An Easily Dissociated 26 S Proteasome Catalyzes an Essential Ubiquitin-mediated Protein Degradation Pathway in Trypanosoma brucei

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The 26 S proteasome, a complex between the 20 S proteasome and 19 S regulatory units, catalyzes ATP-dependent degradation of unfolded and ubiquitinated proteins in eukaryotes. We have identified previously 20 S and activated 20 S proteasomes in Trypanosoma brucei, but not 26 S proteasome. However, the presence of 26 S proteasome in T. brucei was suggested by the hydrolysis of casein by cell lysate, a process that requires ATP but is inhibited by lactacystin, and the lactacystin-sensitive turnover of ubiquitinated proteins in the intact cells. T. brucei cDNAs encoding the six proteasome ATPase homologues (Rpt) were cloned and expressed. Five of the six T. brucei Rpt cDNAs, except for Rpt2, were capable of functionally complementing the corresponding rpt deletion mutants of Saccharomyces cerevisiae. Immunoblots showed the presence in T. brucei lysate of the Rpt proteins, which co-fractionated with the yeast 19 S proteasome complex by gel filtration and localized in the 19 S fraction of a glycerol gradient. All the Rpt and putative 19 S non-ATPase (Rpn) proteins were co-immunoprecipitated from T. brucei lysate by individual anti-Rpt antibodies. Treatment of T. brucei cells with a chemical cross-linker resulted in co-immunoprecipitation of 20 S proteasome with all the Rpt and Rpn proteins that sedimented in a glycerol gradient to the position of 26 S proteasome. The cross-linker resulted in co-immunoprecipitation of 20 S proteasome with all the Rpt and Rpn proteins that sedimented in a glycerol gradient to the position of 26 S proteasome. However, the presence of 26 S proteasome in T. brucei cells, which apparently dissociate into 19 S and 20 S complexes upon cell lysis. RNA interference to block selectively the expression of proteasomes 20 S core and Rpt subunits resulted in significant accumulation of ubiquitinated proteins accompanied by cessation of cell growth. Expression of yeast RPT2 gene in T. brucei Rpt2-deficient cells could not rescue the lethal phenotype, thus confirming the incompatibility between the two Rpt2s. The T. brucei 11 S regulator (PA26)-deficient RNA interference cells grew normally, suggesting the dispensability of activated 20 S proteasome in T. brucei.

The proteasome performs a central and ubiquitous biological function as follows: the degradation of intrinsically short lived regulatory proteins, such as those that control the cell cycle and transcription, as well as the disposal of potentially toxic denatured or misfolded proteins. Protein substrates are generally marked for degradation by their attachment to mult ubiquitin chains catalyzed by a series of enzymes (1, 2). The ubiquitinated protein is then degraded by the 26 S proteasome in an ATP-dependent manner. The latter is a complex between a cylindrical 20 S proteasome and two 19 S regulatory complexes attached to each end of the cylinder (3). The 19 S complex consists of a characteristic set of some 17 heterogeneous protein subunits classified into two subgroups. One subgroup contains six structurally related ATPases, designated Rpt1 to 6 in Saccharomyces cerevisiae, which are encoded by a unique multigene family well conserved during evolution (3) and perform the presumed role of unfolding and translocating the proteins targeted for proteasome degradation. Another subgroup of some 11 non-ATPase subunit proteins, designated the Rnps in S. cerevisiae, are mostly structurally unrelated to one another (3).

The 20 S proteasomes have been universally identified among the eukaryotes as well as some of the archaea and eubacteria (3). There has not been any 26 S proteasome identified in either Thermoplasma acidophilum, Rhodococcus erythropolis, or any other prokaryote. But an archaeabacterial ATPase, homologous to the ATPases in eukaryotic 20 S proteasome, was recently identified in Methanococcus jannaschii and found to activate protein breakdown by bacterial 20 S proteasomes (4). The eukaryotic 20 S proteasome has a similar structure as that of prokaryotes, and high resolution crystal structures have been reported for the 20 S proteasomes of T. acidophilum and S. cerevisiae showing remarkable structural similarities (5, 6).

Trypanosoma brucei is a parasitic protozoan and one of the causative agents of African trypanosomiasis. Recently, we have identified, purified, and characterized the 20 S proteasome from this organism (7), and we cloned full-length cDNAs encoding each of the seven α- and seven β-subunits of this complex. The trypanosome 20 S proteasome exhibits striking morphological similarities to the rat 20 S proteasome under electron microscopy (7). An activated form of the trypanosomal 20 S proteasome was identified and found to contain an additional protein of 26 kDa (PA26). Association with the PA26

1 The GenBank™ database accession numbers of the DNA sequences encoding the 14 subunits of T. brucei 20 S proteasome are as follows: α1, AF193836; α2, AF143815; α3, AF193837; α4, AF169652; α5, AF140353; α6, AJ131148; α7, AF169651; β1, AJ131043; β2, AJ130820; β3, AF169653; β4, AF226673; β5, AF226674; β6, AF148124; and β7, AF290945.
heptamer ring confers enhanced peptidase activities on the trypanosomal 20 S proteasome (8, 9). A functionally similar but structurally diverged protein PA28 has been described previously in vertebrates (10). Despite the many elements of similarity between the proteasomal systems of *T. brucei* and other eukaryotes, our efforts to identify the 26 S proteasome in *T. brucei* have been unsuccessful (8). There could be two causes of this failure as follows: either the 26 S proteasome in *T. brucei* is unstable and falls apart when cell lysates are processed by conventional means, or there may be no 26 S proteasome in *T. brucei*. The second possibility suggests a need for an alternative means of activating the 20 S proteasome in *T. brucei*. The PA26-activated 20 S proteasome in *T. brucei*, which has not yet been identified in any other eukaryotic microorganism including *S. cerevisiae*, could fulfill such a need. However, the PA26-activated 20 S proteasome exhibits only peptidase activity, not protease activity. Furthermore, although poly-ubiquitin genes (11) and ubiquitinated proteins are found in *T. brucei* (12), these are not digested by the PA26-activated 20 S proteasome.\(^2\) In this report, we demonstrate that a 26 S proteasome species is indeed present in *T. brucei*, but it dissociates into the 19 S complex and 20 S proteasome upon cell lysis. By using RNA interference (RNAi) in *T. brucei* (13), we also show that its function is essential for degradation of ubiquitinated proteins and cell viability.

### EXPERIMENTAL PROCEDURES

#### Materials—*T. brucei* strain 427 procyclic form cells were cultivated and harvested as described previously (7). The procyclic form of *T. brucei* strain 29-13, which contains the genes expressing T7 RNA polymerase and tetracycline repressor (14), was a gift from Dr. Paul T. Englund of The Johns Hopkins University School of Medicine. The fluorogenic substrate methyl-3,3'-dithiobispropionimide; RNAi, RNA interference; dsRNA, double-stranded RNA; RT, reverse transcription; FOA, 5-fluoroorotic acid; ATPγS, adenosine 5'-O( triphosphate).

### Dissociable and Essential 26 S Proteasome from Trypanosome

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yeast RPT1 untranslated regions and under the control of the yeast RPT1 promoter.

**Yeast Transformation and Genetic Methods**—Yeast manipulations were carried out using standard procedures (21), unless otherwise noted. Haploid yeast strains carrying HIS3-marked chromosomal insertions of individual RPT genes and containing the corresponding wild type gene on a low copy, URA3-marked plasmids were obtained from Daniel Finley, Harvard Medical School, and have been described previously (20). Yeast cells were transformed with individual T. brucei Rpt expression vectors by the lithium acetate method (22), and the transformants were selected on synthetic minimal (SM, Bio 101, Inc.) medium lacking histidine, leucine, and uracil. For selection on 5-fluoro- orotic acid (FOA), the SM medium was supplemented with 0.1% FOA and 50 μg/ml uracil. The loss of URA3-marked plasmids following FOA selection was confirmed by the failure of the reverted strains to grow in medium lacking uracil.

**Cross-linking the Proteasome Complex within Intact T. brucei Cells and Isolating the Cross-linked Complex by Immunoprecipitation**—The [35S]methionine pulse-labeled T. brucei procyclic form cells, described previously, were suspended in 10 volumes of HEDS buffer (25 mM HEPES, pH 7.8, 1 mM EDTA, 0.25 mM sucrose, and 50 mM KOAc) plus 5 mM of the membrane-permeable chemical cross-linker DTBP and incubated at 26 °C for 30 min. The cells were then washed twice with the same buffer and lysed in the lysis buffer (50 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1% SDS, 1% Nonidet P-40 plus a protease inhibitor mixture) after 10 min at 4 °C. The lysate, preabsorbed with rabbit polyclonal immune serum, was incubated with rabbit antiserum at room temperature for 1 h and precipitated with protein A-Sepharose. The precipitate was heated at 95 °C for 3 min, and the supernatant was boiled in the Laemmli sample buffer. 8-10% of the total protein was loaded per lane. After electrophoresis, the gel slices were stained with Coomassie blue or transferred to nitrocellulose membranes for Western blotting. 

**Results**

**Table I**

|                     |   | ATTP-dependent and lactacystin-sensitive proteolysis of [35S]casein by T. brucei lysate | 10% TCA-soluble radioactivity |
|---------------------|---|----------------------------------------------------------------------------------------|-------------------------------|
|                     |   | Rat erythrocyte crude lysate                                                            | cpm                           |
|                     |   | 2 μM ATP                                                                                 | 7475.3 ± 180.4                |
|                     |   | 2 μM ATP + 8 μM psf                                                                      | 107.5 ± 6.4                   |
|                     |   | 2 μM ATP + 8 μM psf + 2 μM units apyrase                                                 | 87.3 ± 1.7                    |
|                     |   | Rat 26 S proteasome                                                                      | 7581.0 ± 479.5                |
|                     |   | 2 μM ATP                                                                                 | 125.5 ± 10.5                  |
|                     |   | 2 μM ATP + 2 μM psf                                                                      | 122.1 ± 6.7                   |
| T. brucei crude lysate|   | 2 μM ATP                                                                                 | 2418.0 ± 184.0                |
|                     |   | 2 μM ATP + 8 μM psf                                                                      | 576.0 ± 8.5                   |
|                     |   | 2 μM ATP + 8 μM psf + 2 μM units apyrase                                                 | 384.8 ± 15.7                  |
| T. brucei-activated 20 S proteasome |   | 2 μM ATP                                                                                 | 381.5 ± 35.9                  |
|                     |   | 2 μM ATP + 8 μM psf                                                                      | 170.6 ± 67.2                  |
|                     |   | 2 μM ATP + 8 μM psf + 2 μM units apyrase                                                 | 145.3 ± 13.2                  |

* TCA, trichloroacetic acid.

**Northern Blot Analysis and RT-PCR**—Total RNA was extracted from T. brucei cells using the TRIzol reagent (Amerham Biosciences). Thirty μg of total RNA was denatured, separated on 1.2% formaldehyde agarose gel, and blotted onto nitrocellulose membranes in a 20% SSC, 0.5% Denhardt solution with 0.1 mg/ml salmon sperm DNA. After stripping the probes, the same blots were re-hybridized with an α-ubulin gene fragment as a loading control. RT-PCR was performed using gene-specific primers and first strand cDNAs as templates.

**Results**

**T. brucei Lysate Is Capable of Performing an ATP-dependent and Lactacystin-sensitive Digestion of Casein**—Instability of the 26 S proteasome could have frustrated prior efforts to purify it from T. brucei (8). To test this possibility, we monitored 26 S proteasome-like activity in the crude lysate using [methyl-14C]casein as a substrate (16). An appreciable fraction of the radioactivity appeared in the trichloroacetic acid-soluble fraction (Table I). This apparent degradation of casein was inhibited when ATP was replaced with the inactive analogue ATPγS or when the lysate was pretreated to remove existing ATP. Addition of 5 μM lactacystin, a specific inhibitor of the 26 S proteasome (27) and of the 20 S proteasome peptidase activity in T. brucei (28), also blocked degradation (Table I). These characteristics are those expected of the 26 S proteasome.

The activity is, however, less than one-third that in rat erythrocyte lysate on an equal protein weight basis (Table I) and suggests a relatively weak 26 S proteasome-like function in T. brucei.
chased with unlabeled methionine, and the radiolabeled ubiquitinated proteins were immunoprecipitated and analyzed by SDS-PAGE via autoradiography or immunostaining using the anti-ubiquitin antiserum. The amount of \(^{35}S\)methionine-labeled ubiquitinated proteins in \textit{T. brucei} gradually decreased over a 30-h chase period (Fig. 1A). The half-life of the ubiquitinated protein is about 10 h. Lactacystin treatment prevented the turnover for the duration of the chase period (Fig. 1A). Thus, there is an apparent proteasome-mediated turnover of ubiquitinated protein in \textit{T. brucei}, albeit at a relatively slow rate.

When the same time samples of immunoprecipitated ubiquitinated protein were analyzed by immunostaining with anti-ubiquitin antibodies (Fig. 1B), there was no obvious time-dependent change in the amount of ubiquitinated protein; instead, lactacystin caused an increase in the abundance of ubiquitinated proteins. Similar immunostaining of whole cell lysates, rather than immunoprecipitates, with anti-ubiquitin antibody yielded similar results (data not shown). The accumulation of ubiquitinated protein upon treatment with a specific proteasome inhibitor indicates that ubiquitinated protein is continuously synthesized and degraded in the absence of inhibitor.

\textbf{Genes Encoding the Six Proteasome ATPase Homologues Are Present and Expressed in T. brucei—}The presence of 26 S proteasome ATPases Rpt 1–6, were each cloned from \textit{T. brucei} procyclic form cells by RT-PCR. This outcome suggests the presence of the six respective genes and their active transcription in \textit{T. brucei}. The significant sequence identities between \textit{T. brucei} Rpts and the corresponding Rpts from human and yeast (Table I in Supplemental Data) suggest that these \textit{T. brucei} proteins are proteasome components. To elicit specifically reactive antisera, the six recombinant Rpt proteins were each expressed in \textit{E. coli} and purified to apparent homogeneity (see Fig. 1 in Supplemental Data). Although present in insoluble and apparently denatured form, each protein induced production of specific antiserum in rabbits.

\textbf{Five of the Six T. brucei ATPase Homologues Functionally Complement the Corresponding Yeast rpt Deletion Mutants—}Genetic studies in \textit{S. cerevisiae} have demonstrated that, despite their high degree of sequence similarity, the six proteasome ATPases are functionally non-redundant (20). Deletions of individual \textit{RPT} genes in yeast are lethal. To determine whether the six \textit{T. brucei} ATPases function as such \textit{in vivo}, we tested the ability of the individual \textit{T. brucei} homologues to rescue yeast \textit{rpt} deletion mutants. The coding regions of \textit{T. brucei} Rpt cDNAs were each cloned into a LEU2-marked yeast expression vector and transformed into yeast strains bearing a chromosomal deletion of the corresponding \textit{S. cerevisiae} \textit{RPT} gene as well as a \textit{URA3}-marked plasmid carrying a wild type copy of the yeast gene. We imposed selection for loss of the \textit{URA3}-marked plasmids using FOA. In five of six cases, except for \textit{T. brucei} Rpt2, the yeast cells harboring the other five \textit{T. brucei} Rpt expression plasmids grew following loss of the corresponding yeast Rpt expression plasmid (Fig. 2). This implies that the yeast ATPase, coded by the \textit{URA3}-marked plasmid, is redundant and that its function can be provided by the trypanosome homologue carried on the remaining \textit{LEU2}-marked plasmid. Growth of all five complemented strains was equivalent at the normal growth temperature of 30 °C but was diminished to varying degrees at the more restrictive 37 °C (relative growth was wild type = Rpt1 > Rpt4 > Rpt5 > Rpt3 = Rpt6, see Fig. II in Supplemental Data). \textit{T. brucei} Rpt2, on the other hand, failed totally to complement the corresponding yeast deletion mutant. Interestingly, complementation of yeast \textit{rpt} deletion mutants by expression of \textit{Arabidopsis thaliana} Rpt proteins had previously yielded similar results (29). The expression of \textit{T. brucei} Rpt2 protein in yeast, prior to selection on FOA, was verified by immunoblot analysis of a transformant in which a hemagglutinin epitope had been appended to the C terminus of the Rpt2 coding region (results not shown). Each of the six yeast \textit{rpt} deletion mutants was further tested for complementation by each of the six \textit{T. brucei} Rpt homologues and thus extended the cross-species complementation tests to all of the 36 possible combinations. Only the \textit{T. brucei} \textit{RPT} genes that were identified as true homologues by sequence inspection provided complementation (except for Rpt2), confirming the conclusion that, like the yeast ATPases, each of the parasite ATPases plays a distinct and essential role.

\textbf{Rpt Proteins Are Found in a 19 S Complex in the T. brucei Lysate—}To determine whether the 26 S proteasome is present but dissociates into an 20 S proteasome and an either intact or fragmented 19 S regulatory unit upon cell lysis, \textit{T. brucei} lysate was fractionated on a Superose 6 column, and fractions were stained on immunoblots with either rabbit antisera to \textit{T. brucei} Rpt2, Rpt5, o6, and 20 S proteasome. A purified sample of yeast 19 S complex tagged with a FLAG His\(_6\) epitope was included as a marker of molecular mobility of the complex. Results presented in Fig. 3 indicate that both Rpt2 and Rpt5 are located predominantly in the same fractions (fractions 12 and 13) as the 19 S complex, suggesting that they are present.
Addition of ATP to the lysis and fractionation buffers had no apparent effect on the pattern of immunoreactive protein fractions. The fractions collected from the 19 S complex from T. brucei were analyzed in native 4.5% PAGE and monitored for peptidase activity vs. that of the Rpt proteasomes in the gel filtration chromatography (Fig. 3 and glycerol gradient (Fig. 4A), thus suggesting that the Rpts are present in a 19 S-like complex in T. brucei lysate. This conclusion is also supported by data from a control experiment (Fig. 4B), in which rat red blood cell lysate was fractionated using identical glycerol gradient conditions. The fractions collected from the column were separated by SDS-PAGE, blotted, and stained with rabbit antisera to FLAG His6 epitope (for yeast 19 S complex), T. brucei recombinant Rpt2, T. brucei recombinant Rpt5, T. brucei recombinant ab or T. brucei 20 S proteasomes as indicated.

Among fractions 4–6 (Fig. 4A). When the immunoblot was stained with rabbit antiserum against T. brucei recombinant Rpt3, Rpt4, or Rpt5, each of the three proteins was identified exclusively in fractions 3–5 (Fig. 4A). Adsorption of these antisera with an excess of corresponding purified recombinant Rpt antigen caused these immunoreactive bands to disappear (data not shown), implying that the stained bands represent genuine Rpt3–5 from T. brucei. They migrated together in a glycerol gradient to a location accommodating a molecular mass higher than that of the 20 S proteasome, which is distributed primarily among fractions 4–6 in the same gradient (Fig. 4, A and B). In yeast and mammals, the 19 S regulatory complex is known to have a higher molecular weight than the 20 S proteasome (3, 30). T. brucei 20 S proteasome has an estimated molecular mass of 728 kDa (inferred from cDNA sequence analyses). Among the six T. brucei Rpts we have identified and the 11 full-length cDNAs from T. brucei encoding the homologues of Rpn 1–12 that we have just cloned and submitted to the GenBankTM data base (with the exception of Rpn4, see “Discussion”), the 19 S complex from T. brucei can be estimated to have a molecular mass of 873 kDa. These two estimated molecular weights agree well with the position of 20 S proteasome versus that of the Rpt proteins in the gel filtration chromatography (Fig. 3) and glycerol gradient (Fig. 4A), thus suggesting that the Rpts are present in a 19 S-like complex in T. brucei lysate. This conclusion is also supported by data from a control experiment (Fig. 4B), in which rat red blood cell lysate was fractionated using identical glycerol gradient conditions and analyzed with commercially available rabbit antisera against human Rpt1, Rpt2, and Rpn8 for immunoblot staining. The results presented in Fig. 4B indicate that the three mammalian proteins are in the bottom two fractions 1 and 2 where
the 26 S proteasome is located. Substantial quantities of these proteins are also located in fractions 3–5 that are apparently where the rat 19 S complex localizes. Some free Rpt1 and Rpt2 proteins are also located in fractions 3–5 that are apparently–presence in intact cells. We therefore labeled cross-linking may preserve its integrity and thus reveal its and 19 S regulatory complexes upon lysis of rat 19 S complex (30), suggesting that rat 19 S complex may not be as stable as the T. brucei 19 S complex under the same experimental conditions or that pools of Rpt proteins that have not yet entered the complex may be present.

The 26 S Proteasome Is Present in Intact T. brucei Cells—If the 26 S proteasome indeed dissociates into 20 S proteasomes and 19 S regulatory complexes upon lysis of T. brucei, chemical cross-linking may preserve its integrity and thus reveal its presence in intact cells. We therefore labeled T. brucei cells with [35S]methionine and treated them with the membrane-permeable bifunctional cross-linker DTBP (31). The DTBP-treated cells were lysed, and the lysate was immunoprecipitated with the rabbit anti-T. brucei 20 S proteasome antiserum (7). The proteins thus precipitated were boiled in SDS sample buffer in the presence of 5 mM DTT to reduce the disulfide bond in DTBP and dissociate the cross-linked proteins. SDS-PAGE and autoradiography of control samples not subjected to DTBP treatment revealed that anti-20 S proteasome antiserum brings down only the 20 S proteasome protein subunits, which lie within the molecular mass range of 23–34 kDa (Fig. 5A).

With the DTBP pretreatment, however, the same immunoprecipitation procedure recovers many additional proteins. These include a double band in the 36 kDa region, three major bands between 43 and 50 kDa, a pair of bands around 100 kDa, and several other minor protein bands. This band pattern is similar to that of the S. cerevisiae 19 S complex (30), suggesting that cross-linker treatment can stabilize the 26 S complex and enable co-immunoprecipitation of the 19 S complex with the 20 S proteasome. A similar outcome was observed when the rabbit anti-T. brucei Rpt3 antiserum was used in the immunoprecipitation experiment (Fig. 5B). A typical spectrum of protein subunits in the 19 S complex was precipitated by the antiserum without prior cross-linking, suggesting the presence of the Rpt3-containing 19 S complex as an integral entity in the cell lysate. After cross-linking, however, the 20 S proteasome subunit proteins were apparently co-precipitated with the 19 S proteins, suggesting the presence of integral 26 S proteasome in the lysate of cross-linked T. brucei cells. Notably, the pattern of proteins precipitated after cross-linking was very similar regardless of whether the antiserum was directed to the 19 S or 20 S subunit protein (compare the rightmost lanes in Fig. 5, A and B).

In a control experiment, in which the chemical cross-linker was added to the T. brucei lysate instead of the intact cells, there was no detectable co-immunoprecipitation between the 20 S proteasome subunit proteins and the 19 S subunit proteins (data not shown), thus confirming our working hypothesis that dissociation of the 26 S proteasome occurs upon cell lysis.

In order to verify whether the co-immunoprecipitated proteins in Fig. 5, A and B, were indeed subunits of the 19 S and the 20 S complex, respectively, the immunoprecipitate brought down by anti-20 S proteasome antiserum from Fig. 5A was immunoblotted with the rabbit antiserum to T. brucei Rpt4. An immunoreactive protein band co-migrating with the authentic recombinant Rpt4 was seen; its presence depended on the cross-linker treatment (Fig. 6A). Conversely, immunoblot analysis of the anti-Rpt3 antiserum immunoprecipitate in Fig. 5B with a rabbit anti-recombinant T. brucei a6 antiserum shows the presence of an immunoreactive protein migrating at the position of authentic a6 protein from cross-linker-treated cells but not from the untreated cells (Fig. 6B).

We conclude that the 19 S regulatory complex and the 20 S proteasome are combined to form the 26 S proteasome in intact T. brucei cells, and that chemical cross-linking was required to maintain this association during the process of cell lysis and immunoprecipitation.

Isolation and Identification of the 26 S Proteasome from Cross-linker-treated T. brucei Cells—The previous observations imply that DTBP treatment could be used to isolate intact 26 S proteasome in its active form from T. brucei cells. Lysates of DTBP-pretreated T. brucei cells were thus prepared and fractionated by glycerol gradient centrifugation in the absence of DTT. Fractions were examined on native PAGE and stained for fluorogenic peptidase activity as in Fig. 4A (Fig. 7). Portions of each fraction were also separated by SDS-PAGE, and their immunoblots were developed with rabbit antiserum against T. brucei 20 S proteasome, Rpt3, Rpt4, or Rpt5. The results presented in Fig. 7 demonstrate that a substantial proportion of each of the immunoreactive protein bands is now shifted into fractions 1 and 2, where the 26 S proteasome is localized (compare with the data from untreated cells in Fig. 4A). The peptidase activity in these two most rapidly sedimenting fractions is, however, relatively low when compared with that of the 26 S proteasome from rat red blood cells in Fig. 4B. This suggests that cross-linking may have an inhibitory effect on the activity of T. brucei 26 S proteasome, although the peptidase activities in the 20 S and activated 20 S proteasomes appear to be relatively unaffected by DTBP (Fig. 7). The cross-linker may exert little effect on the catalytic activities inside the 20 S cylindrical chamber, but cross-linking the subunits in the 19 S complex.

**Fig. 4.** Fractionation of proteasome complex by glycerol gradient centrifugation. A, lysate of T. brucei procyclic form cells was fractionated by 15–50% glycerol gradient centrifugation. Gradient fractions (bottom fraction number 1) were analyzed by native PAGE, and peptidase activity was visualized with an overlay assay. The fractions were also fractionated by SDS-PAGE, immunoblotted, and stained with rabbit antisera against T. brucei 20 S proteasome (7), T. brucei recombinant Rpt3, T. brucei recombinant Rpt4, or T. brucei recombinant Rpt5, as indicated. B, lysate of rat red blood cells was similarly analyzed. The immunoblots were stained with rabbit preimmune serum or with rabbit antisera against human Rpt1, human Rpt2, and human Rpt8, respectively.

**Fig. 5.** Isolation and Identification of the 26 S Proteasome from Trypanosome. A, T. brucei proteasomes. B, Rat proteasomes.
may block entrance of the peptide substrates into the catalytic chamber. Overall, data in Fig. 7 indicate that the 26 S proteasome can be isolated in its integral form from DTBP-pretreated T. brucei cells.

Effects of RNAi Disrupted Expression of Genes Encoding the Fourteen 20 S Proteasome Subunit Proteins and the Six Rpt Proteins on the Growth of T. brucei Cells—To test whether the 26 S proteasome in T. brucei performs a vital function and...
ments were performed. cDNAs coding for all seven proteins required for ubiquitinated protein degradation were subjected to RNA interference (RNAi) experiments. 3 days after tetracycline induction, the expression of RNAi was highly specific; there was no cross-inhibition on the levels of other mRNAs among any of the 20 individual transfectants (data not shown).

Selective immunoblot analysis was also performed on the lysates of T. brucei RNAi transfectants of α5, α6, Rpt3, and Rpt5. Lysates from each of the four transfectant cell lines grown without and with added tetracycline were compared on immunoblots. The results presented in Fig. 8 demonstrate that the level of each protein is significantly reduced after tetracycline induction of dsRNA synthesis. Inhibited growth of the four transfectant cell lines could thus be ascribed to the absence of α5, α6, Rpt3, and Rpt5 proteins, respectively. Taken as a whole, these data demonstrate that expression of each of the seven α-subunit genes, seven β-subunit genes, and six Rpt genes is essential for growth of T. brucei procyclic form cells.

**Accumulation of Ubiquitinated Proteins in α-, β-, and Rpt-deficient T. brucei Cells**—Since poly-ubiquitinated proteins have been identified as the primary substrates for the proteasome complexes in eukaryotic cells (35) and lactacystin-sensitive turnover of poly-ubiquitinated protein have been observed in T. brucei (Fig. 1), the loss of any of the proteasome α- and β-subunits and the Rpt proteins from T. brucei could result in a cessation of proteasome degradation of poly-ubiquitinated proteins, which could then lead to arrested cell growth. We thus analyzed the profile of poly-ubiquitinated proteins in each of the 20 T. brucei transfectant cell lines on immunoblots and compared their quantities between those without and with prior tetracycline induction within the same transfectant cell line. The poly-ubiquitinated proteins, running in a smeared pattern from the top of the gel in SDS-PAGE as anticipated, were only lightly stained by anti-ubiquitin monoclonal antibody in the lysate from un-induced cells under the present experimental conditions. But after induction of RNAi in the transfectants, the quantities of poly-ubiquitinated proteins are significantly enhanced on all 20 transfectant cell samples (α1, β1 and Rpt1 in Fig. 8C) (for the rest, see Fig. VI in Supplemental Data), suggesting that the loss of any one of the α, β, or Rpt proteins leads to a dysfunction of the proteasome complex in T. brucei, resulting in accumulation of poly-ubiquitinated proteins. This inhibited turnover of ubiquitinated proteins in T. brucei may constitute the basis of arrested cell growth observed in Fig. 8A.

**Expression of Yeast RPT2 Gene Cannot Rescue the Growth of T. brucei Rpt2-deficient Cells**—The failure of the trypanosome RPT2 gene to rescue a yeast rpt2 deletion mutant prompted us to perform an experiment in reverse to test if yeast RPT2 gene could functionally complement the T. brucei Rpt2-deficient cells. The yeast gene was expressed in the T. brucei cell line harboring the pZJM-ThRPT2 RNAi construct for tetracycline-inducible knockout of ThRPT2 gene expression. Northern blot analysis (Fig. 9A) indicate that whereas the level of yeast RPT2 mRNA remains relatively constant throughout the 3-day tetracycline-induction time, the T. brucei RPT2 mRNA begins to diminish visibly on day 2 and becomes essentially undetectable on day 3. Immunoblotting of the cell lysates harvested after different days of induction with rabbit antibodies against the yeast Rpt2 protein indicated that the latter was expressed and maintained at a decreases within a day of incubation and reach an undetectable level after 3 days. The decreases in levels of mRNAs of α2, α4, α6, α7, β2, β3, and β6 subunits and Rpt2 are somewhat less marked, but their quantities begin decreasing following tetracycline addition and reach ~5% of the original level after 3 days. The level of Rpt6 mRNA in the wild type cell was too low to be readily detected by Northern blot analysis. RT-PCR method was used instead to monitor the level of this mRNA, which disappeared totally 1 day after tetracycline induction (see Table V in Supplemental Data). Control experiments indicated that RNAi was highly specific; there was no cross-inhibition on the levels of other mRNAs among any of the 20 individual transfectants (data not shown).
constant level in the transfected cells throughout the 3-day period (Fig. 9B). The cell growth was, however, arrested upon disappearance of T. brucei RPT2 mRNA, despite the abundant presence of yeast Rpt2 protein (Fig. 9C), suggesting that yeast Rpt2 protein cannot functionally substitute for trypanosome Rpt2 protein in a trypanosome cell. Thus, there is no Rpt2 functional crossover between yeast and trypanosome.

The 11 S Regulator Protein from T. brucei (PA26) Is Not Essential for Proliferation of the Procyclic Form—The 11 S proteasome regulator protein (PA26) identified in T. brucei is capable of enhancing the peptidase activity of 20 S proteasomes from T. brucei, rat red blood cells, and yeast (9, 36). The activated 20 S proteasome contributes most of the proteasome-associated peptidase activity in the T. brucei lysate (8). To determine whether this form of the proteasome plays an important role in growth of T. brucei procyclic form cells, RNAi was used to disrupt PA26 expression. Tetracycline induction of a PA26 dsRNA fragment dramatically reduced the level of PA26 mRNA after 1 day (Fig. 10A). Lysates of cells harvested after 10 days of incubation with tetracycline were fractionated by glycerol gradient centrifugation, and fractions collected from the gradient were separated by native PAGE and stained for peptidase activity and protein. Whereas the un-induced cell lysate contains a significant amount of activated 20 S proteasome activity and protein, the tetracycline-induced cells demonstrate no detectable peptidase activity or protein in the region associated with the activated 20 S proteasome in native...
Despite the absence of PA26 mRNA and PA26-activated 20 S proteasomes, however, the growth of PA26-deficient cells proceeded normally (Fig. 10A). Apparently, the function of PA26-activated 20 S proteasome is not essential for the growth of *T. brucei* procyclic form cells.

**DISCUSSION**

We provide here both *in vitro* and *in vivo* evidence for proteasome-mediated turnover of casein and ubiquitinated pro-
teines in T. brucei, which belongs to the function of 26 S proteasome, a composite of 20 S and 19 S assemblies. We had shown previously (7, 8) that T. brucei contains the 20 S and activated 20 S form of proteasomes, and we now document the presence of the genes required for production of the 19 S form; those for the six Rpt ATPase proteins and (as described below) for the full repertoires of established Rpn non-ATPase subunits. Furthermore, the T. brucei cell lysate contains a protein assembly of the anticipated 19 S size with a composition that incorporates the putative T. brucei 19 S subunit proteins. The identity and role of five of the six T. brucei Rpt ATPases are further confirmed by their ability to complement deficiencies of the corresponding yeast homologues. Finally, a functional role for each of the fourteen T. brucei 20 S proteasome proteins and six 19 S ATPases in T. brucei was tested; in every case prevention of individual protein expression led to inhibition of cell growth and an increase in ubiquitinated protein pools. These data strongly substantiate the presence in T. brucei of both 20 S catalytic and 19 S regulatory proteasome components, suggesting the presence in T. brucei the 26 S proteasome.

A conventional means for establishing the presence of 26 S proteasome in eukaryotes has been biochemical purification. By using methods that readily suffice with other organisms, our previous efforts to obtain biochemical evidence for the 26 S proteasome in T. brucei had failed persistently. In an effort to stabilize a structure that we presumed to be present in intact cells, we treated the cells with a membrane-permeable chemical cross-linker. By this means, we were able to document the presence of the 26 S proteasome. This complex is apparently readily dissociated into the 20 S proteasome and the 19 S regulatory complex upon cell lysis, even in the presence of ATP, which serves to impede dissociation in other organisms (15, 37). The ready dissociation of the parasite's 26 S proteasome is clearly a property distinctive to this organism rather than an idiosyncrasy of our methodology; lysis of rat red blood cells under the same condition yielded a substantial amount of intact 26 S proteasome (see Fig. 4B).

Formation of the 26 S proteasome is most likely through binding of the Rpt hexamer ring in the 19 S complex with the outer surface of the Rpt α-ring in the 20 S proteasome (3, 38). The 19 S Rpt proteins of T. brucei are close homologues of those found in other eukaryotes; this is also true of the seven 20 S α-proteins (see Tables I and III in Supplemental Data). In the absence of three-dimensional structural data on the interface between the α-ring and the presumed Rpt ring of any organism, it is difficult to surmise what specific features determine the kinetics or equilibrium of 19 S to 20 S association and dissociation. It is likely that small differences in Rpt or α-subunit primary structures could confer very large differences on the stability of interaction, thus frustrating a direct comparison of primary sequences. Determining the basis of the seemingly weak association between the 19 S and 20 S complexes in T. brucei will require further structure-function analyses. The ready dissociation of the 26 S proteasome in T. brucei lysate may reflect a lesser in vivo stability as well. If so, free 19 S complex may predominate over that in the 26 S form. It has become increasingly apparent in recent years that the functions of the 19 S complex and its proteins go beyond regulating protein degradation. It is possible that the 19 S complex of T. brucei may play non-proteolytic roles, which may require a relatively loose association with the 20 S proteasome.

With the DNA sequence information available from the data base of TIGR Trypanosome Genome Project, we have recently isolated and identified 11 full-length cDNAs from T. brucei encoding 11 distinct Rpn homologues Rpn1–3 and Rpn5–12, bearing significant sequence identities and similarities with the corresponding Rpns from yeast and human.4,5 The molecular mass of each of the Rpn proteins estimated from the full-length cDNAs are as follows: Rpn1, 99.9; Rpn2, 106.6; Rpn3, 38.2; Rpn5, 54.9; Rpn6, 57.3; Rpn7, 45.5; Rpn8, 42.3; Rpn9, 45.9; Rpn10, 35.8; Rpn11, 33.8; and Rpn12, 31.3 kDa. (Rpn4 was recently identified as a transcription factor unassociated with the proteasome (39); no homologue is apparent in T. brucei.) The 11 Rpns we have cloned could represent the complete profile of Rpn proteins in the T. brucei 19 S complex. The predicted molecular weights of T. brucei Rpn and Rpt proteins are typical of 19 S proteins in other organisms and are consistent with the protein profile found in the SDS-PAGE data shown in Fig. 5, A and B. The following identifications are probable: the two protein bands around 100 kDa are Rpn1 and Rpn2; the bands in the 43–50-kDa region are Rpn5–9; and the protein bands in the 36-kDa region are Rpn3 and Rpn10–12.

Since its first discovery (40), RNAi has been proven an efficient reverse genetic approach to study gene function in Caenorhabditis elegans (41, 42) as well as other organisms such as T. brucei (13, 43). Using that technique, we demonstrate that proteasome proteins are essential and are involved in degradation of ubiquitinated proteins. Comparable investigations have been performed on S. cerevisiae using gene disruption of the individual genes encoding the 14 subunits proteins of the 20 S proteasome (44). The results of these investigations indicated that 13 of the 14 subunits are essential to the viability of yeast cells; the only subunit that turned out to be nonessential is a3 (45). The a3-subunit gene deletion was not lethal to the yeast, but the mutant had a longer generation time than the wild type cells. Proteasomes prepared from the a3 disrupted yeast cells contain tryptic and chymotryptic activities equivalent to those of wild type cells, but the chymotryptic activity was not dependent on SDS activation (45), suggesting that a3 disruption may lead to unregulated intracellular proteolysis. Crystallographic analysis of the 20 S proteasome from S. cerevisiae shows the N terminus of a3 to project directly across the pseudo 7-fold symmetry axis, which would require a major rearrangement to open the channel for activation (46). A subsequent observation that deletion of the N-terminal 10 amino acid residues from yeast a3 greatly enhanced the peptidase activities of the 20 S proteasome supported such a conclusion (46). In contrast to the case of yeast, a3-deficiency is lethal in T. brucei and, as with the results from other proteasomal RNAi disruptions, leads to accumulation of poly-ubiquitinated proteins. It is likely that in yeast cells with an a3 disruption other α proteins can occupy the position otherwise reserved for a3 in the α-ring heptamer. Such a substitution in the α3 position may be precluded in T. brucei by structural or functional constraints. An amino acid sequence comparison showed a coiled-coil structure in the C terminus of the a3 proteins from T. brucei, human, and Drosophila but not in yeast a3. This coiled-coil domain was hypothesized to play a role in protein-protein interactions (47). Whether such a structural discrepancy could explain the different consequences of a3 disruption in yeast and trypanosome remains to be investigated.

Finley and co-workers (20) tested the functional effects of structurally equivalent non-conservative mutations in the

4 Z. Li and C. C. Wang, unpublished data.
5 The GenBank TM database accession numbers of the DNA sequences encoding the 11 putative Rpn subunits of T. brucei 19 S proteasome complex are as follows: Rpn1, AF404111; Rpn2, AF404112; Rpn3, AF404113; Rpn5, AF410930; Rpn6, AF404114; Rpn7, AF404115; Rpn8, AF404116; Rpn9, AF404117; Rpn10, AF404118; Rpn11, AF404119; and Rpn12, AF404120.
ATP-binding motif of each of the six RPT genes in *S. cerevisiae* four were lethal and two conferred a strong growth defect. This result implies that these ATPases are not functionally redundant. The complementation study in yeast is thus a stringent test of the functional properties of individual trypanosome Rpt proteins. Although proteins, like those in the 19 S complex, that participate in many structural interactions have co-evolved and may not function well with a distantly related interloper (48), exceptions to this expectation abound among proteins that engaged in multiple conserved interactions (49). The close sequence similarities between *T. brucei* Rpt proteins and those of yeast argued for the feasibility of this approach. Expression of *A. thaliana* Rpt proteins has been found to rescue the viability of yeast strains with a disruption of the homologous gene (29). We obtained results very similar to those seen with Arabidopsis; five of the six trypanosome Rpt proteins (except for Rpt2) could replace the function of the yeast proteins. Failure to complement with *T. brucei* Rpt2 was not due to a deficiency of expression but rather likely to a failure of assembly into the 19 S complex. When an epitope-tagged form of *T. brucei* Rpt2 was expressed in otherwise wild type yeast, yeast Rpt2 assembled into the proteasome, but the *T. brucei* Rpt2 did not (data not shown). In a converse complementation experiment, we tested whether the expression of yeast RPT2 could functionally complement the loss of RPT2 gene expression in *T. brucei* cells and observed that it also failed (Fig. 9A). These mutual failures in rescue between trypanosome and yeast, as well as the failure between Arabidopsis and yeast, may reflect a special role of Rpt2, one that imposes more stringently conserved structural constraints than for the other Rpt proteins. One such specific function, gating the substrate entry into the regulatory complex of proteasome, has been recently assigned to the yeast Rpt2 subunit (50).

The most unanticipated result from these extensive investigations is perhaps the inconsequentiality of blocking PA26 expression. Loss of PA26 activation of 20 S proteasomes had no detectable effect on the growth of *T. brucei* cells or the degradation of ubiquitinated proteins (data not shown). Mammalian 11 S regulator proteins PA28α and PA28β are known to associate with 20 S proteasomes and play essential functions during other specific phases of the trypanosome life cycle. Dissociation and Essential 26 S Proteasome from Trypanosome

Acknowledgments—We thank Dr. Colin D. Robertson of the University of Glasgow for the initial contribution to the cloning of *T. brucei* RPT2 cDNA. We are also grateful to Professor Paul T. Englund of the Johns Hopkins University School of Medicine for providing us the pJLM DNA plasmid and the *T. brucei* strain 29-13. We also thank Professor Daniel Finley of the Harvard Medical School for giving us a variety of *T. brucei* strains and those of yeast argued for the feasibility of this approach. Expression of *A. thaliana* Rpt proteins has been found to rescue the viability of yeast strains with a disruption of the homologous gene (29). We obtained results very similar to those seen with Arabidopsis; five of the six trypanosome Rpt proteins (except for Rpt2) could replace the function of the yeast proteins. Failure to complement with *T. brucei* Rpt2 was not due to a deficiency of expression but rather likely to a failure of assembly into the 19 S complex. When an epitope-tagged form of *T. brucei* Rpt2 was expressed in otherwise wild type yeast, yeast Rpt2 assembled into the proteasome, but the *T. brucei* Rpt2 did not (data not shown). In a converse complementation experiment, we tested whether the expression of yeast RPT2 could functionally complement the loss of RPT2 gene expression in *T. brucei* cells and observed that it also failed (Fig. 9A). These mutual failures in rescue between trypanosome and yeast, as well as the failure between Arabidopsis and yeast, may reflect a special role of Rpt2, one that imposes more stringently conserved structural constraints than for the other Rpt proteins. One such specific function, gating the substrate entry into the regulatory complex of proteasome, has been recently assigned to the yeast Rpt2 subunit (50).

The most unanticipated result from these extensive investigations is perhaps the inconsequentiality of blocking PA26 expression. Loss of PA26 activation of 20 S proteasomes had no detectable effect on the growth of *T. brucei* cells or the degradation of ubiquitinated proteins (data not shown). Mammalian 11 S regulator proteins PA28α and PA28β are known to associate with 20 S proteasomes and play essential roles in immune responses (51). The PA26 from *T. brucei*, bearing insignificant sequence identity but close three-dimensional structural resemblance with PA28α, β or γ (9), has the capacity to activate the 20 S proteasome from *T. brucei*, rat or yeast, in the last of which no 11 S homologue has yet been identified (36). The predominant presence of the activated 20 S proteasome in extracts of both procyclic and bloodstream forms of *T. brucei* suggested that it plays an important function in this organism (8). It may further digest the peptides generated by the 26 S proteasome in *T. brucei* or form a hybrid proteasome (52) with a 20 S proteasome sandwiched between a 19 S regulatory complex and a PA26 heptamer ring. Such a hybrid could be a powerful protein degradation machine, which may, however, lose the 19 S complex upon lysis of cells and thus assume the appearance of a one-ended activated 20 S proteasome. Neither of these speculations, however, can satisfactorily explain the apparent lack of a phenotype in PA26-deficient cells. We plan to test the effect of this deficiency on growth of the bloodstream form of *T. brucei* and on the differentiation of bloodstream into procyclic form to find out whether PA26 has important functions during other specific phases of the trypanosome life cycle.
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An Easily Dissociated 26 S Proteasome Catalyzes an Essential Ubiquitin-mediated Protein Degradation Pathway in *Trypanosoma brucei*

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