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) 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.

Materials and Methods

Cell Lines. LLC-MK2 fibroblasts were obtained from American Type Culture Collection, Rockville, MD (ATCC CCL-7). L6E9 myoblasts were a gift of Dr. R. Docampo (University of Ill-
MK2 cells were infected with were irradiated with 2,000 rads (14) and plated in 4-well Lab-Tek (Uppsala, Sweden). Chromatography columns and resins were from Pharmacia Biotech AB (Uppsala, Sweden).

Inhibition of Trypomastigote Transformation into Amastigotes. LLC-MK2 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, DMNO 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% CO2 atmosphere. The plate was centrifuged and the supernatants were removed and replaced by DMEM (pH 7) containing 10% FCS. 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 × 10^6) were transformed in the presence or absence of protease 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 FACS® system.

Inhibition of Development of Intracellular Parasites. L6E9 myoblasts were irradiated with 2,000 rads (14) and plated in 4-well Lab-Tek macrochamber 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 trypanosomes 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 trypanosomes 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-octylglucosides, 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 trypanosomes, 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–Val–Ty–MCA and Suc–Ala–Ala–Phe–MCA to measure chymotrypsin-like (Cl-L) activity, Cbz–Leu–Leu–Glu–2-naphthylamide to measure pepstatin-like activity (PSPH), and Boc–Leu–Arg–Arg–MCA to measure trypsin-like activity (T-L). Chuzapain activity was measured using Cbz–Phe–Arg–AMC as a substrate.

Purification of T. cruzi Proteosomes. For purification of proteasomes, T. cruzi epimastigotes (Y strain) were used. Parasites were harvested from 31 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 Centricron 10 unit (Amicon, Beverly, MA), and fractionated by fast performance 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 Cl-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 approximately 400–500 M KCl. They were pooled and concentrated in a Centricron 10 unit. The concen-
treated 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 preincubated with preimmune rabbit serum and protein A-Sepharose (Pharmacia Biotech, Uppsala, Sweden), and then incubated overnight with anti-T. cruzi proteasome antibody 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.

**N-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 phenylthiohydantoin 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).

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 eukaryotic cell-permeant inhibitors of cysteine proteases. LC50 of 50% transformation was scored in a double-blind fashion by light microscopy, and results expressed as mean ± SD.
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 morphology of the parasites that had treated with clasto-lactacystin.

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**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.

**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.

**Figure 5.** Effect of lactacystin on amastigote/trypomastigote intracellular transformation. L6E9 irradiated myoblasts were infected with trypomastigotes *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 infected cell cultures. (B) By measuring transglutaminase 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 2.** D shows the amastigote-like morphology of the parasites that had treated with clasto-lactacystin.

Neubauer chamber the number oftrypomastigotes in the culture supernatants. This was measured 48 h after removal of the drugs. (B) By measuring transglutaminase 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.
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
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 bar), or clasto-lactacystin (striped bar), or with medium alone (open bar). 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 immunoprecipitated 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 lactacystin (solid bars), or clasto-lactacystin (striped bar), or medium (open bar) 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.

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. 7A, 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 chymotryptic activity that was inhibited by lactacystin eluted at 400–450 mM of KCl (Fig. 7B). 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. 7C). 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. 8A). The NH_2-terminal protein sequence of the protein from one band (TSL-MAVTFKD) 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. 8B).

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 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).

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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. 9A, 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. 9B).

Discussion

We show here that the proteasome inhibitors MG132 and lactacystin prevented the transformation of trypomastigotes into amastigotes in axenic medium. MG132, a peptide aldehyd, also potently inhibits cysteine proteases, but lactacystin selectively inhibits the peptidase activity of proteasomes. The transient intermediate of lactacystin, clasto-lactacystin B lactone, binds tightly to threonines in the active site of the β subunits of proteasomes (24, 33). Clasto-lactacystin dihydroxy acid (Fig. 2B), 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 transalidase activity. In contrast, higher concentrations of cell-permeant inhibitors of cruzain 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-
polypeptide, or simply contaminants. Alternatively, an unproteasome-associated proteins may be trivial: some extra remodeling, they argue that the role of cruzain is not piv-
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