Relative Functions of the $\alpha$ and $\beta$ Subunits of the Proteasome Activator, PA28*  

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The proteasome is a 700,000-dalton protease composed of 28 similarly sized subunits (1–5). These subunits, which in eukaryotes represent the products of 14 distinct genes, are arranged as a stack of four heptameric rings that form a cylin-

drical particle (6–8). Although all 14 gene products have similar amino acid sequences, they can be divided into two subgroups of seven, termed $\alpha$ and $\beta$, whose respective members’ sequences are more closely related to one another (9).  

Each of the two outer or terminal rings of the proteasome are composed of all seven $\alpha$-type subunits, whereas each of the two inner rings are composed of all seven $\beta$-type subunits (7). Certain archaeabacteria contain a simpler form of the proteasome, which consists of only two gene products, one representing the ancestor of the eukaryotic $\alpha$-type subunits and the other representing the ancestor of the eukaryotic $\beta$-type subunits (9).  

Nevertheless, the archaeabacterial proteasome has the same overall morphology as eukaryotic proteasomes, and the $\alpha$ and $\beta$ subunits occupy the same relative positions within the particle as do those of eukaryotic proteasomes (10, 11). Thus, regardless of source, the proteasome is a multisubunit dimer that exhibits a C2 symmetry about an axis through the two inner rings (7, 11).  

Recently, crystal structures for both archaeabacterial and yeast proteasomes were solved (12, 13). The structures have clarified several issues left unresolved by previous biochemical and molecular studies. For example, it is now clear that the proteasome’s catalytic activities are located on $\beta$ subunits and that at least three of the seven eukaryotic $\beta$ subunits are catalysts. Furthermore, the catalytic nucleophile for each of these distinct subunits is an amino-terminal threonine oriented toward a chamber present in the interior of the cylindrical particle (12–14). Access to this chamber by protein substrates appears to be highly restricted. In archaeabacteria, substrates must traverse a narrow 13-Å portal formed by the center of the terminal ring subunits (12). In yeast proteasomes, this portal is blocked by the amino-terminal portions of the $\alpha$ subunits, and there is no obvious path by which substrates can reach the active sites (13). These findings imply that there must be mechanisms that regulate the influx of substrates through the terminal rings for delivery to the sequestered active sites.  

In eukaryotes, mechanisms that regulate entry of substrates to active sites may be mediated by specific regulatory proteins that bind to the terminal rings of the proteasome. In fact, two proteins, PA700 and PA28, stimulate proteasome activity after binding with such topologies (1, 3, 15–17). PA700 (also known as 19 S cap or ATPase regulator) is a 700,000-dalton multisubunit complex that mediates the proteasome’s ability to degrade ubiquitinated proteins in an ATP-dependent fashion (18–21). This complex contains at least one subunit that binds polyubiquitinated protein substrates (22–24) and six homologous subunits that contain ATP binding domains (16, 25–28). Assembly of the proteasome-PA700 complex as well as subsequent degradation of ubiquitinated proteins requires ATP hydrolysis (18–20, 29). Although the exact role of ATP in either process is unknown, it is reasonable to speculate that ATP may...
be used to translocate the polypeptide chain from binding sites on PA700 to the catalytic sites within the interior of the protein (17).

PA28 is a 180,000-dalton activator of the 20 S proteasome. It is a ring-shaped molecule composed of two gene products, termed α and β (30–32). These subunits have primary structures that are approximately 50% identical (33–35). Previously, work by us and others indicated that the α and β subunits occupy alternating positions in the ring, suggesting that PA28 has a hexameric structure of (αβ)₆ (36, 37). PA28 activates the proteasome by binding to one or both terminal rings, but unlike PA700 does not require ATP for either binding or activation (32). PA28 regulates the proteasome’s hydrolysis of small nonubiquitinated peptides by increasing $V_{\text{max}}$ and decreasing apparent $K_m$, and therefore functions as a positive allosteric effector (30, 31). Previous work in our laboratory has demonstrated an important role for the carboxyl terminus of the α subunit in proteasome activation. Specifically, treatment of PA28 with carboxypeptidases resulted in complete loss of activity due to the loss of one or two amino acids from the carboxyl terminus of the α subunit (36, 38). Thus, a limited structural domain of one of the two subunits appeared to be required for binding of PA28 to the proteasome and resultant proteasome activation. Further support for a key role of the α subunit in PA28 function was obtained in studies showing that the isolated α subunit, prepared as a recombinant protein or electrophoretically separated from denatured native PA28, could activate the proteasome although not as efficiently as the native protein containing both α and β subunits (34, 36, 39, 40). Thus, we hypothesized that the β subunit might act to mediate function of the α subunit, perhaps by increasing the affinity of PA28 for the proteasome.

The purpose of the present work was to further define the structural requirements of the carboxyl terminus of the α subunit in proteasome activation and to examine the functional role of the β subunit in PA28 function. Therefore, we performed mutational analysis of the α subunit to study structure/func
tion relationships for this protein. We have also established the role of the β subunit by reconstituting heteromeric PA28 from it and wild type or mutant α subunits.

**MATERIALS AND METHODS**

Plasmid Construction of the PA28α Mutants—Mutations of rat PA28α were generated by PCR using the previously described PA28α plasmid as a template (35, 36). Mutations were introduced in the 3'-PCR primer, changing the last codon for tyrosine to codons for tryptophan, phenylalanine, serine, lysine, glutamic acid, or to a stop codon. The 5'-PCR primer has a NcoI restriction site at its end and the 3'-PCR primer has a BamHI site. The PCR products as well as the pET16b plasmid (Novagen) were digested with both BamHI and NcoI and blunt ended with Klenow fragment; the resulting plasmid was designated pJVK39. The identity of the PA28α-encoding sequence was verified by sequencing.

Expression of PA28β—For expression of PA28β, pJVK39 was transformed into E. coli BL21 (DE3). Expression was induced with isopropyl-1-thio-β-galactopyranoside (1 mM final concentration). After 3.5 h of induction, the cells were harvested, washed with buffer H (20 mM Tris-HCl, pH 7.6, at 4 °C, 20 mM NaCl, 1 mM EDTA, 5 mM β-mercaptoethanol), collected by centrifugation, and frozen at −70 °C. The cells were thawed on ice and resuspended in 15 ml of buffer H containing 0.3 mg/ml lysozyme and incubated on ice for 20 min. After sonication, insoluble material was removed by centrifugation (40,000 × g for 15 min). PA28β was present in both the soluble and insoluble fraction as determined by Western blot analysis with anti-PA28α antibodies. PA28β was purified from the soluble fraction by column chromatography. The insoluble pellet was resuspended in 8 mM urea and dialyzed extensively against buffer H. The small amount of precipitate that formed during dialysis was removed by centrifugation. The supernatant was purified by ion exchange chromatography. The sample was applied to a 5 × 2.5-cm column of DEAE-Fractogel equilibrated with buffer H containing 150 mM NaCl and eluted with a 100-ml linear gradient of 150–400 mM NaCl in buffer H. The data presented in this study used PA28β purified from the pellet fraction, although similar results were obtained for the originally soluble protein.

Reconstitution of PA28 from Recombinant Subunits—Heterodimeric PA28 was reconstituted from purified recombinant proteins including native α subunits or various PA28α mutants, and PA28β—obtained for the originally soluble protein. Purified proteins at 0.2 mg/ml (i.e., final concentration of 0.1 mg/ml for each protein) were preincubated in buffer H containing 5% glycerol for 16 h at 4 °C. Immunoprecipitation of PA28—Immunoprecipitation of PA28 reconstituted from recombinant subunits was conducted as described previously for native PA28 with minor modifications, using antiserum specific for PA28α (36). Seven μl of serum (except for the precipitation of protein containing the PA28α-Y249 and PA28α-KEKE-Y249 constructs, where 14 μl of serum were used) was preincubated with 300 μl of a 1/6 suspension of protein A-Sepharose CL-4B in TTBS (TBS containing 0.05% Tween 20) for 1.5 h at 4 °C. 15 μl of reconstituted PA28, prepared as described above (0.2 mg/ml in buffer H containing 5% glycerol) and 250 μl of bovine serum albumin (0.2 mg/ml in TTBS) were added to the washed beads. The suspension was shaken gently for 1.5 h at 4 °C, after which the pellet was collected by centrifugation. The equivalent of 8 μl of supernatant was subjected to SDS-PAGE. The pellet was washed three times with TTBS and heated to 100 °C with 100 μl of 2 × SDS sample buffer lacking β-mercaptoethanol. Ten μl of this sample were subjected to SDS-PAGE.

**RESULTS**

The Carboxyl-terminal Tyrosine of PA28α Is Required for Proteasome Activation—Our previous work demonstrated that the carboxyl terminus of the α subunit of PA28 is essential for activation of peptide hydrolysis by the 20 S proteasome (36, 38).
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the KEKE domain of PA28α is not required for proteasome activation—PA28α contains a segment of 28 amino acids (residues 70–97) with an unusually high content of lysine and glutamic acid residues (34, 35). This feature, known as a KEKE motif, has been postulated to participate in protein-protein interactions (41). KEKE motifs are present in numerous proteins including some subunits of the proteasome. Interestingly, however, a KEKE motif is not present in PA28β (35). To investigate the role of the KEKE domain for PA28α function, a mutant in which this domain was deleted was expressed in E. coli, purified, and assayed for proteasome stimulatory activity. As shown in Figs. 3 and 4, this mutant (α-KEKE) bound to the proteasome and stimulated proteasome activity as efficiently as did native PA28α. Thus, the KEKE motif is not required for proteasome activation by PA28α. A second mutant lacking both the KEKE motif and the carboxyl-terminal tyrosine was also generated and analyzed. As expected, this protein had no proteasome stimulatory activity.

PA28β does not activate the proteasome—Our previous studies of the relative roles of the α and β subunits of PA28 for proteasome activation were conducted prior to the successful expression of the β subunit in E. coli. We now have expressed PA28β in E. coli and have purified the protein to homogeneity (Fig. 5). Purified PA28β does not stimulate the proteasome at concentrations up to 10 times greater than those which produced maximal stimulation by PA28α. These and similar results reported during the preparation of this manuscript demonstrate that the β subunit has no direct or independent role in proteasome stimulation by PA28α (39, 40). Despite its inability to stimulate proteasome activity, the β subunit could bind to the proteasome with sufficiently high affinity to be isolated in a complex after glycerol gradient centrifugation (data not shown).

Reconstitution of PA28 from α and β subunits—The results described above demonstrate that the α subunit is necessary and sufficient for proteasome activation. However, they also suggest that the β subunit plays a modulatory role in PA28 function because the native heteromeric protein has a lower K_{act} than the isolated subunit in PA28β subunits of PA28 in proteasome activation, we have developed a procedure, as described under "Materials and Methods," for the reconstitution of PA28 hetero-oligomers from isolated recombinant α and β proteins. Reconstitution was documented by immunoprecipitation of the resulting complexes with antibodies specific for either α or β subunits followed by Western blot analysis of the immunoprecipitates with each of these antibodies (36). The reconstitution of PA28 from wild type α and β subunits is shown in Figs. 6 and 7. Thus, after the reconstitution protocol, these subunits were coimmunoprecipitated with antibodies specific for either protein. These results demonstrate that the recombinant α and β proteins had assembled into a common complex. Additional support for this conclusion was obtained by comparing structural features of the reconstituted PA28 to those of each isolated recombinant protein and to

FIG. 1. Expression of PA28α and PA28α mutants in E. coli. E. coli were transformed with the pET16b vector containing DNA for wild type PA28α or various PA28α mutants. The proteins were expressed and purified as described under "Materials and Methods" and analyzed by SDS-PAGE. Upper panel, Coomassie Blue-stained proteins. Each lane contains 6 μg of protein. Lower panel, Western blot analysis using an antibody prepared against a peptide that specifically recognizes the amino terminus of the PA28α protein (36). Each lane contains 450 ng of protein. U, E. coli extract from uninduced cells; I, E. coli extract from cells induced with isopropyl-1-thio-galactopyranoside; P, purified recombinant protein; N, native PA28 purified from bovine red blood cells.

FIG. 2. Effect of recombinant PA28α and PA28α mutants on the activity of the proteasome. Purified recombinant PA28α proteins from Fig. 1 and purified native bovine PA28 were tested for their ability to activate purified latent 20 S proteasome using the fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC, as described under "Materials and Methods." Each assay contained 0.24 μg of proteasome (1.9 units/assay) and the indicated amounts of PA28 proteins.

µg protein/assay

0 1 2 3 4 5 6 7

PA28 Activity (units/assay)

α Y249S

α Y249FS

α Y249W

α Y249

0 20 40 60 80 100

µg protein/assay

Native PA28

Wild type PA28α

PA28α

α Y249S

α Y249FS

α Y249W
Characterization of PA28α-KEKE. Upper panel, expression of PA28α-KEKE and PA28α-KEKE/-Y249. PA28α lacking the KEKE motif (PA28α-KEKE), or the KEKE motif and the carboxy-terminal tyrosine (PA28α-KEKE/-Y249), were expressed in E. coli and purified as described under “Materials and Methods.” Proteins were subjected to SDS-PAGE and analyzed by Coomassie Blue staining and by Western blotting. U, E. coli extract from uninduced cells (6 μg/lane for Coomassie Blue staining, 400 ng/lane for Western blotting); I, E. coli extract from cells induced with isopropyl-1-thio-β-D-galactopyranoside (6 μg/lane for Coomassie Blue staining, 400 ng/lane for Western blotting); P, purified protein (1 μg/lane for Coomassie Blue staining, 400 ng/lane for Western blotting). N, native PA28 purified from bovine red blood cells (1 μg for Coomassie Blue staining, 100 ng for Western blotting). Middle panel, glycerol density gradient centrifugation of PA28α-KEKE in the presence of the proteasome. PA28α-KEKE (9 μg) and 20 S proteasome (6 μg) were preincubated in a final volume of 8 μl at 37 °C for 5 min and then subjected to glycerol density gradient centrifugation as described under “Materials and Methods.” Samples of the gradient fractions were immunoblotted with an antibody specific for PA28α. Proteins of known molecular weight (thyroglobulin, M₄= 660,000, and catalase, M₄= 240,000) were centrifuged in separate tubes, and their positions are indicated with arrows. Lower panel, reconstitution of PA28α-KEKE and PA28α-KEKE/-Y249 with PA28β. PA28α-KEKE and PA28α-KEKE/-Y249 were subjected to the reconstitution protocol with PA28β as described under “Materials and Methods.” Resulting proteins were subjected to immunoprecipitation using antibodies specific for either PA28α or PA28β. The precipitated proteins (P) and the nonprecipitated proteins (S) were subjected to SDS-PAGE and immunoblotted with antibodies specific for either PA28α or PA28β. Native PA28 from bovine red blood cells (40 ng) is shown in the first lane of each panel.

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Subunit Modulates PA28 Activity—To assess directly the effect of the β subunit on the function of heteromeric PA28, the various reconstituted PA28 molecules described above were analyzed for their ability to activate the proteasome using the fluorogenic substrate Suc-Leu-Leu-Val-Tyr-AMC. Each assay contained 0.1 μg of proteasome (1.0 unit/assay) and the indicated amounts of purified respective PA28 protein.

After the reconstitution protocol, however, the α and β subunits cosedimented and had a distribution profile indistinguishable from that of native PA28 (Fig. 7, panels A and B). These data provide additional strong evidence that the recombinant α and β subunits had reconstituted into a heterohexameric or heteroheptamer complex.

Reconstitution experiments were also performed using wild type β subunit and each of the mutant α subunits described above. As shown in Fig. 6, each α subunit mutated at the carboxyl terminus formed a heteromeric complex with the β subunit. The mutant lacking the KEKE motif also formed a heteromeric complex, but not as efficiently as did the other mutants, as judged by the incomplete coprecipitation of subunits by subunit-specific antibodies (Fig. 3). These results may indicate that α and β subunits interact via the KEKE domain.

The β Subunit Modulates PA28 Activity—To assess directly the effect of the β subunit on the function of heteromeric PA28, the various reconstituted PA28 molecules described above were analyzed for their ability to stimulate the proteasome. PA28 reconstituted from wild type α and β subunits stimulated proteasome activity in an indistinguishable manner from that of native PA28 (Fig. 8). This result indicates that the β subunit functions to decrease the Kₘ for the α subunit, perhaps by...
increasing the affinity of PA28 for the proteasome. PA28 reconstituted from α subunits with tryptophan as the carboxyl-terminal residue stimulated proteasome activity as well as did native PA28. Thus, the β subunit had no effect on this already maximally efficient α subunit. In contrast, PA28 proteins reconstituted from α subunits containing either serine or phenylalanine as the respective carboxyl-terminal residues stimulated the proteasome as well as did native PA28. In these cases, the β subunit restored the ability of α subunit mutants with suboptimal activities to stimulate the proteasome optimally. On the other hand, no proteasome stimulatory activity was detected in heteromeric proteins reconstituted from α subunits lacking the carboxyl-terminal tyrosine, or from those containing either lysine or glutamic acid as the respective carboxyl-

FIG. 6. Reconstitution of heteromeric PA28 from isolated α and β subunits. Wild type PA28α and the various mutants described in the text were prepared and incubated with purified recombinant PA28β as described under “Materials and Methods.” The resulting proteins were subjected to immunoprecipitation using antibodies specific for either subunit (36). The precipitated proteins (P) and the unprecipitated proteins (S) were subjected to SDS-PAGE and immunoblotted with each of the antibodies. IP indicates the specificity of the antibody used for the Western blot analysis. Blot indicates the specificity of the antibody used for the immunoprecipitation.

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with inactivation of PA28. The latter experiments, however, could not establish whether this modification was sufficient for inactivation or whether the removal of the penultimate amino acid (isoleucine) was also necessary for loss of PA28 function. The current analysis of recombinant PA28α mutants lacking only tyrosine has resolved this issue, and demonstrates that the removal of remarkably little structural information results in complete elimination of PA28 function. This information is specific for the chemical properties of the amino acid at the carboxyl terminus because substitution of the tyrosine with charged amino acids such as glutamic acid or lysine resulted in inactive PA28, whereas substitution with tryptophan greatly improved the ability of the isolated α subunit to activate the proteasome.

The current results directly test speculations about functional roles of KEKE motifs (41). These motifs are found in a number of interacting proteins, including PA28α and certain α-type proteasome subunits, thereby raising the possibility that binding might occur via direct interactions between the two KEKE motifs or via an interaction between a KEKE motif of one protein and a distinct structure to which it can bind on the second protein. The current data show that deletion of the KEKE motif from PA28α does not prevent its binding to or stimulation of the proteasome. Thus, direct KEKE-KEKE interactions between PA28 and the proteasome are not required for the binding of these proteins. The PA28α mutant lacking the KEKE domain also formed a multimeric complex, suggesting that the association of α subunits does not depend on this motif. This mutant, however, was poorly reconstituted with the β subunit and it is possible that the KEKE motif plays a role in association between α and β subunits. Additional work will be required to determine the structural and functional significance of KEKE motifs in PA28.

The current results also provide important new information about the role of the β subunit of PA28 in the mechanism of proteasome activation. Although the isolated β subunit cannot directly activate the proteasome, it modulates the stimulatory effect of the α subunit in heteromeric PA28 complexes. Thus, both native PA28 and PA28 reconstituted from recombinant α and β subunits activate the proteasome with a much lower K\text{act} than does PA28α alone. This effect was even more pronounced with two PA28α mutants in which the carboxy-terminal tyrosine was replaced by either phenylalanine or serine. Each of these mutants was a very poor proteasome activator as an isolated protein, but functioned indistinguishably from native PA28 in complete elimination of PA28 function. This information is only tyrosine has resolved this issue, and demonstrates that the affinity of α subunits for proteasome binding. This explanation, however, predicts that isolated β subunits should competitively inhibit proteasome stimulation by the α subunit; we have been unable, however, to detect significant inhibition in this type of experiment.\(^3\)

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