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Experimental Parasitology 109 (2005) 228–236
Experimental Parasitology
www.elsevier.com/locate/yexpr

Schistosoma mansoni: Functional proteasomes are required for development in the vertebrate host

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Received 13 July 2004; received in revised form 6 January 2005; accepted 10 January 2005

Abstract

Proteasomes are multi-subunit proteases involved in several mechanisms and thought to contribute to the regulation of cellular homeostasis. Here, we report for the first time biochemical evidence for the existence of a ubiquitin–proteasome proteolytic pathway in this parasite. Proteasomes from both cercariae and adult worms exhibited a high preference for hydrolysis of the substrate Suc-LLVY-AMC, although in the cercariae extract the rate of hydrolysis was 50% lower when compared to adult worms extracts. The same difference in proteasome activities was observed when endogenous proteins were broken down in the presence of ATP and ubiquitin. Additionally, accumulation of high molecular weight conjugates was observed when cercariae were pre-incubated with proteasome inhibitors. Finally, we present evidence that during experimental schistosomiasis, proteasome inhibitors were able to reduce the number of lung stage schistosomula, reduce the worm burden and consequently decrease the egg output in infected mice.

Index Descriptors and Abbreviations: Schistosoma mansoni; Proteasome; Development; Hepes, 2-hydroxylethylpperazine-1-ethanesulfonic acid; DMSO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; NBT, nitroblue tetrazolium chloride; BCIP, 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt; aa, amino acid(s); SDS, sodium dodecyl sulfate; MG-115 (Z-leucyl-leucyl-norvaline-H), MG-132 (N^{α}-benzyloxycarbonyl-L-leucyl-L-leucyl-leucinal); TBS, Tris-buffered saline; DEAE, diethylaminoethyl; ATP, adenosine triphosphate

1. Introduction

Schistosomiasis is one of the most prevalent parasitic diseases in humans. The disease is endemic in 76 developing countries and it is estimated that 500–600 million people are at risk of the infection (Engels et al., 2002). The parasite *Schistosoma mansoni* gains access to its definitive host by penetration through the skin, and the infection is believed to result from a combination of mechanical and proteolytic processes (Fishelson et al., 1992; McKerrow and Salter, 2002; Stirewalt, 1974).

The development of *S. mansoni* in the vertebrate host requires several coordinated alterations of body morphology. Following invasion, the parasite responds to the altered environmental conditions such as high temperature, osmolarity, and nutrient availability. At the cellular level, such a response constitutes a sequence of events collectively called cercariae transformation (Fishelson et al., 1992; McKerrow and Salter, 2002).

In eukaryotic cells, the turnover of intracellular proteins is primarily mediated by the ubiquitin–proteasome system (Goldberg et al., 1997). Following ubiquitination, proteins are unfolded and degraded by the 26S proteasome, a large multi-subunit complex located in the cytosolic and nuclear compartments. This proteolytic pathway controls a broad array of cellular functions, including cell cycle progression, stage-specific gene transcription, antigen processing, regulation of membrane-anchored and secretory pathway-compartmentalized

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doi:10.1016/j.exppara.2005.01.002

proteins, and protein quality control (Glickman and Maytal, 2002).

The 26S proteasome is made up of two 19S complexes and a proteolytically active 20S proteasome core. The barrel-shaped 20S proteasome is an abundant particle, comprising about 1% of cellular proteins. It is composed of four stacked rings that enclose a central chamber where proteolysis occurs. Its two central β-rings contain multiple proteolytic sites that function cooperatively in protein degradation. In eukaryotes, two of these sites are chymotrypsin-like, two are trypsin-like, and two are caspase-like. This enzyme utilizes the hydroxyl group of the terminal threonine residue of the β-subunit as the catalytic nucleophile that attacks peptide bonds, and completely degrades them in a highly sequential manner into small peptides of 3–20 residues which are further hydrolyzed by other peptidases (Tanaka, 1998).

The 19S complex contains binding sites for ubiquitinated proteins, enzymes that depolymerize the ubiquitin chain and six distinct ATPases that appear to unfold the substrate to facilitate its entry into the 20S particle, therefore providing substrate specificity and regulation (Glickman and Ciechanover, 2002; Pickart, 2001).

Numerous drugs have been reported to inhibit the proteasomes, and most of them directly interfere with the proteolytic activities of the 20S core particle. These inhibitors bind either reversibly or irreversibly to active sites in the core particle, and they display varying rates of inhibition for the 26S proteasome. Among them, the synthetic peptide aldehydes MG-132, PSI (Adams et al., 1998), lactacystin (Dick et al., 1996), NLVS (Bogyo et al., 1997), eponemycin (Meng et al., 1999a) epoxomicin (Meng et al., 1999b), TMC-95A (Goldberg and Rock, 2002), and the peptide boronic acids (Adams et al., 1999) are the most common in current use.

The effect of proteasome inhibitors, such as MG-132 and lactacystin, on protozoan parasites has been described for *Entamoeba invadens*, *Entamoeba histolytica* (Makioka et al., 2002), *Leishmania mexicana* (Robertson, 1999), *Trypanossoma cruzi* (de Diego et al., 2001), *Trypanosoma brucei* (Nkemgu-Njinkeng et al., 2002), *Plasmodium berghei* (Gantt et al., 1998), and *Toxoplasma gondii* (Shaw et al., 2000). In every investigated model these inhibitors interfered with parasite growth, either by inhibiting cell-cycle progression or blocking replication.

The present work aimed to characterize the 20S proteasome from *S. mansoni* in order to determine its possible role in the host–parasite interaction.

2. Materials and methods

2.1. Chemicals

The fluorogenic substrates Suc-LLVY-AMC (*N*-succinyl-Leu-Leu-Val-Tyr-7-amido-4-methylcoumarin), Cbz-GGR-AMC (*N*-Cbz-Gly-Gly-Arg-7-amido-4-methylcoumarin), and Cbz-LLG-βNA (*N*-Cbz-Leu-Leu-Glu-β-naphthyl-amide) were purchased from Sigma (Sigma Chemical, St. Louis, MO 63178, USA). The proteasome inhibitors MG-115 (Z-leucyl-leucyl-norvaline-H), MG-132 (N^{α}-benzyloxycarbonyl-L-leucyl-L-leucyl-leucinal), lactacystin, and epoxomicin were purchased from Affiniti (BIOMOL International LP, Palatine House, MatfordCourt, Exeter, EX28NL, UK). Leupeptin (Acetyl-Leu-Leu-Arg-al) and bovine ubiquitin were purchased from Sigma (Sigma Chemical, St. Louis, MO 63178, USA). For inhibition assays, the proteasome inhibitors MG-132 and MG-115 were dissolved in DMSO, while leupeptin was dissolved in distilled water.

2.2. Parasites

An LE strain of *S. mansoni* is routinely maintained by passage through *Biomphalaria glabrata* snails and BALB/c mice. *B. glabrata*-infected snails were induced to shed cercariae by exposing them under artificial illumination in a 30 °C water bath for 1 h. Schistosomula were recovered from the lungs of infected mice as previously described (Barbosa et al., 1978). Briefly, the lungs of mice were perfused with heparinized RPMI 1640 (pH 7.4, at a temperature of 37 °C) and chopped into small fragments with scissors. These fragments were introduced into glass vessels closed at the lower end by a stainless steel screen of mesh 0.09 mm, which was supported by three legs inside a beaker with culture medium at 37 °C; the schistosomules migrated from the tissue to warm medium and could be collected and counted after 3 h. Adult worms were recovered under aseptic conditions from mice previously infected with cercariae by perfusion of the livers and mesenteric veins.

2.3. Preparation of partially purified S. mansoni 20S proteasomes

Approximately, 2×10^6 cercariae and 5 g of adult worms were used to prepare crude extracts as previously described (Fagan et al., 1987). The protein content of the extracts was estimated using the BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL61105, USA). Total proteins of crude extracts from adult worms (14 mg) or cercariae (14 mg) were layered onto a DEAE–cellulose column (Sigma Chemical, St. Louis, MO 63178, USA) previously equilibrated in 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 10% glycerol, and 1 mM DTT. Under these conditions, 20S proteasomes from both cercariae and adult worms extracts were eluted with 300 mM NaCl in the same buffer, as judged by evaluation of its chymotrypsin-like activity against the synthetic peptide Suc-LLVY-AMC. Active fractions were pooled, dialyzed in the same buffer, and concentrated in Amicon

(Amicon Division, W.R. Grace, CONN, Beverly, MA 01915, USA) using a 300 kDa cut-off membrane. A further enrichment of 20S proteasomes was achieved by ultracentrifugation of the pooled active fractions in a 10–40% glycerol gradient in 50 mM Tris–HCl, pH 7.5, 1 mM DTT, and 5 mM $MgCl_2$ at 80,000*g* for 6 h at 4 °C using a Sorval ultracentrifuge. Active fractions were pooled as described and the protein content was estimated as indicated above.

2.4. Measurement of proteasomal activity

Peptidase activities were assayed using 10 μL (~50 μg) of 20S proteasome-containing fractions from adult worms or cercariae, 90 μL fluorogenic substrates (100 μM diluted in 50 mM Tris–HCl, pH 7.5) and 100 μL reaction mix buffer (50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM DTT, and 1 mM ATP) at 37 °C for 90 min in the presence or absence of different classes of inhibitors: 50 μM MG-132, 50 μM MG-115, 5 μM epoxomicin, 5 μM lactacystin, and 50 μM leupeptin. The chymotrypsin-like, trypsin-like, and peptidylglutamyl peptide hydrolyzing activities were determined by fluorimetric quantification of the substrates Suc-LLVY-AMC, Cbz-GGR-AMC, at 380 nm excitation/440 nm emission, and Cbz-LLG-βNA, at 335 nm excitation/410 nm emission, respectively, using a Quantech fluorometer (Barnstead Thermolyne, Dubuque, IA 52004-0797, USA). The ATP-ubiquitin-dependent proteolysis of soluble proteins in crude extracts from cercariae and adult worms was determined by measuring the free tyrosine content in the trichloroacetic acid-soluble supernatant (Waalkes and Udenfriend, 1957). All proteolytic assays were performed using 250 μg of crude extracts in the presence or absence of 50 μM MG-132, 10 mM ATP, and 200 μg/ml bovine ubiquitin.

2.5. Gel overlay assay and immunoblotting

For detection of chymotrypsin-like activities on the polyacrylamide gel the overlay assay was performed. Approximately, 15 μg of cercariae extracts, 15 μg of adult worms extracts, and 10 μg of partially purified 20S proteasomes from both adult worms and cercariae were resolved by 4% non-denaturing polyacrylamide gels as described by Glickman et al. (1998). After electrophoresis the non-denaturing gels containing the proteasomes were overlaid with 5 ml of 0.1 mM Suc-LLVY-AMC in 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 10% glycerol, 1 mM ATP, and 1 mM DTT, and incubated for 30 min at 37 °C. Proteasome bands were visualized upon their exposure to UV light and photographed using a Polaroid camera. Approximately, 10 μg of partially purified 20S proteasomes from cercariae and adult worms was resolved in a 12% SDS–polyacrylamide gel, and the protein bands were visualized by ammoniacal-silver staining. A replica gel was produced and the protein content was transferred to an Immobilon-NC nitrocellulose membrane (Millipore, Bedford, MA01730, USA). Alpha proteasome subunits were detected by hybridization with a monoclonal antibody raised against six different α-type subunits derived from the human 20S proteasome (BIOMOL International LP, Exeter, EX28NL, UK) at 1:1000 dilution for 3 h in TBS buffer containing 5% skimmed milk and 0.05% Tween 20. Goat phosphatase anti-mouse IgG (Sigma Chemical, St. Louis, MO 63178, USA) was used as the secondary antibody at 1:5000 dilution in the same buffer for 2 h and the immunoblot was developed using the alkaline phosphatase substrates NBT/BCIP (Life Technologies, Gaithersburg, MD 20884-9980, USA) according to the manufacturer's instructions.

2.6. Demonstration of ubiquitin–protein conjugates

Approximately, 10 μg of soluble proteins from cercariae previously incubated at 37 °C for 90 min in the presence of 50 μM leupeptin, 50 μM MG-115, and 50 μM MG-132 was separated by 10% SDS–PAGE and the protein bands were blotted onto Immobilon-NC nitrocellulose membranes (Millipore, Bedford, MA01730, USA). The blocking step was performed overnight at 4 °C in TBS buffer containing 5% skimmed milk and 0.05% Tween 20. Anti-bovine ubiquitin (Sigma Chemical, St. Louis, MO 63178, USA) was used as the primary antibody at 1:1000 dilution during a 2 h incubation at room temperature. Goat phosphatase-conjugated anti-rabbit IgG (Sigma Chemical, St. Louis, MO 63178, USA) was used as the secondary antibody at 1:1000 dilution for 2 h and the immunoblot was developed using the alkaline phosphatase substrates NBT/BCIP (Life Technologies, Gaithersburg, MD 20884-9980, USA). To test the reaction specificity, the immunoblotting was performed either with omission of the primary antibody or hybridization with normal rabbit serum.

2.7. Effects of proteasome inhibitors on S. mansoni infection in mice

Approximately, 2×10^4 cercariae were rinsed in tap water and then incubated for 90 min at 30 °C in one of the following conditions: (1) 1% DMSO (Sigma Chemical, St. Louis, MO 63178, USA), (2) 50 μM MG-115, (3) 50 μM MG-132, and (4) 50 μM leupeptin. Twenty mice per group were infected by immersing their tails in water containing 100 cercariae, pre-treated as described above. The following parameters were monitored in each group: (1) cercariae penetration in mice, (2) number of schistosomula recovered from lungs during the first 9 days p.i., (3) number of eggs eliminated in feces, and (4) number of worms recovered from hepatic perfusion seven weeks after infection.

2.8. Statistical methods

Results are expressed as means ± SEM and differences between means were analyzed by ANOVA, with $P < 0.05$ as the criterion of significance.

3. Results

3.1. Biochemical characterization of S. mansoni 20S proteasomes

The spectra of peptidase activities of partially purified *S. mansoni* 20S proteasomes from cercariae and adult worms were determined by hydrolysis of different fluorogenic substrates (Table 1A). The *S. mansoni* appears to possess all three of the better-characterized proteasome activities: chymotrypsin-like, trypsin-like, and peptidyl-glutamyl-peptide hydrolyzing (also called caspase-like), with a predominant chymotrypsin-like activity in both evolutive forms of this parasite used in this assay. However, the cercariae showed only about 50% of the specific activity in the rate of peptide hydrolysis when compared to those observed for adult worm proteasome.

Among the tested substrates, Suc-LLVY-AMC showed the highest rate of hydrolysis, and was used to assess the proteasome-specific activity by using known proteasome inhibitors (Table 1B). All proteasome inhibitors showed a rate of inhibition of above 80% except in adult worms, in which the inhibition was around 59% in the presence of MG-115 when compared to the control. Leupeptin, a known inhibitor of both serine and cysteine proteases, and also shown to inhibit the yeast, trypanosome, and leishmania proteasomes (Hua et al., 1996; Lee and Goldberg, 1996; Robertson, 1999), did not show any inhibitory effect on *S. mansoni* proteasomes.

Table 1
Partially purified 20S proteasomes from adult worms and cercariae were assayed for activity towards (A) 100 μM Succ-LLVY-MCA, Cbz-GGR-MCA, and Cbz-LLG-βNA and (B) 100 μM Succ-LLVY-MCA in the presence or absence of 50 μM MG-132, 50 μM, MG-115, 5 μM epoximicin, 5 μM lactacystin, and 50 μM leupeptin at 37 °C for 90 min

Substrates	Fluorescence units min^{-1} mg^{-1}	
	Cercariae	Adult worms
A. Peptidase activities of partially purified proteasomes		
Suc-Leu-Leu-Val-Tyr-AMC	300.4 ± 0.03	603.90 ± 0.04
Z-Gly-Gly-Arg-AMC	110.3 ± 0.02	221.24 ± 0.02
N-Cbz-Leu-Leu-Glu-β-NA	80.1 ± 0.02	171.50 ± 0.03
B. Chymotrypsin-like activity is inhibited by proteasome inhibitors		
Suc-Leu-Leu-Val-Tyr-AMC	300.4 ± 0.02	602.8 ± 0.05
+ MG 132	18.8 ± 0.02	25.3 ± 0.01
+ MG115	45.1 ± 0.01	250.8 ± 0.05
+ Epoxomicin	16.5 ± 0.03	25.0 ± 0.01
+ Lactacystin	15.5 ± 0.02	26.8 ± 0.03
+ Leupeptin	305.6 ± 0.03	601.9 ± 0.05

The fluorescence was expressed as indicated (see Materials and methods). The results represent the average of three independent experiments.

Table 2
Approximately 250 μg of proteins from adult worms and cercariae were incubated at 37 °C for 90 min with or without 2 mM ATP, 200 μg/mL bovine ubiquitin, and 50 μM MG-132

Medium	Protein degradation nmole Tyr/mg protein min	
	Cercariae	Adult worm
Crude extracts	0.087 ± 0.001	0.180 ± 0.015
+ATP	0.120 ± 0.001	2.150 ± 0.01
+ATP + ubiquitin	0.217 ± 0.013	3.630 ± 0.03
+ATP + ubiquitin + MG132	0.020 ± 0.001	0.907 ± 0.01

The amount of tyrosine generated by proteolytic cleavage of endogenous proteins was measured. Values are the means of the tyrosine produced in three independent experiments ± SD. ATP and ubiquitin stimulate the degradation of endogenous proteins from *S. mansoni* crude extracts.

To investigate the presence of an ATP–ubiquitin-dependent proteolytic system in this parasite, we examined the rate of cellular protein degradation in crude extracts from cercariae and adult worms. As shown in Table 2, the endogenous proteolytic activity in adult worms was increased 12-fold by stimulation with ATP and was 20-fold higher when stimulated with ATP plus ubiquitin. In cercariae, the endogenous proteolysis was increased 1.4- and 2.5-fold by stimulation with ATP, either alone or in the presence of ubiquitin, respectively.

Addition of MG-132, a peptide aldehyde inhibitor of the proteasome, caused a reduction of 90 and 75% in endogenous protein breakdown stimulated by ATP and ubiquitin in cercariae and adult worms, respectively. These results strongly suggest the presence of a ubiquitin–proteasome-dependent proteolytic system in this parasite and confirm the lower proteasomal activity observed in cercariae when compared to adult worms.

3.2. In gel chymotrypsin-like activity of proteasomes from cercariae and adult worms

To further characterize the peptidase activities of *S. mansoni* proteasomes, the crude extracts and partially purified 20S proteasome from adult worms and cercariae were subjected to non-denaturing PAGE followed by an overlay assay using Suc-LLVY-AMC as the substrate. The 26S and 20S proteasomes active bands were detected in extracts from both adult worm and cercariae. Interestingly, the activity in the cercariae proteasome bands was not so intense in comparison to that observed for adult worms proteasomes (Fig. 1A). These results were confirmed with partially purified 20S proteasomes from adult worms and cercariae (Fig. 1B). It was found that under denaturing conditions, 20S proteasomes from cercariae and adult worms display the characteristic band patterning of molecular weight between 20 and

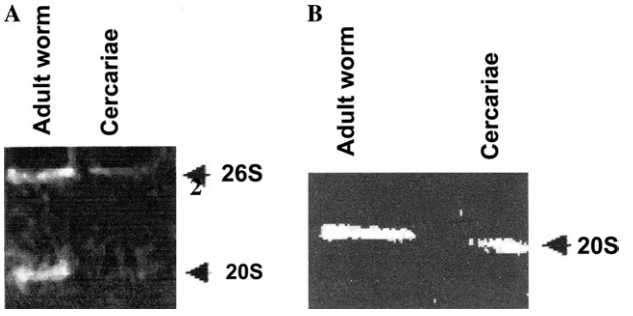

Fig. 1. Non-denaturing polyacrylamide gel electrophoresis of *S. mansoni* proteasomes. Crude extracts (15 μg) from adult worms and cercariae (A) and partially purified 20S proteasomes (10 μg) from adult worms and cercariae (B) were subjected to non-denaturing PAGE and the gel overlaid with the peptide Suc-LLVY-MCA in the presence of 1 mM ATP.

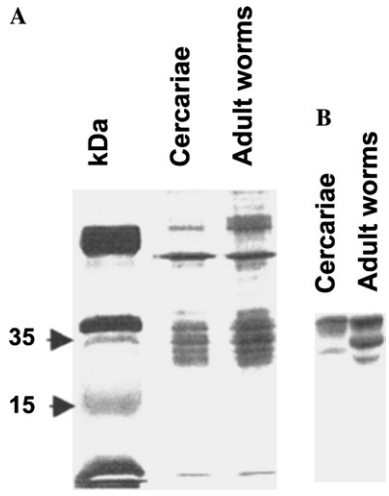

Fig. 2. SDS–PAGE and immunostaining of *S. mansoni* 20S proteasomes. Proteasome active bands from adult worms and cercariae were excised from a non-denaturing gel and subjected to 10% SDS–PAGE. The resolved 20S proteasome subunits were either silver stained (A) or transferred to nitrocellulose membranes and hybridized with a monoclonal antibody raised against human 20S proteasome α-subunits. Protein size markers (kDa) are indicated in the figure.

35 kDa. The results obtained with 20S proteasomes from adult worms and cercariae showed similar patterns of protein subunits (Fig. 2A). When we examined these 20S resolved proteasomes on immunoblots using a monoclonal antibody against human α-type subunits (Fig. 2B), we found that the antibody cross-reacted with *S. mansoni* 20S proteasome subunits. In addition, differences in the antibody reactivity were observed when comparing 20S proteasomes from cercariae and adult worms.

3.3. Proteasome inhibitors increase the level of high molecular weight ubiquitin conjugates in cercariae extracts

To determine if, under proteasome inhibition, ubiquitinated proteins would accumulate, the effects of MG-115, MG-132, and leupeptin on the levels of ubiquitin–protein conjugates were analyzed by immunoblot. The addition to the cercariae suspension of proteasome inhibitors at 50 μM resulted in the accumulation of multiple, high molecular weight bands recognized by the anti-ubiquitin antibody (Fig. 3B). The conjugates were detected even after 90 min of incubation and their levels increased with time. In parallel, a pronounced decrease was observed in the level of free ubiquitin compared to the control (data not shown).

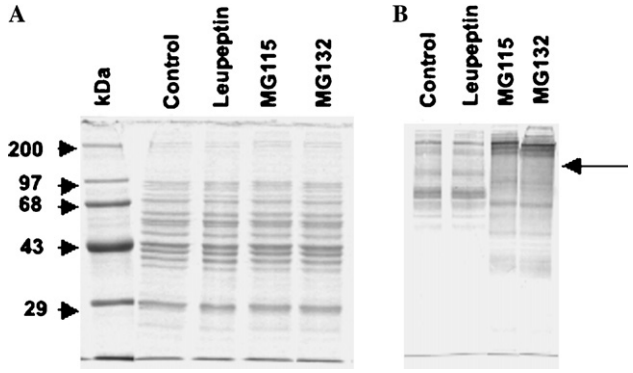

Fig. 3. Demonstration of ubiquitin conjugates from *S. mansoni* cercariae. Cercariae extracts (10 μg) pre-incubated or not for 90 min at 37 °C in the presence of 50 μM leupeptin, 50 μM MG-115, and 50 μM MG-132 were resolved by 10% SDS–PAGE and stained with Coomassie blue (A). A replica gel was blotted with a nitrocellulose membrane, which was further hybridized with anti-ubiquitin antibody and developed with the NBT and BCIP substrates (B). The position of the size marker (kDa) is indicated. Arrow indicates the accumulation of high molecular weight ubiquitin conjugates.

3.4. Effects of proteasome inhibitors on S. mansoni infection in mice

To assess whether the ubiquitin–proteasome system plays a role in *S. mansoni* development in the vertebrate

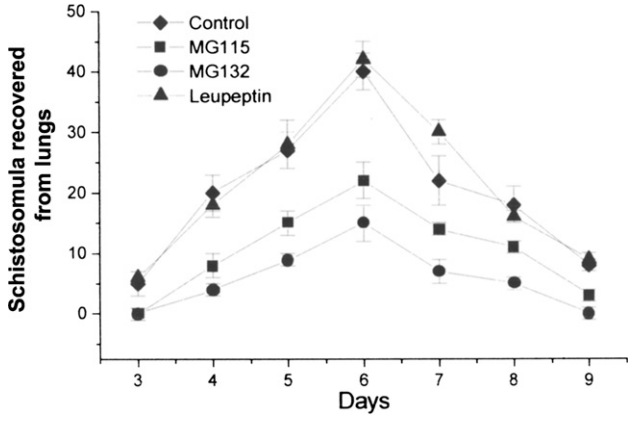

Fig. 4. Proteasome inhibitors reduce the number of lung-recovered schistosomula. Mice were infected with 100 cercariae pre-treated with 50 μM MG-115, 50 μM MG-132, 50 μM leupeptin, and 1% DMSO. After the third day post-infection three mice from each group were sacrificed daily and the number of lung-recovered schistosomula was estimated. The bars represent the averages ± SD of a triplicate determination of schistosomula recovered each day.

Table 3
The number of eggs was determined in three samples of feces for each group and the worm burden reduction rate was estimated according to the formula: % of worm burden reduction = 100 − (worm burden of the treated group × 100/worm burden of the control group)

Group	Cercariae preincubation conditions	Eggs output	Inhibition (%)	Recovered worms	Inhibition (%)
1	Control	135 ± 9	0	728 ± 44	0
2	MG115 (50 μM)	18 ± 2	86.7	242 ± 16	66
3	MG132 (50 μM)	0	100	29 ± 7	96
4	Leupeptin (50 μM)	200 ± 6	0	762 ± 29	0

Data are means ± SD from 20 animals. Effect of proteasome inhibitors on eggs output and worm burden during experimental schistosomiasis.

host, we used cercariae previously incubated with MG-115, MG-132, and leupeptin during experimental schistosomiasis. Although the peptide aldehydes MG-115 and MG-132 had no effect on cercariae penetration in the vertebrate host, these inhibitors affected the number of schistosomula recovered from the lungs of infected mice (Fig. 4). The peak of schistosomula recovery was reached on the sixth day for all groups, and both MG-115 and MG-132 caused a decrease in their numbers by 50 and 72.5%, respectively, on all analyzed days. The reduction in recovered schistosomula was due to specific blockade of proteasome degradation was confirmed by the finding that the effects in the leupeptin-treated group mirrored that of the DMSO control. Additionally, during the experimental infection the proteasome inhibitors MG-115 and MG-132 produced an inhibition in egg output of 86.7 and 100%, respectively (Table 3).

Seven weeks post-infection mice were sacrificed and the number of recovered worms was estimated. Both MG-115 and MG-132 inhibited the worm burden, by 66 and 96%, respectively (Table 3). Interestingly, those worms recovered from mice were unpaired, especially in the MG-132-treated group, and this finding is probably related to the reduction in the number of eggs in the feces (Table 3).

4. Discussion

Although the presence of the 20S proteolytic core has been demonstrated in a broad range of organisms, including *T. cruzi* (Gonzalez et al., 1996), *T. brucei* (Hua et al., 1996), *L. mexicana* (Robertson, 1999), *Entamoeba histolytica* (Scholze et al., 1996), and a cDNA with high degree of homology with the human 26S proteasome subunit S5a was identified in *S. mansoni* (Harrop et al., 1999), neither 26S nor 20S proteasomes have been identified in parasitic helminths. As far as we know, this report constitutes the first characterization of proteasomes from a helminth parasite.

Proteasome peptidase activities against fluorogenic substrates using partially purified 20S proteasome revealed, in both stages, a preference for degradation of Suc-LLVY-AMC by its chymotrypsin-like active site. The reaction specificity was evaluated by hydrolysis of this substrate in the presence of several proteasome inhibitors (Table 1B). In this respect, *S. mansoni* proteasomes are closely related to proteasomes found in higher eukaryotes when compared to their counterparts in protozoan parasites. For instance, in *T. brucei* the trypsin-like activity seems to prevail (Hua et al., 1996) and proteasomes from *L. mexicana* and *T. cruzi* are unusually inhibited by leupeptin (de Diego et al., 2001; Robertson, 1999).

Furthermore, the differences observed in the rate of hydrolysis of fluorogenic peptides (Table 1A), sensitivity to inhibitors (Table 1B) and the lower ubiquitin-dependent proteolytic activity in cercariae extracts when compared to adult worms, suggest the existence of subpopulations of proteasomes during development of this parasite. Accordingly, the analysis of 20S proteasomes by non-denaturing PAGE, followed by overlay with the peptide Suc-LLVY-AMC and immunoblotting using a monoclonal antibody against α-subunits, revealed differences in migration and in the number of labeled subunits when comparing 20S proteasomes from cercariae and adult worms (Fig. 2B). There are a number of reports in the literature describing the existence of proteasome isoforms either within the same life-stage, where they are thought to perform tissue-specific functions, or during phase-transition events, where they are believed to participate in the regulation of several growth-signaling pathways (Cardozo et al., 1995; Haass and Kloetzel, 1989; Hutson et al., 1997; Yuan et al., 1996). In *S. mansoni*, the observed antibody reactivity was subjected to further exploration using proteomic approaches (Castro-Borges et al., manuscript in preparation).

The presence of 26S proteasomes as indicated in Fig. 1A was not directly investigated, but could be inferred by the existence of a proteolytic system whose activity is stimulated by ATP and ubiquitin and reduced by highly specific proteasome inhibitors (Table 1B). In addition, the cercariae's capacity to form ubiquitin–protein conjugates (Fig. 3B), which are the natural 26S proteasome substrates, could be demonstrated by Western blot. Using proteasome inhibitors this proteolytic system was blocked in cercariae resulting in a detectable accumulation of high molecular weight ubiquitin conjugates (Fig. 3B).

The key role played by proteasomes during *S. mansoni* development in the vertebrate host is clearly demonstrated by the finding that proteasome inhibitors such as

MG-115 and MG-132 markedly reduced the worm burden and eggs output in infected mice. The concentrations of the tested inhibitors were comparable to those used in animal (Meng et al., 1999b; Palombella et al., 1994), yeast, and plant systems (Genschik et al., 1998; Lee and Goldberg, 1996; Woffenden et al., 1998).

Thus, our observations suggest that these inhibitors were taken up by cercariae and strongly affected proteasome-mediated degradation of crucial protein molecules, leading to a reduction in the number of schistosomula recovered from lungs.

The ability of these inhibitors to inhibit cercariae proteasomes was clearly confirmed by the results obtained from in vitro assays. The increase in ubiquitin conjugates is usually correlated with an activation of this system by up-regulation of members of the ubiquitin–proteasome proteolytic pathway (Rock et al., 1994), but in this case, the marked increase in polyubiquitin conjugates, as shown in Fig. 3B, appears to correlate with proteasome inhibition, as evidenced by the immunoblotting experiments. Additionally, the inhibitor's ability to bind to cercariae proteasome subunits was further confirmed by in vitro activity assays. After 1 h of incubation, some ubiquitin conjugates had already accumulated in the treated cercariae.

During experimental schistosomiasis, induced by infecting mice with cercariae previously incubated with proteasome inhibitors, a marked reduction was shown in the number of larvae recovered from lungs (Fig. 4), the worm burden and the number of eggs eliminated in feces (Table 3) when comparing the same parameters to those of the control group. The reduction in the worm burden was later confirmed in the portal system. Under the conditions and concentration tested, leupeptin, primarily an inhibitor of cysteine and serine proteases, which is structurally similar to MG-132 and MG-115 and expected to exhibit a comparable degree of diffusion into the cercarial body, did not compromise the parasitic development in the vertebrate host. The reduced recovery of schistosomula from lungs, when cercariae were previously incubated with proteasome inhibitors, may reflect the premature death of most larvae during the skin–lung interval because the reduction in the number of lung schistosomula was found from the third day until the ninth day of recovery. It is worth mentioning that the technique employed for schistosomula recovery permits the capture of live larvae only. The effect of proteasome inhibitors over *S. mansoni* in this study is then limited to the skin and or pulmonary phases and, therefore, it may represent a post-cercarial penetration event.

On the other hand, the possibility of a direct effect of these inhibitors on the viability of cercariae, by promoting the parasite's death and consequently inducing a real decrease in schistosomula recovered from lungs was discarded. The rate of infection was similar in all experimental groups (approximately, 100%) as demonstrated by the absence of remaining viable cercariae in the medium after the time of exposure. The number of tails left in the medium, although not a reliable parameter, was also comparable in all experimental groups. In agreement with this, the results of the in vitro cultivation showed that these inhibitors do not lead to death of this parasite. Accordingly, a direct signal of biochemical integrity of the cercariae, during exposure to the proteasome inhibitor, could be inferred by the ability to continue the conjugation of cytosolic proteins with ubiquitin, resulting in a detectable accumulation of these (Fig. 3B). Taken together, these results support the hypothesis that this particular degradation pathway is an important determinant for the successful development of cercariae in the vertebrate host.

Based on these pieces of evidence, we believe that the reduction in worm burden and, consequently, in egg output observed in the presence of proteasome inhibitors may be due to an early action upon premature stages of the parasite, during or between the skin and pulmonary migration phases. Furthermore, there was a reduction in real terms in the number of recovered larvae and not a retarded migration of these. Proteasome inhibition could have interfered with the levels of one or more proteins that directly or indirectly regulate the developmental mechanism.

The molecular mechanisms by which schistosome larvae gain access to the vascular system by migration through the skin of their definitive host have not yet been precisely defined and it is not known how many and which type of enzymes are involved in this process (Curwen and Wilson, 2003). On the other hand, several studies have revealed, at the microscopic level, that the parasite's body undergoes extensive remodeling associated with its adaptation to the vertebrate host. The emptying of the secretory glands, the shedding of the glycocalyx, and the forming of the new double-unit membrane on the syncytial surface are the first signs of transformation (Stirewalt, 1974). Later on, the disappearance of the gland cells and the reconfiguring of the musculature before intravascular migration may represent processes wherein proteasome-mediated degradation could play an important role (Crabtree and Wilson, 1986). In accordance with this suggestion is the abundance of transcripts coding for proteins of the ubiquitin–proteasome and autophagy pathways and the little evidence for the existence of apoptosis-related molecules found during the *S. mansoni* transcriptome project http://verjo18.iq.usp.br/schisto/.

The factors that control the *S. mansoni* development are of considerable interest for understanding of the metabolic processes, associated with the parasite's biology, involved in schistosomiasis. Interference with the development and differentiation of the larvae in the vertebrate host is an interesting approach in the search for molecular tools aimed at manipulating the parasite for

practical purposes, such as therapeutic intervention. The results presented here not only provide the first demonstration that the ubiquitin–proteasome system plays a crucial role in *S. mansoni* biology, but also that proteasome inhibitors can be used as tools to study the molecular mechanisms governing its developmental program in the vertebrate host.

Acknowledgments

This work was supported by grants from FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo), CAPES, and CNPq. The authors thank Olinda Mara Trevilato for her technical assistance.

References

Adams, J., Behnke, M., Chen, S., Cruickshank, A.A., Dick, L.R., Grenier, L., Klunder, J.M., Ma, Y.T., Plamondon, L., Stein, R.L., 1998. Potent and selective inhibitors of the proteasome: Dipeptidyl boronic acids. Bioorganic and Medicinal Chemistry Letters 84, 333–338.

Adams, J., Palombella, V.J., Sausville, E.A., Johnson, J., Destree, A., Lazarus, D.D., Maas, J., Pien, C.S., Prakash, S., Elliott, P.J., 1999. Proteasome inhibitors: A novel class of potent and effective antitumor agents. Cancer Research 59, 2615–2622.

Barbosa, M.A., Pellegrino, J., Coelho, P.M., Sampaio, I.B., 1978. Quantitative aspects of the migration and evolutive asynchronism of *Schistosoma mansoni* in mice. Revista do Instituto de Medicina Tropical de São Paulo 20, 121–132.

Bogyo, M., McMaster, J.S., Gaczynska, M., Tortorella, D., Goldberg, A.L., Ploegh, H., 1997. Covalent modification of the active site threonine of proteasomal subunits and the *Escherichia coli* Homolog HslV by a new class of inhibitors. Proceedings of the National Academy of Science United States of America 94, 6629–6634.

Cardozo, C., Eleuteri, A.M., Orlowski, M., 1995. Differences in catalytic activities and subunit pattern of multicatalytic proteinase complexes (proteasomes) isolated from bovine pituitary, lung, and liver. Changes in LMP7 and the component necessary for expression of the chymotrypsin-like activity. Journal of Biological Chemistry 270, 22645–22651.

Crabtree, J.E., Wilson, R.A., 1986. *Schistosoma mansoni*: An ultrastructural study of pulmonary migration. Parasitology 92, 343–354.

Curwen, R.S., Wilson, R.A., 2003. Invasion of skin by schistosome cercariae: Some neglected facts. Trends in Parasitology 19, 66–68.

de Diego, J.L., Katz, J.M., Marshall, P., Gutierrez, B., Manning, J.E., Nussenzweig, V., Gonzalez, J., 2001. The ubiquitin–proteasome pathway plays an essential role in proteolysis during *Trypanosoma cruzi* remodeling. Biochemistry 40, 1053–1062.

Dick, L.R., Cruikshank, A.A., Grenier, L., Melandri, F.D., Nunes, S.L., Stein, R.L., 1996. Mechanistic studies on the inactivation of the proteasome by lactacystin: A central role for clasto-lactacystin beta-lactone. Journal of Biological Chemistry 271, 7273–7276.

Engels, D., Chitsulo, L., Montresor, A., Savioli, L., 2002. The global epidemiological situation of schistosomiasis and new approaches to control and research. Acta Tropica 82, 139–146.

Fagan, J.M., Waxman, L., Goldberg, A.L., 1987. Skeletal muscle and liver contain a soluble ATP + ubiquitin-dependent proteolytic system. Biochemical Journal 243, 335–343.

Fishelson, Z., Amiri, P., Friend, D.S., Marikovsky, M., Petitt, M., Newport, G., McKerrow, J.H., 1992. Schistosoma mansoni: Cell-specific expression and secretion of a serine protease during development of cercariae. Experimental Parasitology 75, 87–98.

Gantt, S.M., Myung, J.M., Briones, M.R., Li, W.D., Corey, E.J., Omura, S., Nussenzweig, V., Sinnis, P., 1998. Proteasome inhibitors block development of *Plasmodium* spp. Antimicrobial Agents and Chemotherapy 42, 2731–2738.

Genschik, P., Criqui, M.C., Parmentier, Y., Derevier, A., Fleck, J., 1998. Cell cycle-dependent proteolysis in plants: Identification of the destruction box pathway and metaphase arrest produced by the proteasome inhibitor MG-132. Plant Cell 10, 2063–2076.

Glickman, M.H., Ciechanover, A., 2002. The ubiquitin–proteasome proteolytic pathway: Destruction for the sake of construction. Physiology Review 82, 373–428.

Glickman, M.H., Maytal, V., 2002. Regulating the 26 Sproteasome. Current Topics in Microbiology and Immunology 268, 43–72.

Glickman, M.H., Rubin, D.M., Fried, V.A., Finley, D., 1998. The regulatory particle of the *Saccharomyces cerevisiae* proteasome. Molecular and Cellular Biology 18, 3149–3162.

Goldberg, A.L., Akopian, T.N., Kisselev, A.F., Lee, D.H., Rohrwild, M., 1997. New insights into the mechanisms and importance of the proteasome in intracellular protein degradation. Biological Chemistry 378, 131–140.

Goldberg, A.L., Rock, K., 2002. Not just research tools-proteasome inhibitors offer therapeutic promise. Nature Medicine 8, 338–340.

Gonzalez, J., Ramalho-Pinto, F.J., Frevert, U., Ghiso, J., Tomlinson, S., Scharfstein, J., Corey, E.J., Nussenzweig, V., 1996. Proteasome activity is required for stage-specific transformation of a protozoan parasite. Journal of Experimental Medicine 184, 1909–1918.

Haass, C., Kloetzel, P.M., 1989. The *Drosophila* proteasome undergoes changes in its subunit pattern during development. Experimental Cell Research 180, 243–252.

Harrop, R., Coulson, P.S., Wilson, R.A., 1999. Characterization, cloning and immunogenicity of antigens released by lung-stage larvae of *Schistosoma mansoni*. Parasitology 118, 583–594.

Hua, S., To, W.-Y., Nguyen, T.T., Wong, M-L., Wang, C.C., 1996. Purification and characterization of proteasome from *Trypanosoma brucei*. Molecular and Biochemical Parasitology 78, 33–46.

Hutson, M.R., Rhodes, M.R., Kirby, M.L., 1997. Differential expression of a proteasomal subunit during chick development. Biochemical and Biophysical Research Communications 234, 216–223.

Lee, D.H., Goldberg, A.L., 1996. Selective inhibitors of the proteasome-dependent and vacuolar pathways of protein degradation in *Saccharomyces cerevisiae*. Journal of Biological Chemistry 271, 27280–27284.

Makioka, A., Kumagai, M., Ohtomo, H., Kobayashi, S., Takeuchi, T., 2002. Effect of proteasome inhibitors on the growth, encystation, and excystation of *Entamoeba histolytica* and *Entamoeba invadens*. Parasitology Research 88, 454–459.

McKerrow, J.H., Salter, J., 2002. Invasion of skin by Schistosoma cercariae. Trends in Parasitology 18, 193–195.

Meng, L., Kwok, B.H., Sin, N., Crews, C.M., 1999a. Eponemycin exerts its antitumor effect through the inhibition of proteasome function. Cancer Research 59, 2798–2801.

Meng, L., Mohan, R., Kwok, B.H., Elofsson, M., Sin, N., Crews, C.M., 1999b. Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo anti-inflammatory activity. Proceedings of the National Academy of Science United States of America 96, 10403–10408.

Nkemgu-Njinkeng, J., Rosenkranz, V., Wink, M., Steverding, D., 2002. Antitrypanosomal activities of proteasome inhibitors. Antimicrobial Agents and hemotherapy 46, 2038–2040.

Palombella, V.J., Rando, O.J., Goldberg, A.L., Maniatis, T., 1994. The ubiquitin–proteasome pathway is required for processing the NF-kappa B1 precursor protein and the activation of NF-kappa B. Cell 78, 773–785.

Pickart, C.M., 2001. Mechanisms Underlying Ubiquitination. Annual Review of Biochemistry 70, 503–532.

Robertson, C.D., 1999. The *Leishmania mexicana* proteasome. Molecular and Biochemical Parasitology 103, 49–60.

Rock, K.L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., Goldberg, A.L., 1994. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell 78, 761–771.

Scholze, H., Frey, S., Cejka, Z., Bakker-Grunwald, T., 1996. Evidence for the existence of both proteasomes and a novel high molecular weight peptidase in *Entamoeba histolytica*. Journal of Biological Chemistry 271, 6212–6216.

Shaw, M.K., He, C.Y., Roos, D.S., Tilney, L.G., 2000. Proteasome inhibitors block intracellular growth and replication of *Toxoplasma gondii*. Parasitology 121, 35–47.

Stirewalt, M.A., 1974. *Schistosoma mansoni*: Cercaria to schistosomule. Advances in Parasitology 12, 115–182.

Tanaka, K., 1998. Proteasomes: Structure and Biology. Journal of Biochemistry 123, 195–204.

Waalkes, T.P., Udenfriend, S., 1957. A fluorometric method for the estimation of tyrosine in plasma and tissues. Journal of Laboratory and Clinical Medicine 50, 733–736.

Woffenden, B.J., Freeman, T.B., Beers, E.P., 1998. Proteasome inhibitors prevent tracheary element differentiation in zinnia mesophyll cell cultures. Plant Physiology 118, 419–430.

Yuan, X., Miller, M., Belote, J.M., 1996. Duplicated proteasome subunit genes in *Drosophila melanogaster* encoding testes-specific isoforms. Genetics 144, 147–157.