Reconstitution of proteasome activator PA28 from isolated subunits: optimal activity is associated with an α,β-heteromultimer

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Abstract PA28, a 200 kDa activator of 20S proteasomes, was purified from human placenta and was gel electrophoretically resolved into two different subunits, α and β. In reconstitution experiments, α-subunits alone were found to re-associate forming homooligomers with an M_r of about 200 kDa, which elicit a stimulatory effect on proteasomal peptide-hydrolyzing activity, albeit at a moderate level. Under the same conditions, isolated β-subunits were neither found to associate nor did they display stimulatory activity. Significantly, when both α- and β-subunits were present in the reconstitution assay, heteromultimers formed, concomitant with a marked increase in stimulatory activity when compared with that of α-homooligomers. The reconstituted PA28α,β protein is indistinguishable from purified PA28 by several criteria: it displays the same molecular mass, shows the same abundance of α- and β-subunits and has a similar stimulatory activity toward 20S proteasomes. These results indicate that optimal PA28 activity is associated with a heteromultimeric structure which contains the α- and β-subunits in fixed stoichiometry, most likely as an $\alpha_3\beta_3$-heterohexamer.

Key words: 20S proteasome activator; PA28; 11S regulator; Reconstitution experiments

1. Introduction

Proteasomes are non-lysosomal proteolytic complexes of high molecular mass (~700 kDa), composed of multiple subunits, which are arranged in four stacks of seven-membered rings. These subunits can be grouped into two families, related either to the α- or to the β-subunit of proteasomes from the archaebacterium *Thermoplasma acidophilum* (for a review, see [1]). In mammalian cells, proteasome activity is modulated by specific regulatory proteins which form proteasome-regulator complexes. One of these, designated 19S regulator or PA700 [2], is a large, multisubunit complex. With the 20S proteasome as a core enzyme and 19S regulator complex associated at either terminal ring of the proteinase, the 26S proteasome is formed which is essential for ATP-ubiquitin-dependent protein degradation [3]. Another regulator, termed PA28 [4] or 11S regulator [5], is a 200 kDa protein and is composed of two different types of polypeptide, designated α and β [6]. The subunits are arranged in a ring-like structure and 'cap' one or both of the 20S proteasome's terminal rings [7]. Until now, the in vivo function of PA28 has remained uncertain, since the activator had been found to stimulate proteasomal activity toward small synthetic peptides but not toward larger protein substrates [4,5,8]. Very recently, a cellular function for PA28 in antigen processing has been proposed by demonstrating that overexpression of PA28α in mouse fibroblasts results in an improved antigen presentation [9]. This correlates with in vitro experiments on 20S proteasome digests of viral peptides, where PA28 promotes a shift in cleavage mechanism that leads to a largely enhanced production of immunodominant peptide or potential precursors [10]. While these studies have established a cellular role for PA28, the underlying mechanism of action has remained difficult to interpret, owing in part to a lack of knowledge about the composition and stoichiometry of the activator protein. For example, in a study reporting the properties of recombinant PAα [11] this activator was found to be functionally very similar if not identical to PA28 directly isolated from human tissue and containing both α- and β-subunits. Thus, if PA28α is sufficient to elicit a full stimulatory effect, what is the role of the β-subunit, if any?

To address these questions, we have isolated the α- and β-subunits from purified PA28 and have performed reconstitution experiments which show that optimal activity is associated with an α/β-heteromultimer of fixed stoichiometry, most likely an $\alpha_3\beta_3$-heterohexamer.

2. Materials and methods

DEAE-cellulose (DEAE-Servacel) and DTT were bought from Serva, Heidelberg (Germany). Gel filtration standard proteins, DEAE-Sephacel, Sepharose CL-6B as well as Mono-Q HR 5/5, Superose 6 HR 10/30 and Phenyl Superose HR 5/5 pre-packed columns were obtained from Pharmacia Biotech GmbH, Freiburg (Germany). Prepacked columns were run in conjunction with the Pharmacia FPLC system. The fluorogenic peptide, Suc-LLVY-MCA was from Bachem Biochemica GmbH, Heidelberg (Germany). Alkaline phosphatase-conjugated anti-rabbit IgG came from Sigma Chemie GmbH, Deisenhofen (Germany). SDS/PAGE molecular weight standards and hydroxyapatite (DNA grade) were from Bio-Rad GmbH (Munich, Germany). Polyvinylidenedifluoride (PVDF) blotting membrane was from Millipore (Eschborn, Germany). All other chemicals were of analytical grade.

2.1. Purification of PA28

PA28 was prepared from human placenta by tissue homogenization in a solution of phosphate-buffered, isotonic sucrose, fractional centrifugation at 15000×*g* for 30 min and 100000×*g* for 60 min, and by subjecting the resulting supernatant to the purification scheme described for PA28 from rabbit skeletal muscle [8].

2.2. Purification of 20S proteasome

The proteinase was purified from outdated human blood as detailed in [12].

2.3. Assay of 20S proteasomes and PA28 activator

The activator assays contained 1 μg of proteasome protein in TEAD buffer (20 mM Tris-HCl/1 mM EDTA/1 M NaN_3/1 mM DTT, pH 7.5) without or with activator fractions and TEAD buffer

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Abbreviations: BSA, bovine serum albumin; DTT, dithioerythritol; EDTA, ethylenediaminetetraacetic acid; MCA, 7-amino-4-methylcoumarin; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidenedifluoride; SDS, sodium dodecyl sulfate; Suc, succinyl

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to 100 μl. Samples were pre-incubated for 10 min at 37°C prior to addition of Suc-LLVY-MCA (100 μM final concentration) to a final volume of 200 μl. After incubation for 15 min at 37°C, the reaction was terminated and the reporter group was monitored spectrofluorometrically as in [8].

2.4. Electrophoretic techniques

Electrophoresis under denaturing conditions in SDS-containing, 10–20% (w/v) polyacrylamide linear gradient gels was as described previously [8] or using the discontinuous tricine-SDS-PAGE system [13]. Gels were stained with Coomassie brilliant blue. Semi-dry Western blotting of proteins following PAGE and detection of antigen-antibody complex were carried as in [8].

2.5. Antibodies

Polyclonal antibody to proteasome activator isolated from human placenta was raised in rabbits and isolation of the IgG fraction from whole antiserum was as in [8].

2.6. Isolation of PA28 α- and β-subunits

Separation of the two subunits was as in [8], with the following changes: after the electrophoretic run, excision of the bands containing the separated subunits and electroelution, samples were concentrated to about 1 ml in a Centricon 3 microconcentrator. Protein was precipitated with a 10-fold volume of ice-cold acetone. After overnight standing at −20°C, samples were centrifuged and the supernatants were discarded. Precipitates were dissolved in a small volume of TEAD buffer and insoluble material was spun down (40 000 × *g*, 15 min). The resulting supernatants were used for reconstitution experiments as described below.

2.7. Reconstitution of PA28 from isolated subunits

Single subunits or both at a protein ratio of 1:1 were mixed with an equal volume of glycerol for storage at −20°C. Prior to measuring stimulatory activity toward 20S proteasomes, samples were dialyzed against TEAD buffer.

2.8. Other methods

Protein concentrations were determined by the method of Bradford [22], with Lab-Trol (Dade, Miami, FL, USA) as the standard.

All other experimental details were as given in the table and figure legends.

3. Results

PA28 α- and β-subunits differ only little in their M_r and they migrate very closely upon SDS-PAGE ([8], Fig. 1). As the interpretation of reconstitution experiments critically depends on a quantitative separation of the two subunits, their homogeneity was assessed by Western blotting, using an anti-PA28α,β antibody as a probe. As illustrated in Fig. 2, with purified PA28, both subunits are recognized by the antibody, whereas with isolated subunits, the reaction is confined to a single band, with no additional protein reacting, indicating

Table 1
Effect of native PA28 and of isolated subunits on 20S proteasomal activity

Addition of	Activation of LLVY hydrolysis (fold)
Control	1 ±0.1
PA28 native	16 ±1.3
α-subunit	3.5 ± 0.2
β-subunit	1 ±0.2
α- plus β-subunit	13 ±2.1

1 μg each of the indicated fractions (α- plus β-subunits as a 1:1 mixture) was added to 1 μg of purified proteasome protein. Incubation conditions and determination of LLVY hydrolyzing activity were as detailed in Section 2. The control consisted of 1 μg of BSA which had been subjected to the same steps as those applied for the isolation of PA28 subunits. Values given are means ± S.E.M. from three independent experiments.

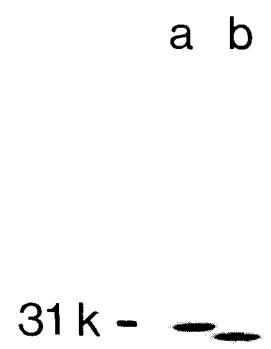

Fig. 1. SDS-PAGE of proteasome activator subunits. 1 μg of activator subunit protein, isolated as detailed in Section 2, was electrophorized on a SDS-polyacrylamide gel with a 10–20% (w/v) continuous acrylamide gradient. Electrophoresis, staining with Coomassie blue and destaining were as detailed elsewhere [8]. a and b, isolated α- and β-subunit, respectively.

that the respective band corresponds to a homogeneous population of either the α- or the β-subunit.

We then examined the ability of isolated PA28 subunits to stimulate peptide-hydrolyzing activity by 20S proteasome. The results of these experiments can be summarized as follows (Table 1). In the presence of purified PA28, proteasomal LLVY-hydrolyzing activity is stimulated 16-fold. The α-subunit protein alone elicits a stimulatory effect (3.5-fold) which is, however, low when compared to that of native PA28. With the single β-subunit protein, no stimulatory activity is detectable. In the presence of both subunits at a 1:1 ratio, stimulation of proteasomal activity is markedly enhanced, attaining a value similar to that elicited by native PA28. As PA28 subunits had been separated on SDS-containing gels and with SDS activating 20S proteasomes [14], it might be argued that residual detergent present in the samples mimics the action of PA28 subunits. This, however, appears highly unlikely as the β-subunit shows no stimulatory activity. Furthermore, BSA, which is not a substrate for 20S proteasomes [12], subjected to the same protocol as that for isolation of the α- and β-subunits, was found to be without effect on proteasomal activity. These results rather suggest that the stimulatory effect measured in the presence of α-subunit protein alone or subunit protein together with an equal amount of β-subunit protein reflects an inherent property of PA28 subunits.

In keeping with earlier findings that, both for purified PA28 [4,5,8] and for recombinant PA28α [11], stimulatory activity is associated with an oligomeric structure, gel filtration experiments (Fig. 3) illustrate that proteasomal activation by the subunit, as well as by a mixture of equal amounts of α- and protein, is associated with multimers of M_r about 200 kD which yield, upon SDS-PAGE (Fig. 3, insets), the constitue

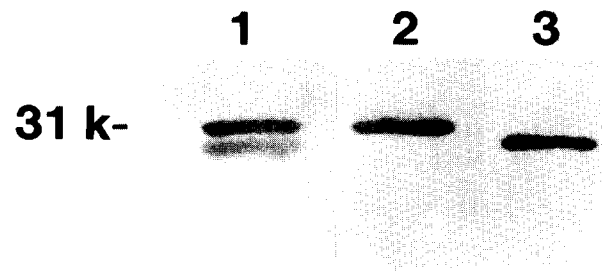

Fig. 2. Western blot analysis of purified PA28 activator and of isolated activator subunits. 2 μg of purified activator protein and 1 μg of isolated subunit were electrophoresed on a discontinuous tricine SDS-polyacrylamide gel prepared according to [13], with a 16.5% T and 3% C separating gel. Following electrophoresis, the gel was semi-dry blotted onto PVDF membranes and probed with rabbit anti-human activator antibody. Primary antibody was visualized with alkaline phosphatase-conjugated goat anti-rabbit IgG as described elsewhere [12]. Lane 1, purified PA28; lanes 2 and 3, isolated α- and β-subunit, respectively.

subunit(s). Significantly, this α/β multimer, which has been reconstituted from the isolated subunits, is resolved into the same two bands of 31 and 29 kDa as found for purified PA28 with this type of analysis [8,15] and, as judged by the intensity of Coomassie staining, the subunits occur at equal abundance, a strong indication that optimal PA28 activity is associated with a heteromultimeric complex containing the α- and β-subunits in a fixed stoichiometry, most likely as an $\alpha_3\beta_3$-heterohexamer. In contrast, upon gel filtration of isolated β-subunits, no stimulatory activity was detectable in the eluate fractions nor was any protein measurable in fractions 26–33, those fractions where active α- or α/β-oligomers are eluted from the column (data not shown).

In conclusion, these experiments have demonstrated that isolated PA28 subunits can re-associate to form two distinct oligomers, a homomultimer exclusively composed of α-subunits displaying moderate stimulatory activity as well as an α/β-heteromultimer, having an activity similar to that found for native PA28, purified from placenta.

4. Discussion

In agreement with earlier reports on PA28 from a number of different species and tissues [6,11,15], human placenta provides a further example of activator protein to be composed of two different types of polypeptide. The experiments described here extend those findings to the identification of PA28 α/β-heteromultimers as the structural organization that optimally stimulates peptide hydrolysis by 20S proteasomes. This is achieved by adding to a polypeptide with the ability to self-associate and displaying moderate stimulatory activity a polypeptide with neither of these properties. This situation is reminiscent of the assembly process of the 20S proteasome, where α-type subunits are thought to first assemble, forming a ring-like structural backbone on which the β-type subunits assemble to give the fully assembled, functional $\alpha_7\beta_7\beta_7\alpha_7$ proteasome [16,17,23]. PA28 is known to elicit its stimulatory activity by associating with the terminal rings of 20S proteasomes [7]. Two of the proteasomal α-subunits, XAPC7 and C9, and constituents of the terminal ring as well as the PA28 α-subunit [18], but not the β-subunit [6] contain KEKE motifs which have been implicated in the interaction between the two protein complexes [18]. The results of our reconstitution experiments support the importance of such motifs for the interaction of PA28 with proteasomes, as the activator β-subunits alone are not able to form active complexes with proteinase but only KEKE-containing α-subunits or α/β-heteromultimers. However, α-subunits alone lead

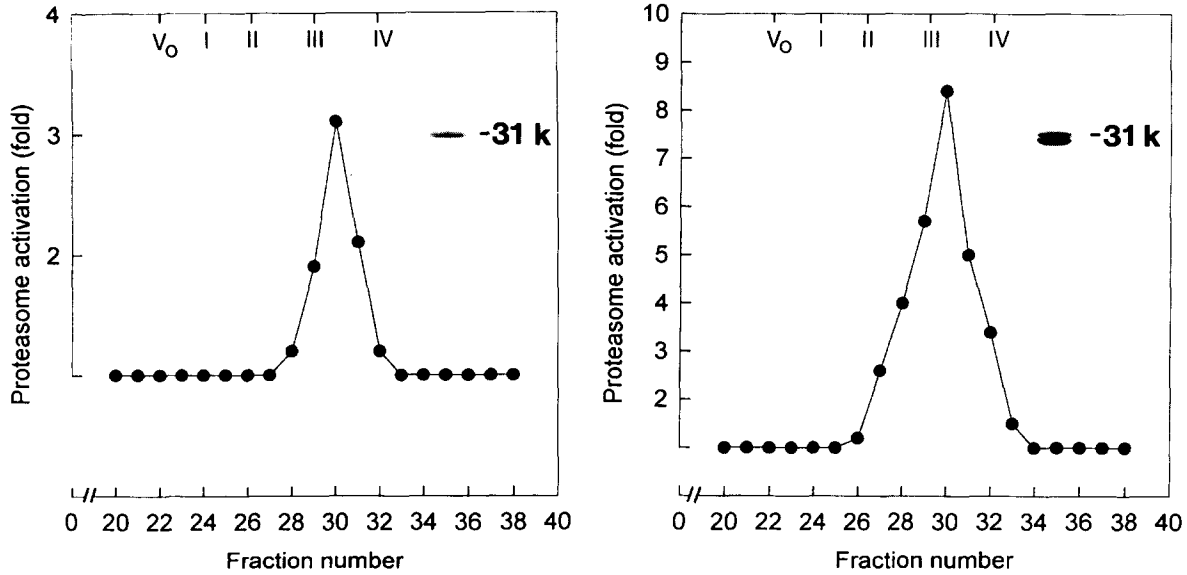

Fig. 3. Molecular sizing chromatography of isolated PA28 subunit α- and of the α- plus β-subunit. A 500 μl sample containing 100 μg of PA28 α-subunit (left panel), and a sample consisting of equal amounts of α- and β-subunit (right panel) were subjected to gel filtration on a Superose 6 HR 10/30 column. TEAD was used as the sample and running buffer. The flow rate was 0.5 ml/min and fractions of 0.5 ml were collected. 25 μl aliquots were assayed for activator as described in Section 2. Insets: Aliquots of the pooled fractions 28–32 were concentrated in Centricon 10 concentrators and analyzed by SDS-PAGE as given in the legend to Fig. 1. The calibration run was performed with protein standards of known molecular mass: V_o, blue dextran (M_r 2 200 000); I, thyroglobulin (M_r 669 000); II, ferritin (M_r 440 000); III, catalase (M_r 232 000); IV, aldolase (M_r 158 000).

only to partial activation (Table 1, Fig. 3), indicating that contact sites between proteasomes and β-subunits mediated in conjunction with α-subunits may also be of importance. This is supported by the finding that the interaction between PA28 and proteasomes is inhibited not with antibodies against proteasome subunits XAPC7 or C9 [19] but with an antibody against subunit C3 [19] or C2 [20], neither of which contains KEKE motifs [21].

To explain the existence of a two-subunit activator protein, it has been hypothesized that the two PA28 subunits may have become specialized with regard to function [15]. Our data, together with those reported for the in vitro activity of recombinant PA28α [11] as well as studies in vivo with cells over-expressing PA28α [9], would be compatible with such a model. Moreover, our results open the possibility that individual PA28 subunits might associate with other cellular proteins such as Ki antigen [6], either to co-ordinately modulate a proteasome-catalyzed degradative process such as antigen processing or to mediate other cellular functions which remain to be identified. The experiments reported here provide a basis for the search for such proteins.

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