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EVIDENCE FOR THE EXISTENCE OF A PROTEASOME IN *TOXOPLASMA GONDII*: INTRACELLULAR LOCALIZATION AND SPECIFIC PEPTIDASE ACTIVITIES

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Summary :

The proteasome is a large intracellular protein complex whose main function is proteolytic removal of damaged proteins. It has recently been shown that the proteasome has a crucial role in the pathogenesis of protozoan parasites. We attempted to characterize the proteasome of *T. gondii* (RH strain). In immunoblot experiments, we showed that MCP231 monoclonal antibody, directed against the human 20S proteasome, labelled homologous proteins in *T. gondii* with a pattern similar to that observed in mammalian cells. The study of *in vitro* proteolytic activities showed that chymotrypsin-like activity (the only activity obtained with archaebacteria) was present in *Toxoplasma*, with K_m and specific activity values close to those observed with eukaryotic cells. Immunofluorescence studies showed that the *Toxoplasma* proteasome predominated in the cytosol.

KEY WORDS : *Toxoplasma gondii*, proteasome, immunoblotting, chymotrypsin-like, trypsin-like, fluorescent antibody.

Résumé : MISE EN ÉVIDENCE DU PROTÉASOME DE *TOXOPLASMA GONDII*: LOCALISATION INTRACELLULAIRE ET ACTIVITÉS SPÉCIFIQUES PEPTIDASIQUES

Le protéasome est un complexe protéique cellulaire dont le rôle essentiel est la protéolyse des protéines anormales. Son implication dans la pathogénicité des protozoaires parasites a pu être démontrée récemment. Notre étude avait pour but de caractériser le protéasome de T. gondii *(souche RH). Par immunoblot, nous avons montré que le protéasome de toxoplasme était détecté à l'aide de l'anticorps monoclonal MCP231, dirigé contre la partie 20S du protéasome humain, le profil d'immunomarquage étant identique à celui obtenu avec différentes cellules de mammifères. L'étude des activités protéolytiques a mis en évidence une activité dite chimiotrypsine-similaire (seule activité retrouvée chez les archéobactéries) dont le K_m et l'activité spécifique sont proches de celles retrouvées chez les cellules eucaryotes. Par immunofluorescence, la localisation du protéasome de toxoplasme apparaît essentiellement cytoplasmique.*

MOTS CLÉS : Toxoplasma gondii, *protéasome, trypsine-similaire, chimiotrypsine-similaire, immunoblot, immunofluorescence.*

INTRODUCTION

The proteasome is a non lysosomal large multi-subunit protease complex that is ubiquitous in eukaryotic cells (Bochtler *et al.*, 1999; Dahlmann *et al.*, 1989). Recent studies have shown that the proteasome is involved in the regulation of key cellular processes such as cell cycle progression, oncogenesis, transcription, development, growth and atrophy of development tissues, substrate flux through metabolic pathways, selective elimination of abnormal proteins, and antigen processing (DeMartino & Slaughter, 1999). The proteasome plays a key role in the regulation of numerous transcription factors (Hilt & Wolf, 1996). Electron microscopy and X-ray crystallography of proteasomes from *Thermoplasma acidophilum* (Baumeister *et al.*, 1998) and *Saccharomyces cerevisae* (Groll *et al.*, 1997) revealed a cylindrical structure composed of four rings with a narrow channel running through the center of the structure. Each ring consists of seven subunits classified as α and β (7α7β7β7α) on the basis of the simpler proteasome found in the archaebacterium *Thermoplasma acidophilum*. Proteins are hydrolyzed inside the cylinder sites located on α-subunits. In eukaryotic cells, the proteasome consists of two complexes, namely the 20S proteasome, which forms the proteolytic core, and the 26S proteasome, which consists of the 20S proteasome and a complex regulator (PA 700) which confers ATP-dependency and ubiquitinated substrate specificity on the enzyme (Ferell *et al.*, 2000). The proteasome, previously described as a multicatalytic proteinase complex, has several distinct peptidase activities. It hydrolyses small peptides whose carboxyl end contains basic, hydrophobic and acidic amino acids (Goldberg, 1995). The first two specificities are often referred to as the trypsin-like (basic) and chymotrypsin-like (hydrophobic) activities (Arribas & Castano, 1993; Orlowski & Wilk, 1988). The *Thermoplasma* proteasome contains only one type of active site and primarily exhibits chymotrypsin-like activity (Dahlmann *et al.*, 1992). Presumably, during evolution from the prokaryote to the eukaryotic proteasome, the active site developed increasing (but not absolute) specificities (Coux *et al.*,

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1996). Proteases play an important role in the pathogenic mechanisms and differentiation of protozoan parasites; proteasome function has recently been found to play a role in the life cycle progression of the parasites *Trypanosoma cruzi* (Gonzalez *et al.*, 1996) and *T. brucei* (Mutomba *et al.*, 1997). Evidence for the presence of proteasomes in *Entamoeba histolytica* (Scholze *et al.*, 1995) and *Leishmania mexicana* (Robertson, 1999) has also been reported. The potential of proteasome inhibitors for the treatment of malaria has been highlighted in an experimental model (Gantt *et al.*, 1998), and Shaw recently reported the inhibition of *Toxoplasma gondii* growth and replication by proteasome inhibitors (Shaw *et al.*, 2000). However, little is known of proteasome distribution and activity in protozoan parasites.

The objectives of this study were to detect proteasomes in *Toxoplasma* by means of immunoblot and immunofluorescence, and to characterize *Toxoplasma* peptidase activity in soluble cell extracts in comparison with HeLa cells

MATERIALS AND METHODS

T. GONDII ISOLATION AND CELL CULTURE

The RH strain of *Toxoplasma gondii* (Sabin, 1941) was maintained by serial passage in IOPS/OF1 Swiss male mice (Iffa Credo, France). Tachyzoites were harvested from peritoneal fluid of mice infected four days earlier. Mice were killed and sterile saline was injected into the peritoneal cavity. Tachyzoites were recovered with a syringe. After two filtration steps on 10- and 5-µM Cyclopore track-etched membranes (Whatman), the recovered fluid was centrifuged for 10 min at 1,000 g. The pellet was washed and resuspended in phosphate buffered saline (PBS) and centrifuged for 15 min at 20,000 g. The pellet was immediately stored at – 20° C for immunoblotting and peptidase assays. NIH-3T3 murine fibroblast cells and HeLa cells were grown on 22-mm^2 glass coverslips (Marienfeld, Nr. 1) in Dulbecco's modified Eagle's medium pH 7.2 (Life Technologies), supplemented with 10 % fetal calf serum, 5 IU/ml penicillin and 5 µg/ml streptomycin at 37° C in humidified 5 % CO_2-air. NIH-3T3 murine fibroblasts were infected with tachyzoites (~ 10^5 parasites/ml). Parasites were harvested shortly after complete host-cell lysis and purified by filtration as described above. Free tachyzoites were centrifuged for 10 min at 600 g at room temperature, then washed and deposited on coverslips for immunofluorescence studies (see below).

PROTEIN AND PEPTIDASE ASSAYS

The synthetic fluorogenic substrates succinyl-leucyl-leucyl-valyl-tyrosyl-4-methylcoumaryl-7-amide (Suc-LLVY-AMC) and succinyl-leucyl-seryl-threonyl-arginyl-4-methylcoumaryl-7-amide (Suc-LSTR-AMC) were purchased from Sigma Chemical Co. The protein concentration was determined by the Bradford microassay method (Bradford, 1976) using γ-globulin as standard. Stock solutions of the peptide substrates were prepared as follows: LLVY and LSTR were diluted to 20 mM in 100 % DMSO and stored at – 20° C. Prior to the proteolytic assay, substrates were diluted in Tris-HCl (50 mM pH 7.5). For the determination of K_m – and activity – values, a wide range of LLVY and LSTR concentrations was used (10 to 2,000 µM). Frozen cells were rapidly thawed and homogenized at 4° C in 100 µl of 25 mM Tris pH 7.5 then frozen again at – 80° C for at least 10 min before rethawing. The combination of hypotonic buffer treatment and repeated freezing caused complete lysis. The protein concentration in the reaction mixture was 25 µg per sample. At the start of the proteolytic assay, 50 µl of sample solution and 50 µl of substrate were added at 37° C to individual wells of 96-well plates (Nunc) and the fluorescence of the cleavage product was detected at an emission wavelength of 440 nm (excitation wavelength 380 nm) in a Wallac Victor spectrofluorimeter. A standard curve of methylcoumaryl-7-amide (AMC) from 0.5 to 100 µM was prepared to express proteolytic activity as picomoles of AMC per minute and milligram of protein.

ANTIBODIES

MCP-231, a mouse monoclonal antibody against 20S human proteasomes, was obtained from Affiniti Research Products (UK). This antibody reacts with the phylogenetically preserved probox 1 motif shared by all α-type subunits (HC2, HC3, HC8, HC9, Iota and Zeta); it reacts with proteasomes of several species, including higher plants, when tested on immunoblots of SDS/PAGE gels, yielding two main bands of 29 and 32 kDa (Hendil *et al.*, 1995; Kopp *et al.*, 1997; Tanaka & Tsurumi, 1997). A rabbit polyclonal antibody against the 20S proteasome "core" was obtained from Affiniti. This antibody was raised by rabbit immunization with a proteasome preparation from human red blood cells. Western blotting shows bands attributable to "core" subunits at 25-30 kDa. This antibody has been shown to react with human, mouse and yeast proteasomes (Kopp *et al.*, 1997; Tanaka & Tsurumi, 1997). Rabbit immunserum against liver rat proteasomes was a generous gift from B. Friguet (Conconi *et al.*, 1996).

POLYACRYLAMIDE GEL ELECTROPHORESIS AND IMMUNOBLOTTING

Immunoblot analysis was performed, in the same experiment, with *Toxoplasma gondii*, HeLa cells and NIH-3T3 murine fibroblast cells. The protein extracts were prepared at 4° C. The cells were lysed in cold lysis

buffer containing 1 % NP40, 0.1 % SDS, 158 mM NaCl, 10 mM Tris pH 7.8, 1 mM phenylmethylsulfonylfluoride and 1 mM Na_3VO_4. The cell suspensions were then centrifuged for 20 min at 12,000 g at 4° C. We tested the supernatant and pellet of each cell type. Pellets were homogenized in 100 µl of 25 mM Tris pH 7.8. The protein concentration was determined as described above. Equal amounts of protein (50 µg) from the supernatant and pellet were separated by electrophoresis on SDS-12.5 % polyacrylamide gel and transferred electrophoretically onto a nitrocellulose membrane. The membrane was incubated for 2 h at room temperature with 0.25 % gelatin in Tris-buffered saline (10 mM Tris pH 7.5, 150 mM NaCl) containing 0.05 % Tween 20 (TBST) to block nonspecific binding sites. The membrane was then incubated for 1.5 h at room temperature with the MCP231 anti-20S proteasome monoclonal antibody at 1:2,000 dilution (Affiniti). After extensive washing with TBST, the membrane was incubated for two hours with horseradish peroxidase-conjugated goat anti-mouse IgG (Dako) diluted 1:5,000, then washed with blocking buffer. Proteins were visualized after chemiluminescence staining, following the manufacturer's protocol (ECL, Amersham).

IMMUNOCYTOFLUORESCENCE AND CONFOCAL MICROSCOPY

Cells grown on 22-mm^2 glass coverslips were used for immunocytofluorescence studies. The cells were washed three times with PBS then fixed with 4 % paraformaldehyde in PBS for 10 min. This and all subsequent steps were performed at room temperature. Permeabilization was obtained with 1 % Triton X100 in PBS for 5 min. Preincubation for 30 min with PBS + 1 % bovine serum albumin, Fraction V (Sigma) + 1 % normal goat serum (GS) was followed by incubation for 1.5 h with proteasome antibody at 1:50 dilution in PBS supplemented with 1 % GS. After washing in PBS supplemented with 1 % BSA, 1 % GS and 0.1 % Triton, the secondary fluorescent antibody (FITC-coupled goat antibody, Sigma) at 1:100 dilution was added for 40 min. Final washes were followed by mounting in Vectashield (Vector, Biosys). Coverslips were examined using a BioRad MRC-1000 device mounted on a Nikon Optiphot II equipped with a 40x objective (Plan apo: NA 1.4). Confocal sections were taken at 0.5-µm focus steps. HeLa cells and NIH-3T3 murine fibrobasts were used for comparison. Labeling was considered specific, as no labeling was observed when the primary antibody was omitted.

RESULTS AND DISCUSSION

IMMUNOBLOTTING

Supernatants and pellets of *Toxoplasma*, HeLa cells and mouse cells (NIH-3T3) containing the same amount of protein (50 µg) were fractionated by SDS-PAGE and blotted to nitrocellulose membranes as described in Material and Methods. We tested a rat brain lysate as specific control, because immunoblot analysis of purified proteasomes and total brain homogenate with anti-proteasome antibody yields the same pattern (three polypeptide bands around 30 kDa) (Mengual *et al.*, 1996). Immunoblotting of cell lysates with a mouse Mab MCP231 yielded three different bands at 29, 30 and 32 kDa with *T. gondii* (Fig. 1). The two most intensely labeled bands had relative molecular weights of 29 and 32 kDa. An identical pattern was observed with the three animal cell types. Although an equal amount of protein was used, supernatants yielded more-intense bands than pellets, confirming that in *Toxoplasma*, as in other eukaryotic cells, proteasomes are mainly present in the cytosolic fraction (Palmer *et al.*, 1996). The MCP231 antibody reacts with a motif common to all α-type subunits (HC2, HC3, HC8, HC9, Iota and Zeta), which is phylogenetically preserved (Hendil *et al.*, 1995). To our knowledge, MCP231 has only been used twice to detect proteasomes in protozoan parasites. Scholze *et al.* (1995) showed that MCP231 crossreacted with a soluble *E. histolytica* extract. Emmerlich *et al.* (1999) observed immunoblot cross-reactivity with MCP231 and *Giardia lamblia* proteins; MCP231 was the only antibody showing cross-reactivity among several other antibodies directed against the proteasome. We observed a similar lack of cross-reac-

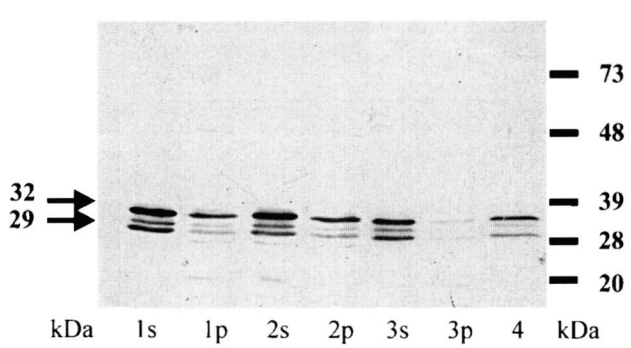

Fig. 1. – SDS-PAGE and immunoblotting of cell lysates with mAb MCP231 (12.5 % acrylamide gel).
1: Human cells (HeLa).
2: *Toxoplasma gondii* cells.
3: Mouse cells (NIH-3T3 fibroblasts).
4: Rat cells (brain homogenate).
s: Supernatant. p: Pellet.
Equal amounts of protein were subjected to SDS-PAGE as described in Materials and Methods.

tivity with the *T. gondii* proteasome when we used an antiserum raised against the 20S human red cell proteasome (see Materials and Methods) in immunoblot experiments. Hua *et al.* (1996) demonstrated that the 20S proteasome of *Trypanosoma brucei* showed no cross-reactivity with an antiserum raised against the human 20S proteasome. In addition, To *et al.* (1997), using polyclonal antibodies raised against the *T. brucei* proteasome, observed no cross reactivity with proteasomes from rat blood cells. These data point to species-specific differences among proteasomes of protozoan parasites and mammalian cells.

Proteolytic activities

Proteolytic activities were measured with fluorogenic peptides as described above. The hydrolytic reaction rate, when tested as a function of the enzyme concentration and incubation time, was linear, indicating that the enzyme was stable in the assay conditions used (data not shown).

The two peptide substrates tested here exhibited different properties with regard to substrate requirements and rate of hydrolysis; Lineweaver-Burke plots of proteolytic activity at different substrate concentrations allowed us to determine the K_m of the two substrates. The derived K_m and specific activity values are listed in Table I. Determination of the K_m value revealed higher binding of the *Toxoplasma* proteasome to the LLVY substrate (K_m = 60 µM) than to the LSTR substrate (K_m = 260 µM), as observed with HeLa cells (K_m = 80 and 240 µM, respectively). The proteasome activities of *Toxoplasma* and HeLa cells had the same magnitude for LLVY (16.7 versus 32 pmol.min^{-1}.mg^{-1}) but very different magnitudes for LSTR (76.2 for *Toxoplasma* versus 1568 pmol.min^{-1}.mg^{-1} for HeLa cells). To determine if the HeLa cell LSTR value was representative of mammalian cells, we also tested *Toxoplasma* cells and NIH-3T3 mouse cells simultaneously, and obtained similar results to those obtained with *Toxoplasma* and HeLa cells (data not shown).

Catalytic activity	[a]K_m (4)		[a]Specific activity (7)	
	HeLa	*T. gondii*	HeLa	*T. gondii*
Chymotrypsin-like Suc-LLVY-AMC	0.08±0.02	0.06±0.01	32±7.3	16.7±5.2
Trypsin-like Suc-LSTR-AMC	0.24±0.05	0.26±0.03	1598±328	76.2±10.07

Proteolytic activities were measured after 2 h of incubation (pH 7.5; 37° C) with 200 µM substrate. Fluorescence was detected at 460 nm (355 nm excitation).

[a]: Values (means ± standard deviation) were obtained from the numbers of experiments shown in parentheses.

Table I. – Proteasome-derived K_m (mM) and proteolytic activities (pmol.min^{-1}.mg^{-1}) for synthetic substrates. Comparison between results obtained with *Toxoplasma* cell lysates and HeLa cell lysates.

Few data are available on proteasome peptidase activities of protozoan parasites, and most have been obtained with purified 20S proteasomes. The chymotrypsin-like activities (LLVY-AMC fluorogenic substrate, expressed in nmol.mg^{-1}.min^{-1}) observed with purified proteasomes of protozoan parasites range from 0 with *Giardia lambia* (Emmerlich *et al.*, 1999) to 6.2-69.2 with *Trypanosoma brucei* (Hua *et al.*, 1996; To *et al.*, 1997); 9.4 with *Leishmania mexicana* (Roberston, 1999) and 20.44 with *Entamoeba invadens* (Gonzalez *et al.*, 1996). We chose to work with crude cell extracts and not with purified proteasomes for the following reasons. Proteasomes in crude cell extracts are more likely to retain their native activities (Driscoll *et al.*, 1992). Indeed, according to various studies, isolation and purification processes activate the proteasome and lead to structural changes (Ma *et al.*, 1992). This could explain why Emmerlich *et al.* (1999) after purification of *Giardia lamblia* 20S proteasomes, failed to observe significant activity on a range of fluorogenic peptides that are hydrolyzed by the proteasomes of other organisms. Published activity values of crude parasite proteasome extracts are scarce. However, protease activities measured with Suc-LLVY-AMC as substrate have been reported for crude extracts of *Entamoeba invadens* (Gonzalez *et al.*, 1996) and *E. histolytica* (Scholze *et al.*, 1995). The observed activities – 0.016 and 0.01 nmol/min.mg, respectively – were close to those we obtained with *T. gondii* (0.011 nmol/min.mg). We found no published studies using LSTR and crude parasite extracts, but our results suggest that proteasome of *T. gondii* has lower trypsin-like activity to that of mammalian cell (1598 pmol.min^{-1}.mg for HeLa versus 76 pmol.min^{-1}.mg for *Toxoplasma*). The proteolytic activity of the *Toxoplasma* proteasome seems closer to that of prokaryotic proteasomes than that of mammalian proteasomes, the former showing only chymotrypsin-like activity (de Mot *et al.*, 1999).

Immunofluorescence microscopy

We tested the Mab MCP231 and two rabbit antisera, one against human proteasomes (Affiniti) and the other against rat proteasomes (generously provided by Pr B. Friguet, Paris VI University). As the rabbit antiserum against rat proteasomes gave the clearest immunofluorescence pattern, we only discuss the results obtained with this antibody. In the same experiment, we simultaneously labeled *Toxoplasma*, HeLa cells and NIH-3T3 murine fibrobast cell crude extracts. The proteasome of NIH-3T3 murine fibroblast cells and HeLa cells was localized in the cytoplasm and the nucleus. The cytoplasm showed fine granular staining, nuclear staining was stronger, and nucleoli were not labeled (Fig. 2A). This pattern is that usually observed with eukaryotic cells (Machiels *et al.*, 1995). With *Toxo-*

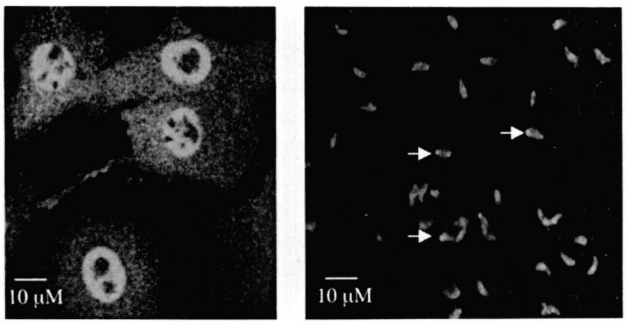

Fig. 2. – Immunofluorescence microscopy of cells cultured *in vitro* and labeled with a rabbit polydonal antibody raised against rat proteasomes.
A: NIH-3T3 murine fibroblast cells.
Note that both the cytoplasm and the nucleus were labeled.
B: Free *T. gondii* from 3T3 cell culture.
Agregates of cytoplasmic fluorescence are arrowed. Absence of nucleal fluorescence was visualized by staining DNA with 4.6-diamidino-2-phenylindole (DAPI) corresponding to blue coloration of the nucleus.

plasma (Fig. 2B), the cytoplasm was labeled while the nucleus was not. In a wide variety of multicellular organisms the proteasome is found in both the cytoplasm and the nucleus (Tanaka *et al.*, 1990; Amsterdam *et al.*, 1993). Subcellular fractionation experiments have generally shown that proteasomes are more abundant in the cytosol than in the nucleoplasm (Russel *et al.*, 1999). Densitometric analysis of western blots obtained in cell-fractionation experiments shows that, for the same amount of bulk protein in each fraction, the level of proteasomes is about 10-fold higher in the cytosol than in the nucleus (Palmer *et al.*, 1996), although the exact ratio varies with the cell type and growth conditions (Peters *et al.*, 1994; Yang *et al.*, 1995). In addition, immunofluorescence experiments with mammalian cells have shown that the proteasome ratio varies in a complex manner through the cell cycle (Nannmark *et al.*, 1996; Knecht *et al.*, 1991; Russel *et al.*, 1999). To detect possible changes in the intracellular localization of the *Toxoplasma* proteasome during mitosis, we stained NIH-3T3 murine fibroblast cells infected by *Toxoplasma* (data not shown). Despite the intracellular multiplication of *Toxoplasma,* we observed no nuclear labeling, suggesting that the *Toxoplasma* proteasome is most abundant in the cytoplasm. Two distribution patterns were seen: fluorescence was dispersed throughout the cell in 90 % of parasites, while it showed aggregates of different sizes and shape in 10 % of parasites (Fig. 2B, arrowheads). The aggregates tended to accumulate at the basal pole of the parasite, where the endoplasmic reticulum is principally located (Hager *et al.*, 1999). This difference in labeling could also be due to the presence of parasites at different stages of the cell cycle (Reits *et al.*, 1997). Thus, although the intracellular distribution of mammalian proteasomes remains somewhat controversial (Hirch & Ploegh, 2000), the *T. gondii* proteasome seems to be exclusively located in the cytoplasm.

CONCLUSION

We report evidence that *T. gondii* possesses a proteasome. In immunoblot experiments, *T. gondii* crude extracts reacted strongly with the monoclonal antibody MCP231 but was only weakly reactive with a polyclonal antibody against the 20S human proteasome. The chymotrypsin-like activity of the *T. gondii* proteasome was similar to that of eukaryotic proteasomes, although the trypsin-like activity of the former was far weaker. Localization studies of the *Toxoplasma* proteasome based on immunofluorescence and confocal microscopy suggested a principally cytoplasmic location and no nuclear labelling. These data point to differences between the *Toxoplasma* proteasome and the mammalian 20S proteasome.

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