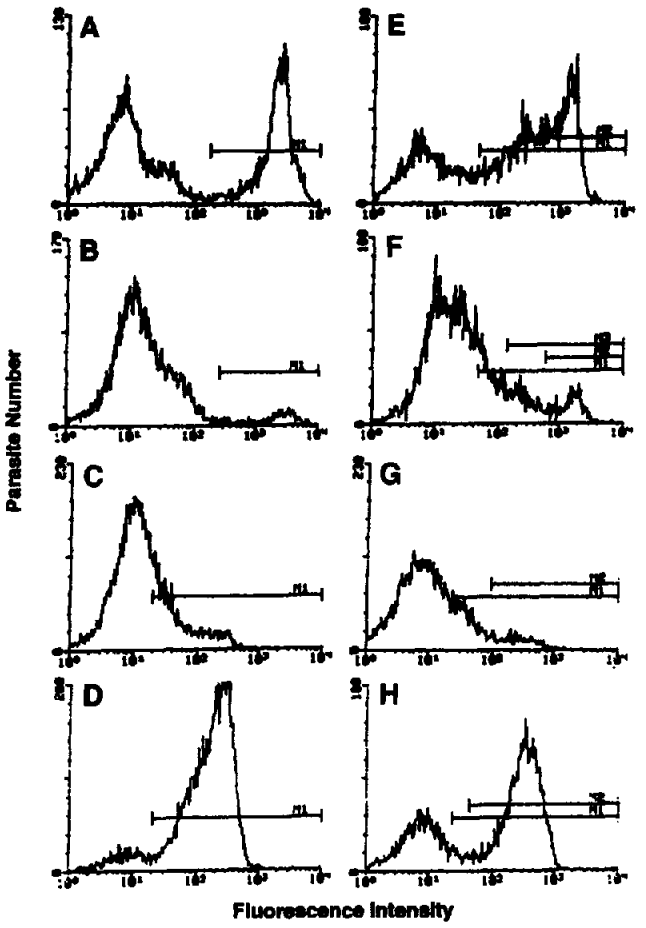


Figure 3. Effect of proteasome inhibitors on the expression of stage-specific epitopes of *T. cruzi*. Parasites undergoing transformation in the presence or absence of the proteasome inhibitors lactacystin (*A, B, C, D*), and MG-132 (*E, F, G, H*), were analyzed by FACS®. Trypomastigotes were incubated for 4 h in the transformation medium alone or medium containing inhibitor, and then reincubated in DMEM 10% FCS in the presence (*B, D, F, H*) or absence (*A, C, E, G*) of inhibitors. At the end of the incubation, the parasites were washed and stained by immunofluorescence with mAb 2C2 (*A, B, E, F*) or 3C9 (*C, D, G, H*), and analyzed by FACS®. The mAb 2C2 detects Ssp-4, an amastigote-specific epitope, and mAb 3C9 detects Ssp-3, a trypomastigote-specific epitope.

otic cells. Fig. 2 *D* shows the amastigote-like morphology of the parasites that had treated with clasto-lactacystin.

The proteasome inhibitors also delayed the expression of stage-specific antigens, as shown by FACS® analysis of parasite samples taken at the end of the transformation process. In control samples, a large proportion of the amastigote-like organisms acquired the amastigote-specific Ssp-4 epitope, and lost the trypomastigote-specific Ssp-3 epitope (13), while most parasites incubated with lactacystin or MG-132 retained the Ssp-3 epitope, and were Ssp-4 negative (Fig. 3).

Effect of Protease Inhibitors on the Intracellular Transformation of T. cruzi. In one series of experiments, trypomastigotes were preincubated with 10 μM lactacystin or clasto-lactacystin for 1 h at 37°C, washed by centrifugation, and added to cultured myoblasts. The mean number of intracellular

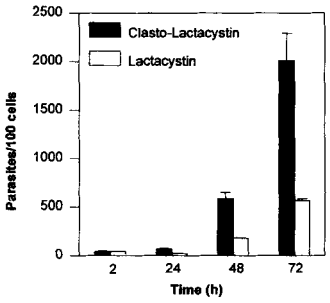


Figure 4. Effect of lactacystin on cell invasion by *T. cruzi*. L6E9-irradiated myoblasts were infected with trypomastigotes that had been preincubated for 1 h at 37°C with 10 μM lactacystin or clasto-lactacystin. After 2 h incubation at 37°C, the trypomastigotes were removed, and the L6E9 cells were washed with DMEM. One set of cells was fixed with 4% paraformaldehyde in PBS for 30 min. The extracellular trypomastigotes were detected by immunofluorescence with a polyclonal antibody to *T. cruzi*, and the total number of parasites was determined by staining with Hoechst dye after permeabilization of the L6E9 cells with cold methanol for 10 min. The number of intracellular parasites was calculated by subtracting the extracellular from total number of parasites. The remaining infected cell cultures were reincubated at 37°C. At 24, 48, and 72 h, triplicate wells were washed and stained with May–Grunwald–Giemsa. The slides were examined under light microscopy and the number of intracellular amastigotes in 100 cells was counted. Results are expressed as mean ± SD.

parasites 2 h after infection was not significantly different for trypomastigotes treated with lactacystin (41.7 ± 5.4) or with clasto-lactacystin (41.1 ± 1.2), indicating that proteasome activity was not required for cell invasion. Nevertheless, at 24, 48, and 72 h after infection the number of intracellular amastigotes was much lower in cells infected with lactacystin-treated trypomastigotes (Fig. 4).

Next, we studied the effect of lactacystin on the intracellular transformation of the dividing amastigotes into trypomastigotes, an event that occurs between 40 and 48 h after infection. In the following set of experiments, the myoblasts were treated 48 h after infection with lactacystin or clasto-lactacystin. After 2 h incubation, the drugs were removed, the cells were thoroughly washed and reincubated at 37°C. At various times thereafter, trypomastigotes were collected in the culture supernatants and counted. In the

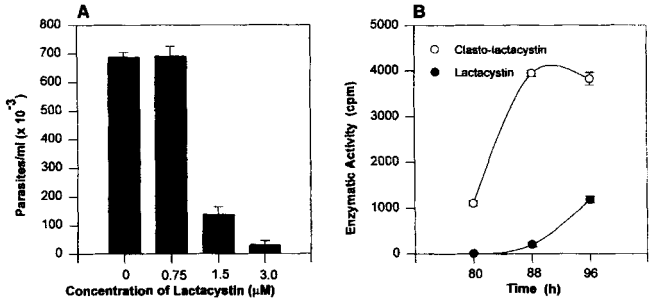
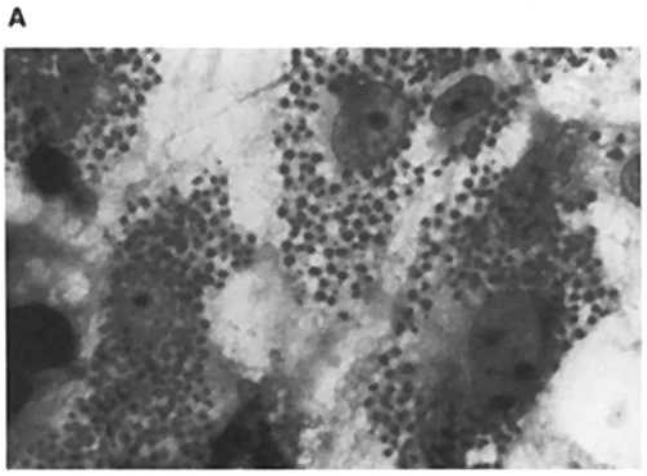


Figure 5. Effect of lactacystin on amastigote/trypomastigote intracellular transformation. L6E9 irradiated myoblasts were infected with *T. cruzi* trypomastigotes. At 48 h after infection, lactacystin or clasto-lactacystin was added. After 2 h of incubation at 37°C, the cultures were washed and reincubated at 37°C for various periods of time. The effect of the drugs on parasite development was evaluated as follows. (*A*) By counting in a Neubauer chamber the number of trypomastigotes in the culture supernatants. This was measured 48 h after removal of the drugs. (*B*) By measuring transialidase activity in extracts of infected cells 72, 80, 88, and 96 h after infection, i.e., 24, 32, 40, and 48 h after removal of the drugs. All experiments were performed in triplicate and values expressed as mean ± SD.



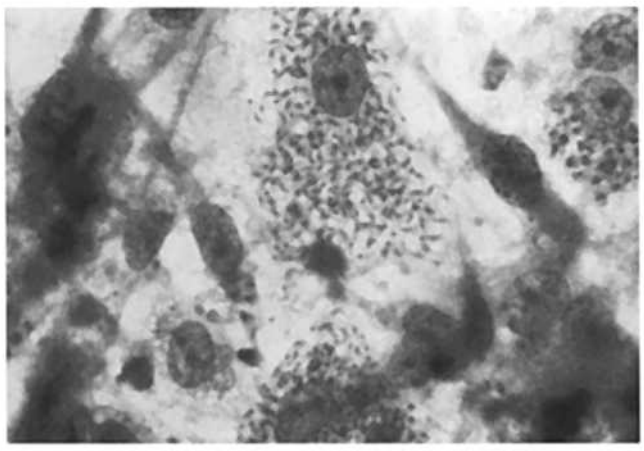


Figure 6. Morphology of *T. cruzi* infected cultures treated with lactacystin L6E9-irradiated myoblasts were infected with *T. cruzi* trypomastigotes. At 48 h after infection, lactacystin or clasto-lactacystin was added. After 2 h of incubation at 37°C, the cultures were washed and reincubated at 37°C for another 48 h. The infected cultures were fixed and stained with May–Grunwald–Giemsa and examined by light microscopy (*A*) Myoblasts treated with lactacystin showing typical amastigotes. (*B*) Myoblasts treated with clasto-lactacystin showing trypomastigotes and intermediate forms.

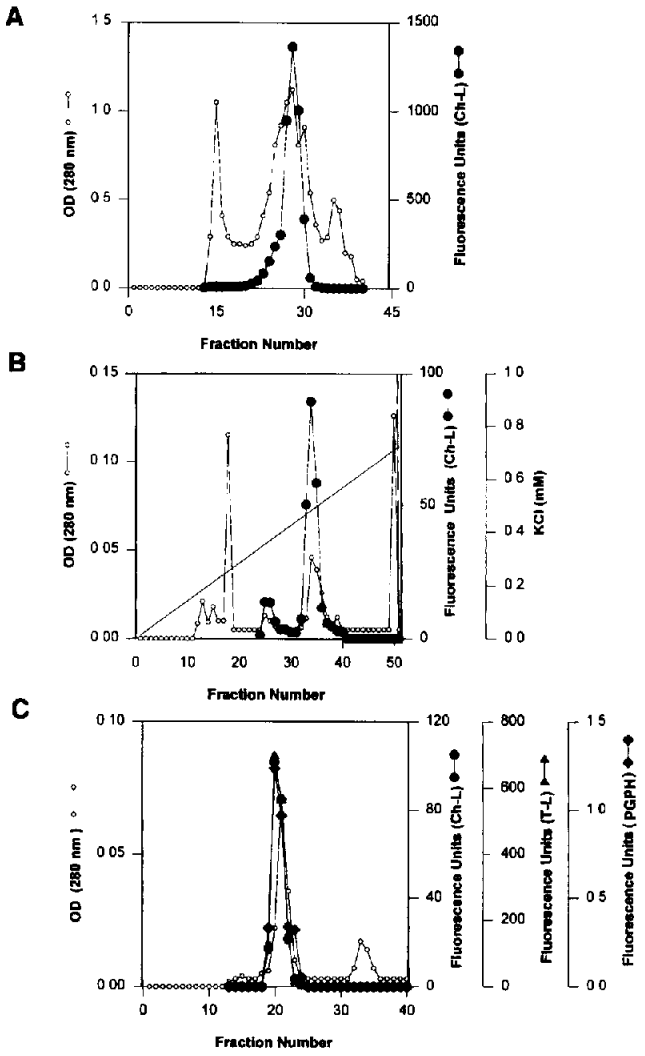


Figure 7. Purification and characterization of *T. cruzi* proteasomes. (*A*) Gel filtration on Superose 6. The chymotrypsin activity in fractions 17–24 was totally inhibited by lactacystin but unaffected by E-64. (*B*) Anion-exchange chromatography of pooled fractions 17–24 on a Mono Q column. Bound proteins were eluted using a 0–1 mM KCl linear gradient. Fractions that displayed Ch-L activity that was inhibitable by lactacystin, but not by E-64, were eluted at approximately 400–500 mM KCl. (*C*) Gel Filtration on Superose 6. Fractions eluted from the Mono Q at 400–500 mM KCl were loaded onto Superose 6 16/30 . Proteolytic activities under the major protein peak were measured with the following fluorogenic peptides: Suc–Leu–Leu–Val–Tyr–MCA for Ch-L activity (*Ch-L*), Boc–Leu–Arg–Arg–MCA for T-L activity (*T-L*) and Z–Leu–Leu–Glu–βNA for peptidylglutamyl peptide hydrolase (*PGPH*). All activities were strongly inhibited by lactacystin but not by E-64.

cultures treated with lactacystin at concentrations of 3 and 1.5 μM, significantly fewer trypomastigotes were released from the cells as compared with controls treated with clasto-lactacystin or medium alone (Fig. 5 *A*). We also assayed extracts of infected cells for the presence of transialidase, an enzyme expressed only in trypomastigotes. In cultures treated with clasto-lactacystin or medium alone, the expression of transialidase starts 80 h after infection, and increases until the end of intracellular parasite differentiation. In lactacystin-treated cultures, the expression of transialidase was inhibited (Fig. 5 *B*). Finally, one set of infected cells was stained 90 h after infection and examined by light microscopy. While 90% percent of cells treated with lactacystin contained typical amastigotes, about 80% of myoblasts treated with clasto-lactacystin contained trypomastigote-like or intermediate flagellate forms (Fig. 6). Analogous

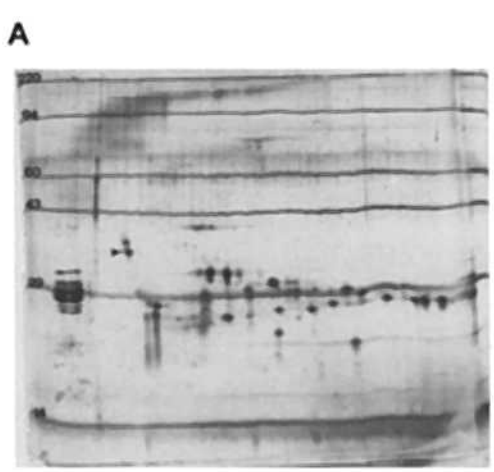


Figure 8. (*A*) Composite of SDS-PAGE (first track on the left) and two-dimensional gel analysis of *T. cruzi* proteasomes. The arrow points to an added control protein (*pI 5.2*). On the left are the MW markers. Gels were silver-stained. (*B*) Electronmicroscopy of *T. cruzi* proteasomes. Bar, 100 nm

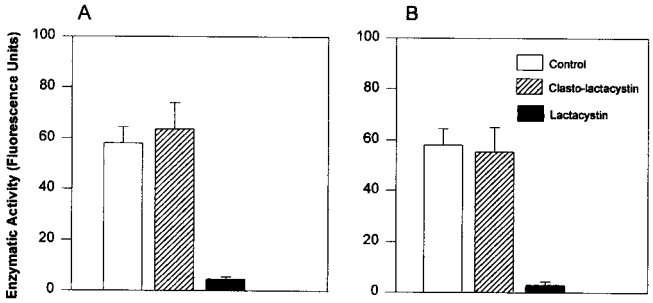


Figure 9. In vivo and in vitro inhibition of *T. cruzi* proteasomes by lactacystin. (*A*). Trypomastigotes were incubated for 3 h in transformation medium containing 10 μM lactacystin (*solid bars*), or clasto-lactacystin (*striped bars*) or with medium alone (*open bars*). Samples of parasites (3×10^7) were washed with PBS, resuspended in 200 μl of 20 mM Tris, sonicated, and centrifuged. Supernatants were immunoprecipitated with polyclonal antibodies raised against *T. cruzi* proteasomes. Immunocomplexes were collected using protein A–Sepharose, and the Ch-L activity associated with the beads was measured. When parasites were treated with medium and immunoprecipited with preimmune serum, no Ch-L activity was detected. (*B*) As additional controls for the specificity of the immunoprecipitation reaction, untreated parasites were sonicated, treated with lactacysin (*solid bars*), or clasto-lactacystin (*striped bars*), or medium (*open bars*) and immunoprecipitated as above. The Ch-L activity of the immunoprecipitates was then measured. All experiments were performed in triplicate, and results expressed as mean ± SD.

experiments were performed with the cell-permeant cysteine proteinase inhibitors E-64d (31) and Cbz–Phe–Ala–FMK at concentrations of 10 μM. They had no effect on the transformation of intracellular amastigotes into trypomastigotes, or on the expression of transialidase (data not shown).

Identification of the Lactacystin Target in T. cruzi. We used two approaches to identify the target of lactacystin in *T. cruzi*. First, we isolated the lactacystin-inhibitable chymotrypsin activity from crude extracts of parasite. As shown in Fig. 7 *A*, a broad peak of chymotrypsin activity was detected following filtration of the extracts in a Superose 6 column. However, only the activity in the shoulder peak (fractions 17–24), containing proteins of higher molecular mass, was inhibitable by lactacystin, but not by E-64. In later fractions the chymotryptic activity was inhibited by E-64 but not by lactacystin. The lactacystin-inhibitable fractions were then subjected to anion-exchange chromatography in a Mono Q column. A peak of chymotrypsin activity that was inhibited by lactacystin eluted at 400–450 mM of KCl (Fig. 7 *B*). Pooled fractions from this peak were then filtered through another Superose 6 column. A major symmetrical OD peak of 670 kD was eluted from the column. It contained the three characteristic peptidase activities of eukaryotic proteasomes, T-L, Ch-L, and PGPH (Fig. 7 *C*). All activities were inhibitable by lactacystin. Using Suc–Leu–Leu–Val–Tyr–AMC as a substrate, the specific activity of the Ch-L activity was 1.5 μM/mg/h. At concentrations up to 50 μM, the cruzain inhibitors Cbz–Phe–Ala–FMK and Cbz–(S-Bz)Cys–Phe–CHN_2 did not affect the Ch-L activity of the purified proteasomes.

Using SDS-PAGE under denaturing conditions the 670 kD molecules were resolved into subunits with molecular masses between 25–35 kD. By isoelectrofocusing, their isoelectric points varied between 4.5 and 8.5 (Fig. 8 *A*). The NH_2-terminal protein sequence of the protein from one band (TSIMAVTFKD) is identical to that of the β-subunit of PRE3, a PGPH activity from yeast proteasomes (32). Electron microscopy of negatively stained preparations revealed characteristic images of proteasomes, i.e., hollow cylinders 18 nm in length and 12–15 nm in diameter (Fig. 8 *B*).

To identify the target of lactacystin in vivo, we incubated samples of trypomastigotes for 2 h in transformation medium in the presence of lactacystin, clasto-lactacystin, or medium alone. The parasites were washed, and sonicated

extracts were immunoprecipitated with a rabbit antiserum to purified *T. cruzi* proteasomes, or with normal rabbit serum. Immunoprecipitates were then assayed for chymotrypsin activity. As shown in Fig. 9 *A*, the immunoprecipitated proteasomes from parasites that had been incubated with lactacystin were inactive. The control immunoprecipitates from parasites treated with medium alone or clasto-lactacystin had Ch-L activity that was inhibited by lactacystin, but not by E-64. No enzymatic activity was detected in samples immunoprecipitated with normal rabbit serum. As additional controls of the specificity of the immunoprecipitation, trypomastigote extracts were treated with lactacystin or clasto-lactacystin and then immunoprecipitated as described above. The immunoprecipitates originating from extracts treated with lactacystin were inactive (Fig. 9 *B*)

Discussion

We show here that the proteasome inhibitors MG132 and lactacystin prevented the transformation of trypomastigotes into amastigotes in axenic medium. MG132, a peptide aldehyde, also potently inhibits cysteine proteases, but lactacystin selectively inhibits the peptidase activity of proteasomes. The transient intermediate of lactacystin, clasto-lactacystin β lactone, binds tightly to threonines in the active site of the β subunits of proteasomes (24, 33). Clasto-lactacystin dihydroxy acid (Fig. 2 *B*), the product of hydrolysis of the active β lactone, had no activity in parasite transformation. Lactacystin does not inhibit serine or cysteine proteases of mammalian cells (24), and did not affect the activity of cruzain, the major *T. cruzi* lysosomal enzyme. We further ascertained that proteasomes are the targets of lactacystin in trypomastigotes by two independent criteria. First, proteasomes were isolated to apparent homogeneity from crude extracts of parasites using a lactacystin-based assay to follow purification. Second, while immunoprecipitates of proteasomes present in extracts of clasto-lactacystin-treated parasites had Ch-L activity, the immunoprecipitates from lactacystin-treated parasites were inactive.

We also studied the effect of lactacystin on the infectivity of *T. cruzi* trypomastigotes to myoblasts. In these experiments, we tried to minimize or exclude possible effects of the drug on the target cells. For example, when studying the attachment and penetration phases of infection, drug-treated parasites were washed before incubation with the myoblasts. We found that lactacystin had no effect on invasion, an active process that requires parasite energy (34), and is associated with calcium fluxes in the parasite (35). However, the intracellular development of the lactacystin-treated parasites was arrested. It cannot be deduced from these results whether lactacystin inhibited only the trypomastigote/amastigote transformation. There is a distinct possibility that lactacystin inhibited amastigote proliferation as well, since the eukaryotic cell cycle is regulated by proteasomes. In any case, these experiments also show that the effects of lactacystin persisted during the intracellular development of the parasite. Lactacystin is an irreversible inhibitor of proteasomes, and the half-life of *T. cruzi* proteasomes may be long. Alternatively, drug treatment may have irreversibly affected a proteasome-dependent essential parasite function.

Lactacystin also prevented the transformation of amastigotes into trypomastigotes that occurs at the end of the intracellular phase. In these experiments, myoblasts infected 48 h previously with trypomastigotes were exposed for 2 h to 1–3 μM of lactacystin. The effect was striking: as compared with clasto-lactacystin-treated cells, the lactacystin-treated cells released fewer trypomastigotes into the culture medium, contained more amastigotes in their cytoplasm, and displayed much less transialidase activity. In contrast, higher concentrations of cell-permeant inhibitors of cruzipain had no effect on the amastigote/trypomastigote transformation. The small concentrations of lactacystin used, the short duration of drug treatment, the specificity of the observed effects, and the lack of effect of cysteine protease inhibitors argue strongly that the prime targets of lactacystin are the transforming parasites rather than the myoblasts.

These results show that proteasome activity is necessary for remodeling, but the substrates that are degraded have not been identified. They probably include proteins that maintain the old shape, most likely cytoskeletal elements, a set of proteins and enzymes involved in the old metabolic pathways, and stage-specific surface proteins. In addition to these housekeeping functions, the cleavage of key regulatory proteins by proteasomes may provide the central switching mechanism that initiates the stage-specific changes (36).

In eukaryotic cells, the substrates destined for degradation are recognized by specific E2–E3 Ub–protein ligases (37). However, very little is known about the Ub–proteasome system in protozoan parasites. Southern and Northern blots of DNA and RNA from various strains of *T. cruzi* revealed large variations in the number of Ub genes (38). Its genome may contain more than 100 Ub coding sequences, a number much larger than in other organisms. These are encoded in five polyUb genes and five Ub fusion genes, whose transcription is altered under stress conditions. There is a significant increase in steady-state levels of Ub mRNA between the midlog phase cultures of noninfective epimastigotes of *T. cruzi*, and the stationary phase cultures that contain the morphologically distinct, infective metacyclics (39). It is noteworthy that heat–shock elements are present in the intergenic regions preceding the polyUb genes. Perhaps the expression of the Ub genes in *T. cruzi* is regulated by the shifts in environmental pH and temperature, and by other stress conditions that lead to stage-specific remodeling. In yeasts that bear mutations in proteasomes, sensitivity to stress is increased, and under stress conditions the mutants accumulate ubiquitinated proteins.

Other proteases have been identified in *T. cruzi* (40–42). One of them, cruzain, a lysosomal cathepsin L-like cysteine protease, also plays a role in growth and differentiation of the parasite (28–30). Studies in different laboratories have shown that synthetic inhibitors of cruzain, including Cbz–

Phe–Ala–FMK and Cbz–(S-Bz)Cys–Phe–CHN2, inhibit *T. cruzi* infectivity. However, different from lactacystin, the cysteine protease inhibitors prevent parasite penetration into the heart muscle cells (28). As shown here, relatively high concentrations of Cbz–Phe–Ala–FMK and Cbz–(S-Bz)–Cys–Phe–CHN2 did not affect the remodeling of *T. cruzi* in axenic medium or inside cells. Although our findings do not exclude a role for cruzain and other lysososmal enzymes in the extensive proteolysis that must accompany remodeling, they argue that the role of cruzain is not pivotal during these phases of parasite development.

Some publications report the presence of proteasomes in *Trypanosoma* (43, 44) and *Entamoeba* (45), but their function has not been studied. We found that the structural features and architecture of the *T. cruzi* proteasomes were similar to those of other species. By SDS-PAGE the cylindrical 20S structure was resolved into the typical 6–8 bands of 25–35 kD. However, more than 20 proteins, with widely diverse pIs, were seen in *T. cruzi* proteasomes analyzed by two-dimensional PAGE. It is generally accepted that the 20S proteasome is a dimer of 14 subunits arranged $\alpha_7\beta_7\beta_7\alpha_7$. In the yeast *Saccharomyces cerevisiae* there are fourteen genes encoding 7 α and 7 β subunits, and the dendrogram representing the alignments of all eukaryotic proteasome sequences yields only 14 subgroups containing a single yeast member. The explanation for the large number of *T. cruzi* proteasome-associated proteins may be trivial: some extra spots could represent posttranslational modifications of a polypeptide, or simply contaminants. Alternatively, an unusual feature of *T. cruzi* is that its proteins are frequently encoded by several tandemly arranged genes that are polycistronically transcribed from a single promoter and are concurrently expressed. Sequence variation of genes found in one such transcription unit could add to the apparent subunit heterogeneity. Further studies are necessary to clarify this issue.

The present paper demonstrates that proteasome activity is essential for *T. cruzi* remodeling. Very similar results were recently obtained with other protozoan parasites. In a rodent malaria model Sinnis, P., B. Gutierrez, M. Briones, and V. Nussenzwerg (manuscript in preparation) showed that lactacystin did not prevent the penetration of the *Plasmodium berghei* crescent-shaped sporozoites into hepatocytes, but strongly inhibited their transformation into the round hepatocyte stages and subsequent development. Eichinger, D., V. Nussenzweig, and J. Gonzalez (manuscript in preparation) demonstrated that lactacystin prevented the encystation of *Entamoeba invadens*. *Trypanosoma*, *Entamoeba*, and *Plasmodium* belong to phyla widely separated in evolution. Therefore, it is likely that the mechanisms governing stage-specific morphological changes in protozoa are conserved, and proteasome-dependent, and that proteasome inhibitors may have a broad range of targets. Encouraging features for attempting to develop this class of chemotherapic agents are that some parasites, such as *Plasmodium*, undergo constant and rapid remodeling in the mammalian host. Thus, effective drugs need not be administered for prolonged periods of time to arrest parasite development. Furthermore, the accurate discrimination between the old and new proteins that coexist within the same cell during remodeling of protozoa may require specialized features of the proteasome/Ub system. Proteasomes from intracellular protozoan parasites may also differ significantly in structure from those of the host cell, rendering the infected cells susceptible to destruction by cells of the immune system. Hopefully, some of these approaches to therapy will yield to experimental attack.

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