PA28, an Activator of the 20 S Proteasome, Is Inactivated by
Proteolytic Modification at Its Carboxyl Terminus*

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PA28, a protein activator of the 20 S proteasome, was previously identified in soluble extracts of bovine red blood cells (Ma, C.-P., Slaughter, C. A., and DeMartino, G. N. (1992) J. Biol. Chem. 267, 10515–10523). To test whether this regulatory protein is as widely distributed as the proteasome, PA28 content and activity were examined in various eukaryotic tissues by immunoblot analysis and by functional assays of tissue extracts. PA28 protein was present in all sources examined. PA28 activity, however, was not detected in many of these sources, including those with the highest level of PA28 protein. To determine the biochemical basis of this result, PA28 was purified from extracts of rat liver, which had high levels of PA28 protein but no PA28 activity. The resulting purified PA28 had no detectable activity but had native and subunit molecular weights indistinguishable from the active PA28 of bovine red blood cells. Using the inactivation of purified PA28 as an assay, a protein that inactivated PA28 without altering its apparent molecular weight on SDS-polyacrylamide gel electrophoresis was identified, purified, and characterized from bovine liver. It had biochemical and catalytic characteristics similar to those of lysosomal carboxypeptidase B. When leupeptin, an inhibitor of lysosomal carboxypeptidase B, was included in the buffers used for the preparation of PA28, PA28 activity was detected in tissues which otherwise failed to demonstrate this activity. A similar result was obtained when extracts were prepared in a manner that minimized disruption of lysosomes. Other carboxypeptidases such as carboxypeptidase Y and pancreatic carboxypeptidase B also inactivated PA28 without altering its apparent molecular weight. Active PA28 binds to the proteasome to form a protease-activator complex that can be isolated after velocity sedimentation centrifugation through glycerol density gradients. Carboxypeptidase-inactivated PA28 failed to form such a complex, suggesting that the carboxyl terminus of PA28 is required for binding to the proteasome. These results indicate the importance of the carboxyl terminus of PA28 for proteasome activation.

The proteasome is a multicatalytic protease found in all eukaryotic cells examined to date (1–4). The proteasome has been identified in both cytoplasmic and nuclear compartments and appears to play a central role in non-lysosomal pathways of intracellular protein degradation, including those mediated by ATP and ubiquitin (4). Recent evidence also suggests a possible role for the proteasome in the processing of antigens for the class I major histocompatibility complex molecules (5). The involvement of the proteasome in multiple proteolytic pathways suggests that it is subject to complex and possibly multiple regulatory mechanisms. This could be achieved through the interplay of different proteasome with different regulatory proteins, resulting in multiple proteasome-regulator complexes with unique catalytic and regulatory features. In fact, several distinct proteasome regulatory proteins have recently been identified and characterized. These proteins include activators (6, 7) and inhibitors (8–10) of the various hydrolytic activities of the multicatalytic proteasome, as well as proteins that confer upon it additional regulatory properties such as a requirement for ATP and specificity for ubiquitinated substrates (11, 12). Despite the important implications of these studies for proteasome regulation, little is known about the relative interaction of the different regulatory proteins with the proteasome. Furthermore, the tissue distribution of these regulatory proteins, which have been identified and studied only in red blood cells, is not known. Because such information might provide insight about their cellular roles, we sought to determine the tissue distribution of PA28, a specific proteasome activator recently identified, purified, and characterized in our laboratory (6). The current work shows that PA28 is indeed widely distributed and therefore is likely to be an important regulator of proteasome function. This work also describes the basis for the apparent lack of detectable PA28 activity in some tissue extracts and suggests an important role of the carboxyl terminus of PA28 in its mechanism of action on the proteasome.

**MATERIALS AND METHODS**

**Purification of PA28 and the Proteasome from Bovine Red Blood Cells—**PA28 and the proteasome were purified from bovine red blood cells as described previously (6, 13).

**Assays for Proteasome and PA28 Activities—**PA28 and proteasome activities were measured as described previously (6). In brief, the proteasome was assayed by measuring its hydrolysis of synthetic fluorogenic peptides: Suc-Leu-Leu-Val-Tyr-AMC, Z-Val-Leu-Arg-MNA, Z-Leu-Leu-Glu-βNA, or Z-Gly-Gly-Leu-AMC, at pH 8.0, 30 °C. One unit of activity is defined as the change in concentration of fluorescent product of 1.0 nmol/min under standard assay conditions (6). The activity of PA28 was measured by its activation of the proteasome in these same assays. One unit of PA28 activity is defined

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*The abbreviations used are: SUC-, succinyl; Z-, benzyloxycarbonyl; MNA, Z-Leu-Leu-Val-Tyr-AMC, Z-Val-Leu-Arg-MNA, Z-Leu-Leu-Glu-βNA, or Z-Gly-Gly-Leu-AMC; PAGE, polyacrylamide gel electrophoresis; MES, 4-morpholinethanesulfonic acid; DTT, dithiothreitol.

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Proteolytic Inactivation of Proteasome Activator, PA28

**Preparation of Antibodies against PA28**—Polyclonal antibodies were prepared in rabbits against purified bovine PA28, isolated from SDS-gels.

**Immunoblotting and Electrophoretic Methods**—Immunoblotting and SDS-PAGE were performed as described previously (13). Gels were 12.5% acrylamide. Molecular weight standards included bovine serum albumin (M_r = 69,000), ovalbumin (M_r = 45,000), and carbonic anhydrase (M_r = 29,000).

**Assay for PA28-inactivating Activity**—The inactivation of PA28 was assessed by measuring residual PA28 activity after preincubation of purified PA28 with the inactivating protein. The preincubation contained purified bovine red blood cell PA28 (0.2 µg), 100 mM MES buffer, pH 5.2, 2 mM DTT, and inactivating protein or other factors, as described in the text, in a final volume of 15 µl. After preincubation at 30 °C for 10 min, the reaction was stopped by addition of 1.0 ml of a solution containing 50 mM Tris-HCl, pH 5.0, 1 mM DTT, and 50 µM Suc-Leu-Leu-Val-Tyr-AMC (or other proteasome substrate). The activity of the PA28 in this solution was determined by addition of 0.8 µg of purified proteasome, as described above (6). One unit of PA28-inactivating activity is defined as the inhibition of one unit of PA28 activity.

**Assays for Peptidase and Protease Activities**—The peptidase activity of the PA28-inactivating protein was also measured directly using various synthetic peptide substrates in assays similar to those described for the proteasome. The assays consisted of 50 mM MES buffer, pH 5.2, 1 mM DTT, 50 µM substrate, and 50 µl of column fractions, or purified enzyme in a final volume of 1.0 ml. Reactions were carried out at 30 °C, and the release of free MNA (or other reporter group) was monitored directly by fluorescence. 1.0 unit of proteolytic activity is defined as the increase in product concentration of 1.0 nM/min. Carboxypeptidase activity was assessed by measuring the hydrolysis of the synthetic peptides, Z-Gly-Arg and Z-Gly-Tyr, as described previously (14). Protease activity was assessed by measuring the degradation of [methyl-^14C]casein, as described previously (6, 8).

**Purification of a Protein That Inactivates PA28**—Bovine livers were obtained at a local slaughterhouse and cooled on ice. An extract from partially purified lysosomes was prepared as described previously (15). For a given preparation, a 200-g portion of liver, freed of connective tissue, was homogenized with 4 volumes of 0.25 M sucrose in a Dounce homogenizer. The homogenate was centrifuged at 30,000 g for 10 min. The supernatant was recentrifuged at 30,000 g for 10 min. The resulting supernatant was centrifuged at 16,300 g for 20 min. The pellet was gently resuspended in 50 ml of 0.3 M sucrose and then centrifuged at 9,500 g for 10 min. The resulting pellet, representing a fraction enriched for lysosomes, was homogenized in 100 ml of a buffer consisting of 5 mM potassium phosphate, pH 7.6, 1 mM DTT, and centrifuged at 30,000 g for 45 min. The supernatant was dialyzed for 16 h against the phosphate buffer. The soluble lysosomal extract was applied to a column containing 10 g of hydroxyapatite. The bound proteins were eluted with 320 ml of a linear phosphate gradient (5–200 mM) composed of the phosphate buffer. Five-µl samples of the 5-ml column fractions were assayed for the ability to inactivate purified PA28 by the assay described above. The data are expressed as a percentage of the untreated PA28 activity. PA28-inactivating activity was identified in the eluted column fractions at a position corresponding to approximately 100 mM phosphate. Fractions 28–38 from the hydroxyapatite column were pooled and dialyzed against a buffer containing 20 mM Tris-HCl, pH 7.6, 20 mM NaCl, 1 mM EDTA, and 5 mM β-mercaptoethanol. The dialyzed sample was applied to a 1.3 × 17-cm column of DEAE-Sepharose (EM Separations) equilibrated with the same buffer. The bound proteins were eluted with 200 ml of a linear gradient of NaCl (20–200 mM) prepared in the column buffer. Samples of the 5-ml fractions were assayed for both PA28-inactivating activity and Z-Leu-Arg-MNA hydrolyzing activity. Each activity bound to the ion-exchange gel and eluted coincidently at a position corresponding to approximately 50 mM NaCl (see Fig. 4B). Fractions 8–12 from the DEAE-Fractogel column were pooled and dialyzed against 50 mM sodium acetate buffer, pH 5.5. The dialyzed sample was concentrated to a final volume of 3 ml and applied to a column of Sephacryl S-100 (50 × 2.5 cm), equilibrated with 50 mM sodium acetate, pH 5.5, 100 mM NaCl. Samples of the 5.5-ml fractions were assayed for PA28-inactivating activity and for Z-Leu-Arg-MNA hydrolyzing activity. Fractions 40–44 from the Sephacryl column were pooled, concentrated, and stored in column buffer containing 20% glycerol at −70 °C for further characterization.

**RESULTS**

Identification of Inactive Forms of PA28 in Tissue Extracts—To determine the tissue and species distribution of PA28, the content of this protein from different sources was examined by two methods. First, crude soluble extracts of various tissues from rat, cow, and rabbit were subjected to immobiloblotting using an antibody prepared against purified bovine red blood cell PA28. Second, small scale, partial purifications of PA28 from these same extracts were conducted and monitored by assay for PA28 activity. The immunoblotting data, shown in Fig. 1 for rat tissues, indicate that all tissues examined contained an immunoreactive protein at a molecular weight of approximately 28,000. Similar results were obtained for rabbit and bovine tissues (data not shown).
These results suggest that PA28, like the proteasome, is widely distributed. In most of these samples, additional immunoreactive bands with apparent molecular weights of 45,000 and 55,000, were also observed. These bands were detected even after preabsorption of the antisera with the respective tissue extract, but they were not detected when equivalent blots were probed with non-immune or preimmune sera. Furthermore, antibodies that were affinity purified against PA28 also cross-reacted with the same higher molecular weight bands (data not shown). Therefore, it is currently unclear whether these higher molecular weight bands represent modified forms of PA28 or proteins distinct from, but immunologically related to, PA28. In contrast to the identification of PA28 protein in all tested tissues, detection of PA28 activity among the same sources varied greatly. In fact, tissue extracts which showed the highest PA28 content by immunoblotting (e.g., kidney and liver) had no detectable PA28 activity. The lack of PA28 activity could be accounted for by an inactive form of the protein. Alternatively, some extracts might contain proteins that interfere with PA28 function in the assay. In order to distinguish between these possibilities, PA28 was purified from rat liver, a tissue in which no PA28 activity could be detected. The same purification scheme employed for active PA28 (6) was used and was monitored by immunoblotting. Inactive PA28 behaved similarly to active PA28 on the various chromatographic columns used for purification. Although the purified liver PA28 had subunit and native molecular weights (28,000 and 180,000, respectively) indistinguishable from those of active PA28, it had no detectable PA28 activity (Figs. 2 and 3). These results demonstrate that PA28 is present in some tissue extracts as an inactive protein.

**Identification and Purification of a Protein That Inactivates PA28**—One explanation for these results is that some tissues contain a factor that inactivates PA28. This factor might be a physiological modulator of PA28 function (i.e., inactive PA28 might exist in intact cells prior to homogenization) or it could function during or after preparation of the tissue extract. To test these possibilities, purified active PA28 was incubated with extracts from bovine liver, a tissue which contained PA28 protein, but had no demonstrable PA28 activity. PA28 activity was lost in a time-dependent fashion during the incubation (only in the presence of the liver extract), even though the amount and apparent molecular weight of PA28 protein, detected by immunoblotting, were not significantly altered. A preliminary characterization of the PA28-inactivating activity indicated that it had an acidic pH optimum, and was inhibited by the sulfhydryl alkylating agent, iodoacetate, and by the protease inhibitor, leupeptin. These results suggested that the inactivating factor might be a lysosomal peptidase. In fact, soluble extracts of a subcellular fraction enriched for lysosomes from bovine or rat liver were also highly enriched for PA28-inactivating activity. Inactivation of purified PA28 was used as an assay for the purification of the responsible protein from bovine liver lysosomes. The purification, the details of
were also assayed for protease and peptidase activities using chromatography on hydroxylapatite which are provided under "Materials and Methods," involved column fractions were assayed for the ability to inactivate PA28 and for the hydrolysis of the synthetic peptide Z-Leu-Arg-MNA.

Based on the gel filtration chromatography, it had an apparent native molecular weight of 50,000 (Fig. 4C). SDS-PAGE of this sample showed a single protein band with an apparent molecular weight of 27,000 (Fig. 5). These results indicate that the PA28-inactivating protein is a homodimer of the 27,000-dalton protein.

The purified protein inactivated PA28 in a time-dependent manner. Fig. 5 shows these data for the loss of Suc-Leu-Leu-Val-Tyr-AMC hydrolyzing activity. Similar rates of PA28 inactivation were observed using other peptidase activities of the proteasome (data not shown), providing further evidence that PA28 modulates the multiple peptidase activities of the proteasome in a coordinated fashion. The pH optimum for PA28 inactivation by the inactivating protein was 5.5.

The copurification of PA28-inactivating activity with Z-Leu-Arg-MNA hydrolyzing activity indicated that these activities were accounted for by the same enzyme and that PA28 inactivation resulted from proteolytic modification. In order to demonstrate further that the PA28-inactivating protein was a proteolytic enzyme, its ability to hydrolyze various synthetic peptide substrates and large proteins was assessed. The PA28-inactivating protein hydrolyzed a variety of synthetic peptides. Its hydrolysis of Z-Gly-Tyr (170 units/µg) and Z-Gly-Arg (116 units/µg) demonstrated that it was a carboxypeptidase. The protein also hydrolyzed several aminoterminally blocked di- and tripeptides including Z-Leu-Arg-MNA and Z-Leu-Leu-Arg-MNA, activities also displayed by some carboxypeptidases. Maximal rates of hydrolysis of all of these substrates required the presence of sulfhydryl reducing agents such as β-mercaptoethanol or DTT, suggesting that the enzyme was a sulfhydryl protease. Additional evidence for this conclusion is provided in Table I. The pH optimum for peptide hydrolysis was 5.5, the same value as that for PA28 inactivation. No detectable hydrolysis of large protein substrates such as casein or lysozyme was observed under a variety of assay conditions, indicating that the protein had very poor endopeptidase activity. The inactivation of PA28 by the purified inactivating protein without detectable change in PA28 molecular weight also demonstrated the lack of significant endopeptidase activity (Fig. 3).

A variety of protease inhibitors inhibited both PA28-inactivating activity and the hydrolysis of Z-Leu-Arg-MNA (Table I). The peptide aldehyde protease inhibitors, leupeptin, antipain, and chymostatin, each inhibited the PA28-inactivating protein at concentrations in the micromolar range. Several inhibitors of serine-type proteases (aminopropylbenzenesulfonfylfluoride, and 3,4-dichloroisocoumarin), had no effect on either PA28-inactivating activity or Z-Leu-Arg-MNA hydrolysis. Sulphydryl alkylating agents, such as iodoacetate, inhibited each activity. These various results provide further evidence that the two activities are accounted for by the same enzyme and indicate that the PA28-inactivating protein is a carboxypeptidase of the sulfhydryl class.

PA28 Is Inactivated by Limited Proteolysis at Its Carboxy Terminus—To further demonstrate that PA28 can be inactivated by limited proteolysis at its carboxy terminus, PA28 was treated with purified peptidases. Two carboxypeptidases, bovine pancreatic carboxypeptidase B and yeast carboxypeptidase Y, each inactivated PA28 without detectably altering its molecular weight on SDS-PAGE (Table II). Aminopeptidase M, on the other hand, had no effect on PA28 activity. The endoprotease trypsin rapidly inactivated PA28, but resulted in the extensive degradation of the protein to small peptide fragments (data not shown). This latter result further demonstrates the limited nature of the action of the carboxyterminus of the protein.
The proteasome-PA28 complex (6). The gradients were then analyzed for PA28 content using an anti-PA28 antibody. The results of the experiment are shown in Fig. 6. The effects of purified PA28-inactivating protein on PA28. Purified PA28 was incubated in the presence (O) and absence (C) of purified PA28-inactivating protein (0.1 μg) at pH 5.2, 30 °C for the indicated times. Aliquots were assayed for PA28 activity remaining, as described under "Materials and Methods." Additional support for the conclusion that proteolysis occurred at the carboxyl terminus of PA28 was obtained by subjecting both active and inactive PA28 to Edman degradation. Both forms of PA28 were blocked to amino acid sequencing at the amino terminus, indicating that no proteolytic modification occurred at the amino terminus.

Inactive PA28 Fails to Bind to the Proteasome—Although the results presented above indicate that the carboxyl-terminal region of PA28 is necessary for PA28 activation of the proteasome, they do not define its role in this function, because PA28 could have separate domains responsible for binding and activation. To examine the basis for inactivation of PA28, active and carboxypeptidase-inactivated forms of PA28 were incubated with the proteasome and then subjected to velocity sedimentation centrifugation through glycerol density gradients, a method previously shown to isolate the proteasome-PA28 complex (6). The gradients were then analyzed for PA28 content using an anti-PA28 antibody. The results of the experiment are shown in Fig. 3. In the absence of the proteasome both active and carboxypeptidase-inactivated PA28s sedimented at similar positions corresponding to their expected native size (Fig. 3, A and D). Active PA28 cosedimented with the proteasome (Fig. 3C). However, carboxypeptidase-inactivated PA28 did not cosediment with the proteasome, indicating that the inactivated PA28 could not bind to the proteasome (Fig. 3B). These results suggest that a small region of the carboxyl terminus of PA28 is required for binding to the proteasome. The present data do not indicate whether the carboxyl terminus is also sufficient for proteasome activation, and additional work will be required to resolve this issue.

Discussion

The current study was initiated to examine the tissue distribution of PA28, a protein activator of the proteasome. Immunoblot analysis detected PA28 in every examined tissue in rat, cow, and rabbit. These results suggest that PA28, like its regulatory target, the proteasome, is widely distributed, and may, therefore, play an important general role in the control of proteasome function. In some tissue extracts, proteins with molecular weights higher than purified PA28 cross-reacted with the anti-PA28 antibodies. Although these proteins may be unrelated to PA28, it is also possible that they

![Graph](image_url)
represent modified forms of the protein. For example, a proteasome inhibitor was recently shown to be ubiquitinated (16). Perhaps other proteasome regulators such as PA28 undergo the same or similar modifications. Obviously, additional work will be required to determine the basis and possible significance of the current observations.

An unexpected finding of the current work was the discovery of inactive forms of PA28 in extracts of many tissues. The results presented here strongly suggest that the basis for this finding is the limited proteolysis of PA28 at its carboxyl terminus. We used an assay based on the inactivation of purified PA28 to purify and characterize a protein with this property. A review of the literature showed that the purified PA28-inactivating protein had biochemical and catalytic properties very similar to those of lysosomal carboxypeptidase B (17-23). These properties included: 1) a lysosomal localization, 2) an acidic pH optimum, 3) classification in the sulfhydryl protease family, 4) inhibition by peptidase inhibitors such as leupeptin, 5) hydrolysis of synthetic peptides previously shown to be cleaved by lysosomal carboxypeptidase B (22, 23), 6) undetectable endopeptidase activity against PA28 and other large proteins, and 7) a native molecular weight of 50,000, with a homodimeric subunit structure (17, 23). Regardless of the exact identification of the purified inactivating protein with respect to previously described enzymes, the evidence that it acts by proteolytically modifying the carboxyl terminus of PA28 is strong. Furthermore, other carboxypeptidases, including pancreatic carboxypeptidase B and carboxypeptidase Y from yeast, also inactivated PA28 by limited proteolysis.

The exposure of PA28 to lysosomal carboxypeptidase B, or related peptidases, likely resulted from the disruption of lysosomes during tissue homogenization. In fact, tissues with high lysosome content such as liver and kidney, had no detectable PA28 activity despite their high level of PA28 protein. When tissue extracts were prepared by homogenization in buffers that minimized the disruption of lysosomes (for example, by gentle homogenization in 0.25 M sucrose), active PA28 could be observed in tissues such as liver.2 Therefore, the physiological significance of PA28 inactivation by lysosomal carboxypeptidase B is unclear. Nevertheless, peptidases with related specificities may function to inactivate this protein in intact cells.

Regardless of the physiological significance of PA28 inactivation by proteolytic processing, this modification has identified a critical structural requirement of PA28 for its regulation by proteolytic processing, this modification has identified a critical structural requirement of PA28 for its regulation of the proteasome. Specifically, inactivated PA28 failed to bind to the proteasome. This result suggests that the carboxyl terminus of PA28 is necessary for PA28 interaction with the proteasome and that this interaction is required for proteasome activation. It is unclear from current results, however, whether the carboxyl-terminal domain of PA28 also is sufficient for proteasome activation. Additional studies, currently in progress, will be required to determine whether other structural domains of PA28 serve this function.

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