Structural and Functional Characterizations of the Proteasome-activating Protein PA26 from Trypanosoma brucei*

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The activated 20 S proteasome, which has been found only in mammalian cells, is composed of two heptamer rings of an activator protein on each end of the 20 S proteasome and is inducible by interferon-γ. A 20 S proteasome has been recently identified in a protozoan pathogen Trypanosoma brucei, but there has been no experimental evidence yet for the presence of a 26 S proteasome. Instead, an activated form of 20 S proteasome was isolated from this organism, which has significantly enhanced peptidase activities. It consists of an additional activator protein with an estimated molecular mass of 26 kDa (PA26) (To, W. Y., and Wang, C. C. (1997) FEBS Lett. 404, 253–262). The profile and sequences of tryptic peptides from PA26 were determined by mass spectrometry; no matches were found in the data base. The peptide sequences were used in reverse transcriptase-polymerase chain reaction to isolate a full-length cDNA clone encoding PA26. The protein sequence thus derived from it indicates little sequence identity with those of mammalian activator proteins PA28 α, β, or γ. There is only a single copy of PA26 gene in T. brucei. Purified recombinant PA26 polymerizes spontaneously to form heptamer ring with an outer diameter of 8.5 nm. The ring binds and activates 20 S proteasomes from T. brucei as well as rat, whereas human PA28α can neither bind nor activate T. brucei 20 S proteasome. The former is thus apparently more ubiquitous than PA28 in its capability of binding to and activating 20 S proteasomes. Its presence in T. brucei may also suggest a more ancient origin of proteasome activator proteins and a much wider involvement in protein degradation among other eukaryotic organisms than was originally envisaged.

Proteasome is a multicatalytic protease complex present in prokaryotes as well as eukaryotes. The proteasome-mediated proteolysis removes abnormal proteins and short-lived regulatory proteins, such as cyclins and transcription factors, during cell cycle (1–4). The 20 S proteasome from archaea, Thermoplasma acidophilum, serving as a structural model for eukaryotic 20 S proteasomes (5), has a cylindrical structure consisting of four stacked rings of seven subunits each. There are only two distinctive subunits in archaeobacterial proteasome, α and β. α-Subunits form the two outer rings, whereas β-subunits, which are the catalytic subunits, form the two inner rings (6). Similar 20 S proteasome identified in the actinomycete Rhodococcus erythropolis (7) contains two α-type and two β-type subunits, which can be assembled efficiently in vitro in any combination (8). The 20 S proteasomes in eukaryotes have a greater subunit complexity and, in a given source, are composed of 14 different subunits: 7 distinct α subunits in the α-ring, and 7 distinct β subunits in the catalytic β-ring (9).

Recently, the crystal structures of both archaeobacterial and yeast 20 S proteasomes have become available (6, 9). There is a narrow 13-Å portal at the center of T. acidophilum 20 S proteasome that apparently provides an access for protein substrates to the cylindrical chamber (6). In yeast 20 S proteasome, however, the portal is blocked by the amino-terminal portions of α subunits. There is no obvious path by which protein substrates can reach the active sites (9). These findings suggest a need for opening the portal and facilitating influx of substrates into eukaryotic 20 S proteasomes, which turns out to be mediated by specific regulatory proteins that bind to the terminal α-rings of proteasome. There are at least two different pathways leading to activation of eukaryotic 20 S proteasomes. It can be either through binding to a 19 S protein complex at both ends of the proteasome to form the 26 S proteasome or by binding to a heptamer ring of an activator protein PA28 at each end of the proteasome to yield the activated 20 S proteasome (1, 4, 10, 11). A hybrid with a 19 S complex at one end and a PA28 heptamer at the other end has also been identified (1). The 26 S proteasomes are capable of performing ATP–dependent degradation of ubiquitinated proteins and have been identified among all eukaryotes thus far with a possible exception in Trypanosoma brucei, a primitive protozoan pathogen (12). The activated 20 S proteasome is only capable of digesting peptides in vitro (13) and has been found only among mammalian cells until our recent identification of an activated 20 S proteasome species in T. brucei (see below) (14). The 19 S complex is a multisubunit complex that binds to ubiquitinated proteins and hydrolyzes ATP (13, 15). This complex contains at least one subunit that binds polyubiquitinated proteins and six homologous subunits that contain ATP binding domains (1, 16). PA28 has no hydrolytic activity of its own and is without a homologue in yeast (17, 18). It is generally conceived to be involved in major histocompatibility complex class I antigen processing, because

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY085602.

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synthesis of PA26 is strongly induced by interferon-γ (IFN-γ) (19). There are three isoforms of PA26: PA28α, PA28β, and PA28γ, sharing about 50% amino acid sequence identity (20). The α and β isoforms form a complex with 20 S proteasome in the form of a heptamer ring of three PA28α and four PA28β or three PA28β and four PA28α (21, 22). The crystal structure of recombinant human PA28α has been recently resolved in the form of a self-assembled heptamer ring (23). It contains a central channel that has an opening of 20 Å diameter at one end and 30 Å diameter at the presumed proteasome-binding surface. Presumably, binding to such a ring structure may cause conformational changes that could open the pore in proteasome α-ring to allow the passage of peptide substrates.

*T. brucei*, a member of the Kinetoplastidae family, is the causative agent of African sleeping sickness (24). It is generally in mammalian 20 S proteasomes. However, diameter of the portal S proteasome are similar to those of archaebacterial, yeast, and *T. brucei* -subunits and 7 distinctive β-subunits by the number of proteins separated on two-dimensional gels (14), but its profile of peptidase activity differs from that of other eukaryotic 20 S proteasomes (12). Instead of the primary chymotrypsin-like activities commonly observed among mammalian 20 S proteasomes, it exhibits mainly trypsin-like activity. There has not yet been any biochemical evidence suggesting the presence of a 26 S proteasome in *T. brucei* (14). Instead, an activated form of 20 S proteasome, similar to the mammalian activated 20 S proteasome, was identified and isolated from *T. brucei* (14). This activated 20 S proteasome demonstrated enhanced peptidase activities, up to 100-fold of the original level. It consists of the 20 S proteasome and an extra protein with an estimated molecular mass of 26 kDa. The extra protein was separated from 20 S proteasomes, purified, and found capable of reconstituting the activated 20 S proteasome in *vitro* with purified 20 S proteasome. This protein, designated “proteasome activator protein with a molecular mass of 26 kDa” (PA26), was further analyzed in the present investigation. Molecular masses of the mixture of tryptic peptides of PA26 were determined by mass spectrometry, but did not match any protein in the data base. Hence, de novo sequencing of PA26 tryptic peptides was performed by tandem mass spectrometry, which enabled us to clone the encoding gene and to engage in further structural and functional characterizations of the recombinant PA26.

**EXPERIMENTAL PROCEDURES**

**Materials**—*T. brucei* strain 427 procyclic form and bloodstream form were cultivated and harvested as described previously (12). Red blood cells were collected from Wistar rats. Glycerol was from Fisher Scientific. The fluorogenic peptides succinyl-Leu-Leu-Val-Tyr-4-methyl-7-amidocoumarin (LLVY-MAC), Pro-Phex-Arg-MAC (PPFR-MAC), and Cbz-Gly-Gly-Arg-MAC (GGGR-MAC) were purchased from Sigma. Immobilon-P polyvinylidene difluoride membrane was from Millipore. Molecular weight standards for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were a group of broad range protein markers from Bio-Rad. Molecular mass markers for calibrating gel filtration column were purchased from Sigma. The monoclonal antibody against hexahistidin was from Babco. Horseradish peroxidase-conjugated donkey anti-rabbit IgG, the random primer labeling system, and RedivueTM [α-32P]dUTP were from Amersham Pharmacia Biotech. Reagents for electrophoresis were obtained either from Sigma or Amersham Pharmacia Biotech. All high performance liquid chromatography (HPLC) grade solvents were obtained from Fisher. The rest of the chemicals used in current study were of the highest purity commercially available.

**Mass Spectrometric Analysis**—Protein spots were excised from Coomasie Blue-stained two-dimensional gel and digested with trypsin as described previously (26). Peptides were extracted by washing the gel with HPLC grade water followed by three washes in 50% acetonitrile, 5% trifluoroacetic acid. The combined supernatants were dried in a SpeedVac and redissolved in the same solvent prior to analysis in mass spectrometer. The peptide extracts were further separated by reverse phase HPLC on a Vydac C18 column (1.0 mm × 15 cm). Each of the HPLC fractions was collected, concentrated, and analyzed by mass spectrometry. Molecular masses of the tryptic peptides were determined with a matrix-assisted laser desorption ionization (MALDI) delayed extraction (DE) reflection time-of-flight (TOF) instrument (Perceptive Biosystem, Voyager-DE STR Biospectrometry Workstation, Framingham, MA) equipped with a nitrogen laser (337 nm), which has a typical mass resolution, M/ΔM, of ~8000. Peptides were cocrystallized with equal volumes of matrices consisting of saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 1% trifluoroacetic acid. The MALDI spectra were internally calibrated with trypsin autolysis products to obtain accurate monoisotopic masses of all the tryptic peptides (~20 ppm). Peptide mass values were used for gene and protein mass data base search using MS-Fit (27). For de novo peptide sequencing by MALDI- post source decay (PSD)-DE, the peptides displayed the highest pseudomolecular ion abundance in the MALDI spectra of HPLC fractions were each subjected to PSD analysis on the same MALDI instrument to determine individual peptide sequences. All PSD spectra were manually interpreted.

For determination of molecular mass of the polymerized PA26 complex, the procedures employed were similar to that described previously (28, 29). The experiment was performed on an electrospray ionization (ESI-orthogonal-TOF mass spectrometer at the University of Min-}

scribed above except that the annealing temperatures were 55 °C and 60 °C for the forward and reverse reactions, respectively.

Genomic Southern Blot—The genomic DNA was isolated from procyclic forms of *T. brucei* as described (32). A sample of 3.5 μg of purified genomic DNA was digested using various restriction enzymes and electrophoresed on a 0.8% agarose gel, transferred to an Immobilon-P polyvinylidene difluoride membrane (33), and prehybridized in 5X Denhardt’s containing 5X SSC (0.15 M NaCl and 15 mM sodium citrate, pH 7.0), 50% formamide, 0.1% SDS, and 0.1 mg/ml denatured, fragmented salmon sperm DNA for 2 h at 42 °C. It was then hybridized overnight at 42 °C in the same freshly prepared solution containing 10^6 dpm/ml [32P]dCTP-labeled DNA probe. The membrane was washed twice with 1X SSC plus 0.1% SDS at room temperature for 15 min, once with 0.1X SSC plus 0.1% SDS at 42 °C for 15 min, and once with 0.1X SSC plus 0.1% SDS at 65 °C for 15 min prior to autoradiography at −70 °C overnight.

Samples of genomic DNA (3.5 μg) from *Trypanosoma cruzi* (Dr. Jerry Manning, University of California, Irvine), *Leishmania donovani* (Dr. Richard Locksley, University of California, San Francisco), *Trypanosoma brucei* (University of California, San Francisco), *Trichomonas foetus*, and *Giardia lamblia* (Dr. Alice L. Wang, University of California, San Francisco) were each digested with EcoRI, *XhoI*, NdeI, and BclI, respectively, and electrophoresed on a 0.8% agarose gel. The rest of the procedures of blotting and hybridization were as described above except for washing the membranes only once with 5X SSC plus 0.1% SDS at room temperature for 15 min and once with 2X SSC plus 0.1% SDS at room temperature for another 15 min.

Construction and Expression of a cDNA Encoding Hexahistidine-tagged PA26—The PA26 cDNA was amplified by PCR using primers N (CATCATCATCATCATCATCACCCGACCGGCCCGACCTC) and C (GGCTCTAGATCAATCCATGATCGTCTCC) to yield a DNA fragment containing 6 extra histidine codons at the 5'-end of open reading frame behind the initiation codon and a XhoI site at the 3'-end of full-length cDNA. This fragment was cloned into a pBae expression vector (34), which was cleaved with *XhoI*, NdeI, and BclI, respectively, and electrophoresed on a 0.8% agarose gel. The rest of the procedures of blotting and hybridization were as described above except for washing the membranes only once with 5X SSC plus 0.1% SDS at room temperature for 15 min and once with 2X SSC plus 0.1% SDS at room temperature for another 15 min.

Electrophoresis and Immunoblotting—SDS-PAGE in 12.5% gels was performed as described (12). For two-dimensional gel electrophoresis, the procedure was as described in a previous report (26). Immunoblotting was carried out by a previously described procedure (12).

Reconstitution of Activated 20 S Proteasomes—20 S proteasomes were purified from *T. brucei* procyclic forms and rat red blood cells, respectively, as described previously (12). Samples of purified 20 S proteasome (2.5 μg) were each incubated with varying levels of purified recombinant His6-PA26 or purified recombinant human PA28α, kindly provided by Dr. Christopher Hill (University of Utah). Incubation was performed at 37 °C for 20 min in TSDG buffer (10 mM Tris-HCl, pH 7.4, 25 mM KCl, 10 mM NaCl, 1 mM MgCl₂, 0.2 mM EDTA, 1 mM diethiothreitol, 2 mM ATP, and 20% glycerol) containing 1 mM TLCK and 1 mM phenylmethylsulfonyl fluoride. After sonication, the cell lysate was cleared by a centrifugation at 10,000 × g for 20 min and passed through a Ni²⁺-agarose column equilibrated with TBS. The column was washed with 15 volumes of TBS plus the protease inhibitors and followed by 10 volumes of the same solution plus 25 mM imidazole. The protein still bound to the column was eventually eluted with 6 volumes of TBS containing 0.5 M imidazole.

Electrophoresis and Immunoblotting—SDS-PAGE in 12.5% gels was performed as described (12). For two-dimensional gel electrophoresis, the procedure was as described in a previous report (26). Immunoblotting was carried out by a previously described procedure (12).

De novo amino acid sequence determination of PA26 from *T. brucei* was excised from the two-dimensional gel and digested with trypsin. The tryptic peptide mass values determined in MALDI-TOF were submitted for gene, protein, and expression sequence tag mass data base search using MS-Fit (27), but no matching protein entries were found, indicating that PA26 is a unique protein. De novo peptide sequencing was then pursued with MALDI-PSD-DE, and the sequences of nine peptides were determined and presented in Table 1.

Based on the amino acid sequences of two of the nine peptides, 1 and 7 (the underlined sequences in Table 1 were used for designing primers), both the corresponding sense (F) and antisense (R) degenerated oligonucleotides were synthesized. Two possible pairs of 1F with 7R and 1R with 7F were tried in RT-PCR (see “Experimental Procedures”). The first pair produced a cDNA fragment of 216 bp, which was subcloned and sequenced; the deduced amino acid sequence coincided with that of peptides 1 and 7 at the NH₂ and COOH termini, respectively, and also contained the sequences of peptides 5, 6, and 9 in between (Table I). Two specific primers, M5′ and M3′, derived from the 216-bp cDNA fragment were then paired with oligo(dT)18 and the SL sequence (31) in RT-PCR and produced a 672- and a 408-bp cDNA fragment, respectively. The former included the sequences of peptides 2, 4, 6, and 7, whereas the latter contained the sequences of peptides 1, 3, 5, 8, and 9. The two combined cDNA fragments consist of a full-length open reading frame of 231 amino acids, with a calculated molecular weight of 25,243.93 for the PA26T isoform (see below) and a pl of 5.87 (Fig. 2) similar to the estimated molecular mass and pl of the designated PA26 protein spot from two-dimensional gel (Fig. 1B). Sequence alignments of the cloned PA26 with α, β, and γ isoforms of mammalian PA28 show exceedingly low

RESULTS

The Activated 20 S Proteasome from *T. brucei* Contains One More Protein Spot than the 20 S Proteasome on Two-dimensional Gel—In order to determine the precise difference in subunit compositions between the 20 S proteasome and the activated 20 S proteasome of *T. brucei*, two-dimensional gel electrophoresis was performed. The result shows that the subunit patterns of 20 S proteasome (Fig. 1A) and activated 20 S proteasome (Fig. 1B) are essentially identical except for one additional protein spot from the activated 20 S proteasome. This protein has an estimated molecular mass of 26 kDa and a pl of 5.8 on the gel, suggesting that it may be the activator protein (PA26) of *T. brucei* 20 S proteasome (14).

Determination of the Partial Amino Acid Sequence of PA26 by Mass Spectrometry and Cloning of the Full-length cDNA Encoding PA26—The extra protein spot from *T. brucei* activated 20 S proteasome (Fig. 1B) was excised from the two-dimensional gel and digested with trypsin. The tryptic peptide mass values determined in MALDI-TOF were submitted for gene, protein, and expression sequence tag mass data base search using MS-Fit (27), but no matching protein entries were found, indicating that PA26 is a unique protein. De novo peptide sequencing was then pursued with MALDI-PSD-DE, and the sequences of nine peptides were determined and presented in Table 1.

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Table I
Partial peptide sequences from the tryptic digest of PA26 determined by mass spectrometry

| Peptide Sequence | Source | Identity or Homology |
|-----------------|--------|---------------------|
| Q/K(I/L)(I/L)R   | Human  | 10% sequence homology |
| FGE(R/K)E(I/L)R  | Human  | 10% sequence homology |
| FGE(R/K)E(I/L)R  | Mouse  | 10% sequence homology |
| FGE(R/K)E(I/L)R  | Rat    | 10% sequence homology |
| FGE(R/K)E(I/L)R  | Mouse  | 10% sequence homology |
| FGE(R/K)E(I/L)R  | Rat    | 10% sequence homology |
| FGE(R/K)E(I/L)R  | Mouse  | 10% sequence homology |
| FGE(R/K)E(I/L)R  | Rat    | 10% sequence homology |

Fig. 2. Multiple sequence alignments of PA26 with mammalian PA28α, β, and γ. Identical amino acid matches among all the aligned proteins are shaded. The extra letter V beneath the PA26 sequence in position 49 indicates the dimorphic position where either Thr or Val has been identified in PA26. Sequences of the nine peptides determined by mass spectrometry, which are listed in Table I. In PA26 determined by mass spectrometry, which are listed in Table I, peptides 1 and 3. We found by mass spectrometry (Table I, peptides 1 and 3). We found by mass spectrometry (Table I, peptides 1 and 3).
 stead. Thus, a BclI/BanI/EcoRI restrictive digestion of T. brucei genomic DNA should yield only a single 878-bp hybridizing band in genomic Southern blot, whereas an isomorphic PA26 gene with the threonine codon would yield two hybridizing bands of 232 and 646 bp (see Fig. 3C). Results from such an experiment, presented in Fig. 3D, demonstrate that there are two visible hybridizing bands from the digested genomic DNA with estimated sizes approximating 878 and 646 bp, respectively. Thus, both PA26V and PA26T genes are apparently present in the sample of genomic DNA. The anticipated 232-bp band from PA26T gene is not visible in Fig. 3D, presumably due to the presence of only a relatively short hybridizing segment (142 bp) in this DNA fragment. The data thus suggest apparent PA26 gene dimorphism among the T. brucei cells, which, by the previous indication of a single copy PA26 gene in T. brucei, suggests a mixed population of two distinct PA26 genotypes in the sample of T. brucei strain 427 procyclic forms maintained in our laboratory.

Genomic Southern blots on the genomic DNAs from T. cruzi, L. donovani, T. foetus, and G. lamblia were performed with the full-length PA26 cDNA probe and washed under much less stringent conditions (see “Experimental Procedures”). The results (data not shown) indicated no detectable hybridization band from any of the DNA digests and thus suggested that a homologous PA26 gene is not present in the genome of any of the four other protozoan pathogens.

Recombinant PA26 Can Self-assemble to Form a Heptamer Ring—Recombinant human PA28α was reported capable of self-assembly in vitro into a heptamer ring (22, 23, 37). Its crystal structure showed that the COOH terminus of each subunit protein is important for polymerization as well as interactions between the heptamer ring and mammalian 20 S proteasome, whereas the NH2 terminus is apparently not involved (23). We tried to monitor if self-assembly of recombinant T. brucei PA26 also occurs by first placing a histidine tag at the NH2 terminus of recombinant protein to facilitate its purification. Two isoforms of the tagged recombinant protein, His6-PA26T and His6-PA26V, were expressed in transformed E. coli

| TABLE II | Percentage sequence identities (similarities) among the proteasome activator proteins |
|----------|---------------------------------|
|          | Human | Mouse | Rat  | Human | Mouse | Rat  |
| T. brucei| 24.2  | 23.4  | 23.4 | 17.3  | 17.1  | 16.9 |
| Human    | 95.1  | 98.0  | 96.0 | 93.7  | 97.3  | 92.5 |
| Mouse    | 98.0  | 98.8  |      | 97.1  | 97.3  |      |

**Activator Protein of Trypanosome Proteasome**

**FIG. 3. Determination of the copy number of PA26 gene in T. brucei by genomic Southern blot.** Genomic DNA (3.5 μg) of T. brucei was digested using different restriction enzymes and electrophoresed on 0.8% agarose gel. Panel A, resolved DNA bands were transferred to nitrocellulose membrane and hybridized with a 32P-labeled 693-bp full-length PA26 cDNA. Different amounts of full-length PA26 cDNA, run on the same agarose gel, were included in the same blot as standards. Panel B, densities of the hybridized standards were traced using a LKB densitometer. The genomic DNA band digested with BclI/EcoRI (panel A, lane 2) was placed on the standard curve, and the quantity of DNA in this band was estimated accordingly. Panel C, a tentative restriction map of the PA26 gene in T. brucei derived from the data in panel A. The open reading frame is indicated in a hatched box. Panel D, the BclI/BanI/EcoRI-digested pBAce vector containing the cDNA (0.2 μg) encoding PA26V (lane 1) or PA26T (lane 2) and the BclI/BanI/EcoRI-digested genomic DNA (10 μg) of T. brucei strain 427 (lane 3). Resolved DNA bands were transferred to nitrocellulose membrane and hybridized with a 32P-labeled 693-bp full-length PA26 cDNA. A BclI/BanI/EcoRI restrictive digestion of cDNA encoding PA26V yields a 1400-bp hybridizing band (lane 1), whereas cDNA encoding PA26T yields two hybridizing bands of 920 and 480 bp (lane 2). The same digestion of PA26V gene should yield an 878-bp band, whereas that of PA26T gene is expected to yield a 646- and a 232-bp band (lane 3).
The recombinant protein was purified by passing through a Ni²⁺-agarose column. The final molecular mass of the His₆-PA26 ring was determined by ESI-TOF mass spectrometry to be around 1800, 6000, and 8000, whereas the monomer mass was found linked to Cys-83 of the protein by sequencing the corresponding tryptic peptide using MALDI-DE-PSD. It is estimated that the molecular mass of 170 kDa, which is 6.5 times higher than the anticipated value for His₆-PA26, was due to the additional disulfide linkage. The photos indicate the presence of single ring structures with an estimated diameter of 8.5 ± 0.5 nm, smaller than that of mammalian PA28α ring structure (10.5–15 nm) and that of T. brucei 20 S proteasome (11 nm). The resolution of the photos, however, did not allow a clear indication on whether the ring structure is a heptamer or a hexamer, thus leading to a mass spectrometric analysis of the polymer.

ESI-TOF mass spectrometry was used to measure the molecular mass of His₆-PA26 ring formed apparently via non-covalent linkages (26, 27). The ESI mass spectrum of His₆-PA26T, PA26(Tmodified)₅, PA26T₁PA26(Tmodified)₆, and PA26(T modified)₇, respectively. De-convolution for the ions in the m/z range of 8000 showed three hexamers of His₆-tagged PA26T, PA26(Tmodified)₄, PA26T₁PA26(Tmodified)₆, and PA26(Tmodified)₆. The monomer and the hexamers were the minor components and most likely resulted from partial dissociation of various His₆-PA26T hexamers during ionization under high de-clustering voltage (250 V). These ions carried less charges than those of the heptamer and were not observed at lower de-clustering voltages. The same results were also observed for the His₆-PA26V isoform. Therefore, the results from mass spectrometry confirm that PA26 forms heptamer.

Reconstitution of Activated 20 S Proteasomes between His₆-PA26 and either T. brucei or Rat 20 S Proteasome—Although His₆-PA26 can self-assemble to form a heptamer ring, one crucial question is whether the ring can function in a manner similar to that of the native PA26 in binding to T. brucei 20 S proteasome and enhancing its peptidase activity (14). Different amounts of His₆-PA26T or His₆-PA26V were thus incubated with purified T. brucei 20 S proteasome. Products from the incubation were analyzed in native PAGE, and their peptidase activity stained with fluorogenic peptides. The results show similar stoichiometric increases of the peptidase activity of T. brucei 20 S proteasome with increasing amount of His₆-PA26T or His₆-PA26V from 1 μg to 20 μg (Fig. 8A, upper panel), suggesting that both isoforms of recombinant His₆-PA26 perform the same function as native PA26. When the same experiment was repeated on purified 20 S proteasome from rat, the two isoforms of His₆-PA26 not only enhanced its peptidase activity but also stimulated the proteasome complex to a form that is similar to that of the native PA26 in binding to T. brucei 20 S proteasome.
activity but also showed at least 100 times higher activity than that observed on *T. brucei* 20 S proteasome (Fig. 8, B, upper panel, and C).

The unpredicted finding that PA26 can enhance the peptidase activity of rat 20 S proteasome much more efficiently than that of *T. brucei* 20 S proteasome led us to test potential effects of mammalian PA28 on *T. brucei* 20 S proteasome. A sample of purified recombinant human PA28α was obtained from Dr.
Christopher Hill of the University of Utah and incubated with T. brucei and rat 20 S proteasome as described previously. Results recorded in the bottom panel of Fig. 8B indicate that human PA28α is equally effective as His6-PA26 in enhancing the peptidase activity of rat 20 S proteasome (Fig. 8C). However, the peptidase activity of T. brucei 20 S proteasome is hardly affected at all by human PA28α (Fig. 8, A, middle panel, and C). This lack of effect was also reflected in the unchanged His6-PA26 activation of T. brucei 20 S proteasome in the presence of excess human PA28α, suggesting a failure in competing with PA26 for binding to T. brucei 20 S proteasome (Fig. 8, A, bottom panel, and C). Thus, while PA26 can apparently bind to the 20 S proteasomes from both T. brucei and rat and exert its activating effects, PA28α cannot even bind to T. brucei 20 S proteasome. PA26 could be thus a relatively simple prototype activator protein that may possess a ubiquitous capability of binding to and activating a variety of 20 S proteasomes.

**DISCUSSION**

In our present investigation, a full-length cDNA encoding the activator protein PA26 of 20 S proteasome from T. brucei, has been cloned and expressed. The protein bears no significant sequence homology with any known proteins in the data base, and demonstrates little sequence identity or similarity with the three isoforms of mammalian proteasome activator PA28α, β, and γ. Despite the lack of sequence homology, however, PA26 is capable of forming a heptamer ring structure like PA28α (23) and activate the 20 S proteasome from rat with a near identical efficiency as that of PA28α (Fig. 8C). This sharing of common functions between two distinctive proteins may suggest sharing of similar three-dimensional structures and epitopes involved in intermolecular bindings and proteasome activation. The most extensive intermolecular interactions among PA28α monomers in the heptamer ring structure involve a parallel association between helix 2 of one monomer and helix 4 of the neighboring molecule burying a solvent-accessible surface area of 1,750 Å² from each molecule (23). A similar intermolecular interaction among PA26 monomers for a heptamer formation would be possible, given appropriate folding of peptide chains to allow substantial surface areas for intermolecular binding, which is most likely independent of particular protein sequences since it relies only on mixtures of polar and hydrophobic associations (23). PA28α depends on sequences 141–149 and 240–249 at its COOH terminus for binding and activating mammalian 20 S proteasome (23). A comparison between PA28α and PA26 within these two regions indicates IEDON-NFGV versus LGSVEKSOS and RGETKGMY versus RTGS-DHMVS, representing only loop sequence homologies at best. They are probably inadequate for explaining activation of rat 20 S proteasome by PA28α and PA26 in a nearly identical manner (see Fig. 8C). The sequence data fail also to explain why PA28α is incapable of either binding or activating the 20 S proteasome from T. brucei. Further investigations will be necessary for clarifying these complex structure-activity relationships.

Segment 70–97 in PA28α, which is rich in Lys and Glu residues and constitutes a random loop in the crystal structure (37), was originally postulated to interact with the substrate of proteasome (39). However, subsequent studies indicated that deletion of the “KEKE” motif from PA28α had no effect on its proteasome stimulatory activity (40). This 28-amino acid KEKE segment is also missing from PA26. Its capability in activating rat 20 S proteasome thus provides further support to the previous conclusion by Song et al. (40) that the KEKE loop in PA28α is not involved in binding and activating mammalian proteasome. On the other hand, however, the presence of KEKE loop in human PA28α could have constituted a barrier of its binding to T. brucei 20 S proteasome and may thus explain the experimental results (Fig. 8C).

It is known that expression of PA28α, β, and γ in mammalian cells is specifically induced by IFNγ (19). This finding has led to a generally accepted notion that IFNγ-induced increase of activated 20 S proteasome in mammalian cells leads to enhanced production of major histocompatibility complex class I peptide antigens (41, 42), although a specific demonstration on production of class I peptides by activated 20 S proteasome is not yet available. Since a similarly activated 20 S proteasome has not been found in yeast, and homologues of PA28 genes have not been found in the yeast genome, it is assumed that the activated 20 S proteasomes are present only among the more advanced eukaryotes required for antigen presentations. It was thus somewhat of a surprise to identify an activated 20 S proteasome with a distinctive activator protein in T. brucei, which is regarded as a more primitive eukaryote than yeast. More surprisingly, a homologue of PA26 gene is also missing from the yeast genome.2 Apparently, proteasome activator proteins may have a much more ancient origin than that was initially postulated.

The relatively large pore size of T. brucei 20 S proteasome, apparently larger than that in the yeast 20 S proteasome (9), mimics those observed among prokaryotic 20 S proteasomes (12) and suggests that the former may have certain prokaryotic characteristics. It is possible that the proteasome in T. brucei may function like that in T. acidophilum (6) and R. erythropolis (7), where neither 26 S proteasome nor activated proteasome has been identified. The activated 20 S proteasome in T. brucei is expected to have an even larger pore size to facilitate substrate access (23, 37) and result in much enhanced peptidase activity (14). The ratio between 20 S and activated 20 S proteasomes, likely dictated by the level of PA26 in T. brucei, may thus regulate the peptidase activity level of T. brucei proteasome in vivo. The equilibrium between the 20 S proteasome-PA26 complex versus the dissociated form may play a pivotal role in regulating the overall protein degradations in T. brucei. The relatively poor efficiency of PA26 in activating T. brucei 20 S proteasome in vitro (see Fig. 8C), comparing with its high efficiency in activating rat 20 S proteasome, may lend some support to this postulation. A relatively loose association between PA26 and 20 S proteasome in T. brucei may provide a highly sensitively regulated machinery capable of responding to the slightest fluctuations in the level of PA26. A major effort is currently under way in identifying potential exogenous factors that may either induce or repress expression of PA26 in T. brucei. It is also not impossible that homologues of PA26 may be present in T. acidophilum and R. erythropolis to perform a similar function.

Since both 20 S and activated 20 S proteasomes from mammalian cells have demonstrated only peptidase activity without protease activity in vitro (19), we tested radiolabeled casein on the purified activated 20 S proteasome from T. brucei and found no sign of degradation of the protein.2 It thus, as in the case of bacteria (6, 7), remains unclear how degradation of protein by proteasomes is initiated and proceeded to the stage of peptide formation in T. brucei, since there has not yet been any biochemical evidence for the presence of 26 S proteasome in T. brucei (14). For the time being, one can only assume that PA26 performs a regulatory function in controlling proteasomal degredation of peptides generated from a yet unidentified protein degradation machinery in T. brucei.

One distinctive aspect of the life cycle of T. brucei is that it resides in mammalian bloodstream. The bloodstream form of T.

2 Y. Yao and C. C. Wang, unpublished results.
activator protein in bloodstream T. brucei and activate the 20 S proteasome. However, in a recent collaboration with Dr. John Mansfield (University of Wisconsin), we observed that T. brucei bloodstream forms harvested from C57BL/6-IFN knockout mice (45) contained the same level of activated 20 S proteasomes as those harvested from the wild-type mice.2 Expression of PA26 is thus not under a similar control by IFN as is PA28 (19). This discrepancy suggests that PA26 and PA28 are well separated in the phylogenetic pedigree in terms of their regulation of expression. The single-copy PA26 gene in T. brucei has been apparently evolved and amplified to at least three separate genes and placed under the regulation of IFN. However, since a close PA26 homologue is not found among the genomes of yeast, T. cruzi, L. donovani, T. foetus, and G. lamblia, one cannot rule out the possibility that horizontal transfer of a proteasome activator gene from the host to T. brucei might have occurred in the past. Evolution of the same gene may have then taken different routes between the host and the parasite and resulted eventually in totally different polypeptide sequences as well as distinctive regulatory mechanisms on their expression.

The dimorphism of PA26, which has apparently originated from two different PA26 genotypes of T. brucei procyclic cells, raises some interesting points. The population of cells harboring a threonine residue at position 49 of PA26 has a TIR motif in the protein (see Fig. 2), which is the specific substrate epitope for threonine phosphorylation by protein kinase C (46). Protein kinase C has been identified in T. brucei (47). Another threonine-phosphorylating enzyme, the mitogen-activated protein kinase KFR1, has been also found in T. brucei (48), which can be activated by IFNγ. The glycolipid anchor for variant surface glycoproteins in bloodstream form (49) and procylins in procyclic form of T. brucei (50) can be hydrolyzed by membrane phospholipase C to yield diacyl glycerides in the membrane structure (51). They could trigger the signal transduction pathway leading to protein kinase C activation in T. brucei (52). The phosphorylated PA26T could have an effect on hematopoietic, affinity of binding to 20 S proteasome, or potency in activating proteasome function, thus placing the proteasome function under at least a partial control by one of the potential signal transduction pathways (53). This phosphorylation may also alter the pI value of the protein and should have been reflected in its migration in the two-dimensional gel. However, a careful examination of Fig. 1F revealed no indication of more than a single protein species around the PA26 spot. Either there was no in vivo phosphorylation of PA26T or the phosphate group(s) may have been removed during protein isolation due to potential presence of phosphatase activity in the crude lysate. Further investigation by including various phosphatase inhibitors during isolation of PA26 will be necessary to clarify this issue.

In summary, we have identified a unique protein PA26 from T. brucei that can apparently form a heptamer ring structure and bind to T. brucei as well as rat 20 S proteasomes, resulting in activating proteasome peptidase activity. The presence of such a protein in a relatively primitive eukaryote like T. brucei may suggest the presence of proteasome-activating proteins in many other eukaryotes or even some prokaryotes and their involvement in regulating proteasome functions in a variety of living organisms.

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