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cDNA cloning of p42, a shared subunit of two proteasome regulatory proteins, reveals a novel member of the AAA protein family

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Abstract **We have employed cDNA cloning to deduce the complete primary structure of p42, a protein previously identified as a common subunit of two proteasome regulatory proteins: PA700, a 700 000-Da multisubunit complex that binds to the proteasome and promotes the ATP-dependent degradation of ubiquitinated proteins, and modulator, a 250 000-Da PA700-dependent proteasome activator. Computer analysis reveals that p42 is a novel member of a large protein family characterized by a conserved 200 amino acid domain which contains a consensus sequence for ATP binding. Five other members of this family, termed AAA proteins (ATPases associated with a variety of cellular activities) are also subunits of PA700. Gel filtration chromatography was employed to determine the qualitative and quantitative distribution of p42 in crude soluble lysates of bovine red blood cells. These studies demonstrated that p42 was found in two multi-protein complexes: the 26S proteasome (formed from the 20S proteasome and PA700) and the modulator. These results establish the identity of a new protein involved in the regulation of proteasome function and indicate that this protein is found in at least two different protein complexes.**

Key words: Proteasome; PA700; Sug2p; AAA protein family; ATPase; Modulator; p42

1. Introduction

The proteasome is an intracellular protease found in some bacteria and archaebacteria, and in all eukaryotic cells examined to date [1–4]. This 700 000-Da protease consists of 28 subunits arranged to form a cylinder-shaped complex by four stacked rings, each containing seven subunits [1,2,5]. The proteasome's multiple catalytic sites are located on subunits of the two inner rings and face the interior of the channel that runs through the center of the cylinder [6,7].

The proteasome plays a number of important functions in cellular physiology. It is responsible for the constitutive degradation of most cellular proteins [8], the conditional degradation of proteins that function to repress or activate cellular processes such as transcription or cell cycle progression [9–13], and the processing of antigens for presentation by class I major histocompatibility complexes [8]. Most of the well-characterized physiological substrates of the proteasome must be covalently attached to polyubiquitin chains prior to their degradation [14]. The purified proteasome, however, cannot selectively degrade ubiquitinated proteins. This function is conferred upon the proteasome by regulatory proteins that are subunits of a 700 000-Da multi-subunit complex called PA700 [15–18]. PA700 binds to the proteasome's terminal rings to form a supramolecular-weight complex (M_r = 2 000 000) that has been called the 26S proteasome [1]. Both the assembly of the 26S proteasome from proteasome and PA700 components and the subsequent degradation of ubiquitinated proteins by this complex require ATP hydrolysis, although the mechanistic basis for the relationship between ATP hydrolysis and either of these processes is unknown [15–17,19]. A detailed understanding of the degradation of ubiquitinated proteins will require knowledge of the structure and function of individual subunits of PA700. For example, one subunit has been shown to bind polyubiquitin chains and therefore probably provides at least part of the basis for selective recognition and binding of ubiquitinated substrates [20]. Recent work has shown that six PA700 subunits are members of the AAA (ATPases associated with a variety of cellular activities) protein family [21–23]. This large protein family (> 50 members) is characterized by a conserved 200 amino acid domain that contains a consensus sequence for ATP binding, and is distinct from other ATP-binding proteins such as the ABC transporters and some ion pumps [23]. It is reasonable to assume that one or more of the AAA PA700 subunits participates in the ATP-dependent functions of proteasome/PA700 complex (such as complex assembly and proteolysis), although the relative roles of individual proteins are not known.

In our ongoing characterization of subunits of PA700, we recently identified a subunit which, based on partial amino acid sequence, seemed to be a new member of the AAA protein family [24]. Interestingly, this protein was present in two different proteasome regulatory complexes: PA700, and a new protein called modulator, which functions as a PA700-dependent proteasome activator [24]. Although our initial data identified p42 as a member of the AAA protein family, the data were not extensive enough to allow conclusions about the exact relationship of this protein with other family members. We now report the complete primary structure of p42, deduced from sequencing of a human cDNA clone. The data show that p42 is a novel member of the AAA family and allow comparison of the primary structures of six members of this family in the PA700 complex.

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The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL and NCBI Nucleotide Sequence Databases with the following accession number: D78275.

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2. Materials and methods

2.1. Purification of PA700 and modulator, and isolation of p42

PA700 and modulator were purified from bovine blood as described previously [24]. The p42 subunit of each of these proteins was isolated by HPLC [22,24].

2.2. Automated Edman degradation of p42

Amino acid sequences of isolated p42 were obtained by automated Edman degradation of peptides generated by endolysylpeptidase C, as described previously [24].

2.3. Cloning and sequencing

We have been determining the nucleotide sequences of approximately 30000 clones which were randomly selected from a human cDNA library (T. Fujiwara et al., unpublished data). Poly(A$^+$) RNAs of human tissues were purchased from Clontech. The cDNA library was constructed by directional cloning using the ZAP-cDNA synthesis kit from Stratagene. Bluescript phagemids were rescued by in vivo excision and plasmid DNAs were isolated by a modified alkaline-lysis mini-preparation procedure using an automatic plasmid isolation system PI-100 (Kurabo). All DNA sequencing was performed by the dideoxy nucleotide chain-termination method using an autocycle sequencing kit (Pharmacia). Analysis was done with an ALF automated DNA sequencer (Pharmacia). We compared the oligopeptide sequences obtained from a bovine p42 subunit with our private DNA databank using the TFASTA program. This program translates the nucleotide sequences in all six reading frames and does a Pearson and Lipman search for similarity between p42 oligopeptide sequences and the translated peptide sequences from this DNA databank. As the result, we found a clone identified as GEN-331GO7 from a human aorta cDNA library which showed significant peptide sequence similarity with two of the peptides, and therefore was a putative clone of p42. In order to amplify the missing 5′ sequence, we performed 5′-rapid amplification of the cDNA ends with the 5′Amplifinder RACE kit (Clontech) with slight modifications. In brief, 0.1 μg of human aorta mRNA was reverse-transcribed with random hexamers by Superscript II (Gibco BRL Life Technologies) to synthesize cDNAs which were amplified by the first polymerase chain reaction (PCR) with P1 primer (5′-AAACATTTCTCTGATCAAACGAGCAG-3′) and anchor primer as described in the manufacturer's protocol. Then 1 μg of the 50 μl first PCR product was amplified by a second PCR with a nested P2 primer (5′-ACCAATGTACTTGTCTACAA-TAGAAC-3′) and anchor primer. The product was analyzed by 1% agarose gel electrophoresis. The 5′ RACE product was purified, inserted into pT7Blue(R)T-vector (Novagen) and sequenced.

2.4. Gel filtration of bovine red blood cell extracts

Bovine blood was obtained from a local abattoir, and was washed extensively with phosphate-buffered saline as described previously [25]. 5 ml of packed cells were lysed with 3 volumes of a buffer consisting of 20 mM Tris-HCl, pH 7.6, 20 mM NaCl, 1 mM β-mercaptoethanol, 5 mM $MgCl_2$, and 1 mM ATP at 4°C. The lysate was centrifuged at 30000×g for 20 min and 3 ml of the supernatant was applied to a column (115×2.5 cm) of Sephacryl S-300 equilibrated and eluted with the lysis buffer. The eluted fractions were assayed as described in the text.

2.5. Immunoblotting

Immunoblotting was conducted by standard methods as described previously [24]. Anti-TBP1 antibodies were described previously [25]. Anti-p42 antibodies were prepared in mice against the yeast protein and were a generous gift from Stephen Johnston (University of Texas Southwestern Medical Center). These antibodies were specific for their respective antigens as determined by immunoblotting of PA700 subunits resolved by a two-dimensional separation procedure (data not shown).

3. Results

3.1. Determination of the primary structure of human p42, a novel member of the AAA protein family

p42 was previously identified as a common subunit of two proteasome regulatory proteins: PA700 and modulator [22,24]. Amino acid sequence analysis of five endolysylprotease C fragments of bovine p42 comprising 97 amino acids indicated that p42 was a member of the AAA protein family [24]. We have subsequently obtained sequences of 9 additional endolysylprotease C peptides of p42 (6 from the modulator and 3 from PA700) totalling 87 amino acids. These partial sequence data suggested that p42 was a novel member of the AAA family (Figs. 1 and 2). Therefore, in order to establish the identity of p42 and its structural relationship within the AAA protein family, we deduced its complete primary structure by cDNA cloning. Clone identification was accomplished using the strategy described in section 2. The nucleotide sequence and the deduced amino acid sequence for human p42 are shown in Fig. 1. The sequence contains 1582 nucleotides including the entire coding region as well as 5′- and 3′-noncoding regions. We assigned the ATG nucleotides numbered 1–3 in Fig. 1 as the initiation codon. The deduced amino acid sequence encodes a protein of 389 amino acids with a calculated molecular weight of 44160; this value is in

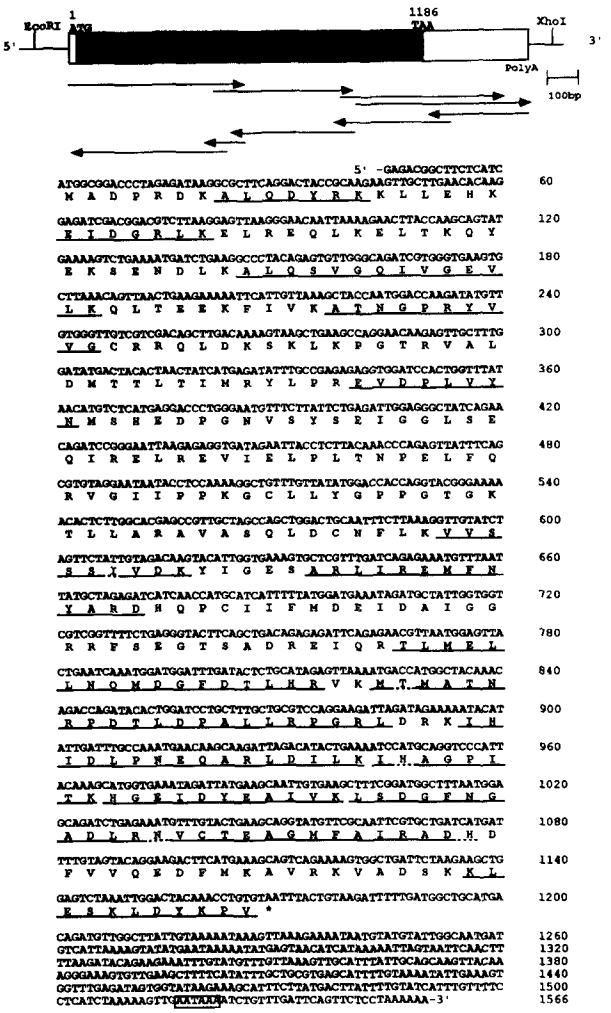

Fig. 1. Nucleotide and deduced amino acid sequences of p42. The nucleotide sequence of human p42 was determined as described in section 2. The deduced amino acid sequence is indicated in single letter format. The underlined amino acid sequences represent bovine peptides determined by Edman degradation. Discrepancies between the sequenced bovine peptides and the deduced amino acids from the human clone are denoted by dashed lines.

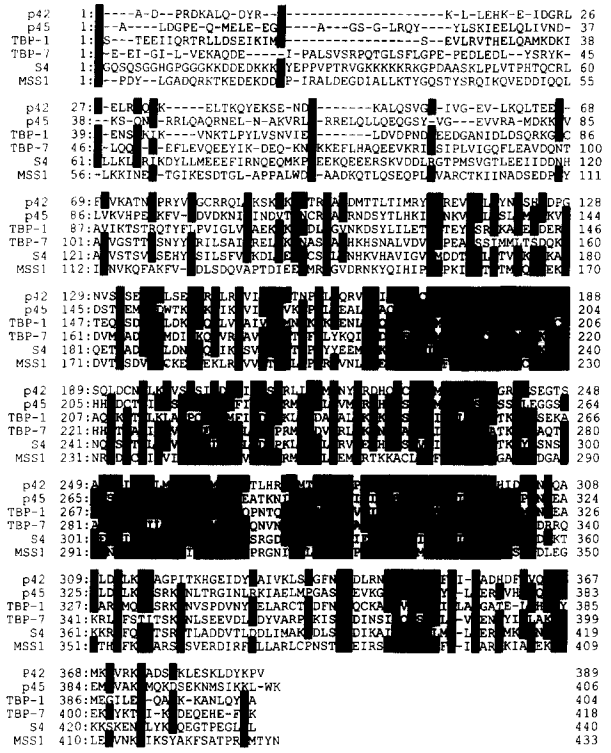

Fig. 2. Amino acid sequence alignment of six AAA proteins of PA700. The amino acid sequences of the six AAA proteins identified as subunits of PA700 are aligned. Positions with at least four identical residues are highlighted with a darkened background. Human p42, this report; human p45 [39]; human TBP1 [28]; human TBP7 [32]; human S4 [31]; human MSS1 [29].

excellent agreement with the molecular weight of 42 000 estimated by SDS-PAGE. The deduced sequence contained all 14 peptides determined by Edman degradation. Of the 178 amino acids identified in these peptides, 173 were in agreement with those deduced from the sequenced cDNA. The five discrepant amino acids might be explained by species differences between bovine and human. Computer-assisted comparison of the sequence of p42 with amino acid sequences of proteins in current data bases confirmed that p42 was a member of the AAA protein family, as suggested previously from partial amino acid sequence data (Fig. 2). It was not, however, identical to any previously described protein nor was it the obvious human homolog of a previously described AAA protein in another species. Thus, p42 represents a novel member of the AAA protein family (see section 4). Fig. 2 shows the amino acid sequence alignment of the six family members that are subunits of the proteasome activator, PA700. These proteins, like all members of this family, are most similar in a central domain of approximately 200 amino acids that contains a putative ATP binding site as defined by Walker et al. [26]. Little sequence similarity occurs among these proteins at their amino or carboxyl termini.

3.2. *Identification of p42 in multiple protein complexes in extracts of red blood cells*

The identification of p42 and TBP1 as common subunits of PA700 and the modulator raised questions regarding the origin of the modulator and the relative functions of p42 and TBP1 between the two complexes ([24] and see section 4). In order to extend our original findings and to address questions about the relationship between PA700 and modulator, we sought to establish the qualitative and quantitative distribution of p42 in crude cell extracts. Therefore, bovine red blood cells were osmotically lysed in a buffer containing ATP and the soluble portion of the lysate was fractionated by gel filtration chromatography on Sephacryl S-300 in the same ATP-containing buffer. The eluted fractions were assayed for proteasome activity using Suc-Leu-Leu-Val-Tyr-AMC, a specific proteasome substrate, and were also subjected to immunoblotting using anti-TBP1, anti-p42, and anti-proteasome antibodies. Fig. 3 shows that nearly all of the proteasome activity was located in fractions at the void volume of the column ($M_r \geq 1\,500\,000$). Analogous experiments using Sephacryl S-400 showed a similar activity profile with a peak corresponding to a molecular weight of approximately 2 000 000, the expected size of the 26S proteasome (data not shown). Some proteasome activity appeared as a small shoulder on the main activity peak and this activity is accounted for by proteasome complexed with the activator PA28. Immunoblotting of the column fractions with anti-proteasome antibodies confirmed the bimodal distribution of proteasome protein indicated by the two peaks of activity (data not shown). Immunoblotting of the column fractions with the anti-p42 and anti-TBP1 antibodies showed that each of these proteins also had a bimodal distribution. Most of the p42 and TBP1 coeluted with each other and with the main peak of proteasome activity (Fig. 3). These results are expected because the 26S proteasome is probably composed of a complex between proteasome and PA700. The distribution pattern of the p42 and TBP1 proteins showed no significant peak of these proteins in the molecular weight range of 700 000, suggesting that little free PA700 occurred in these extracts. This probably results because the ATP-containing buffer stabilizes the association between the proteasome and PA700. In fact, when the same experiment was conducted in buffers without ATP, significantly less p42 and TBP1 protein were detected in 26S proteasome peak and correspondingly more p42 and TBP1 were detected in the molecular weight range of 700 000 (data not shown). Second peaks of p42 and TBP1 protein were detected in the column fractions of the experiment shown in Fig. 3. These peaks were also coincident with one another and occurred at fractions corresponding to a molecular weight of approximately 250 000, the expected size of the modulator, a protein previously shown to contain p42 and TBP1 as subunits. Unfortunately, modulator activity could not be assayed directly in these fractions because they were contaminated with PA28, a potent ATP-independent activator of the proteasome. Nevertheless, the results indicate that significant amounts of cellular p42 and TBP1 are present in the modulator protein, and that this protein existed in the intact cells.

4. Discussion

The current work establishes the primary structure of a novel member of the AAA protein family. This protein, which we have termed p42, was identified previously as a common subunit of two proteins that regulate proteasome activity [24]. Members of the large AAA protein family exhibit a high degree of sequence similarity in a central domain of approximately 200 amino acids characterized by a consensus sequence for ATP binding [23]. Because AAA proteins share little sequence similarity outside this domain, any functional relation-

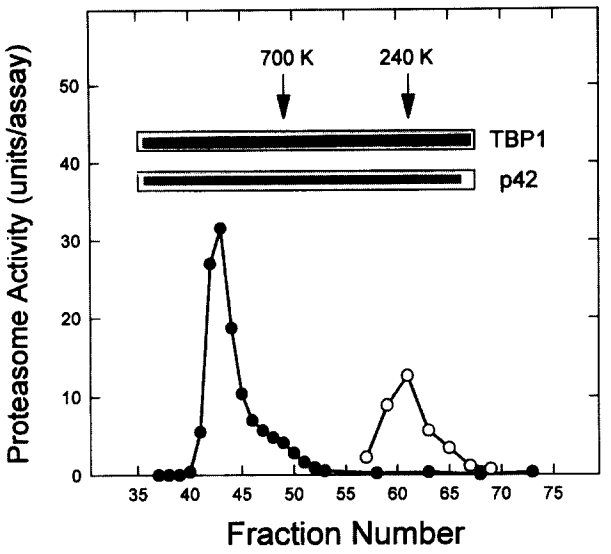

Fig. 3. Distribution of p42 and TBPL in soluble extracts of bovine red blood cells fractionated by gel filtration chromatography. Soluble extracts of bovine red blood cells were prepared and subjected to gel filtration chromatography on Sephacryl S-300. 20 μl of the column fractions were assayed for hydrolytic activity against the substrate: Suc-Leu-Leu-Val-Tyr-AMC (●). The fractions were also subjected to immunoblotting using antibodies against TBP1 and p42 (Sug2p). Purified modulator (50 μg) was chromatographed separately under the same conditions and assayed for activity as described previously (○). Molecular weight markers (thyroglobulin, M_r = 660 000 and aldolase, M_r = 240 000) were chromatographed separately and their elution positions are indicated by arrows.

ship among family members may be limited to their binding and/or hydrolysis of ATP. Interestingly, a subclass of AAA proteins contains two ATP-binding domains [23]. Although the functional significance of this feature is unclear, mutational studies on one of these proteins, the *N*-ethylmaleimide-sensitive fusion protein (NSF), indicate that the two domains have distinct roles [27].

Six members of the AAA protein family, including the p42 protein described here, are subunits of PA700, a 700 000-Da 20-subunit complex that binds to the proteasome and regulates its ability to degrade peptide and protein substrates. In addition to p42, the AAA subunits of PA700 include: TBP1 [28], MSS1 [29,30], S4 [31], p45 (Sug1p) [22], and TBP7 [22,32,33]. It is currently unclear whether each PA700 molecule contains each of these AAA proteins, or whether there are subpopulations of PA700 with qualitatively different compositions of these subunits. Recent work has shown that the cellular levels of some of these proteins change differentially in response to physiological stimuli, suggesting that subpopulations of PA700 do exist [34]. However, as shown here and in previous work, PA700 is not the only complex into which these proteins can assemble.

Regardless of the exact number of PA700 forms, the existence of six homologous proteins in PA700 raises important questions regarding their roles in PA700 function. Although it is reasonable to assume that one or more of the AAA proteins is responsible for the ATPase activity exhibited by the intact PA700 molecule [22] and that this activity is mechanistically linked to ATP-dependent functions of the proteasome/PA700 complex (including assembly of the proteasome/PA700 complex, and degradation of ubiquitinated proteins), it is unclear whether the individual subunits play distinct roles in different ATP-dependent functions or whether they play similar multiple roles in these functions. Additional work to determine the exact composition and architecture of PA700 should clarify these issues. Interestingly, the modulator, which contains two AAA protein subunits, p42 and TBP1, has no detectable ATPase activity compared to the ATPase activity of PA700 (R.J. Proske and G.N. DeMartino, unpublished observations). This finding may indicate that only some of the AAA proteins actually bind and hydrolyze ATP. Alternatively, the ATPase activity of these proteins may be positively regulated by other subunits of PA700 or they may be negatively regulated by the third, non-AAA subunit of the modulator [24]. One AAA protein from yeast, the probable homolog of human S4, has been expressed and the isolated protein catalyzes ATP hydrolysis [35].

The current work confirms and extends our previous observations regarding the distribution of p42 and TBP1 in cell extracts. These proteins were each found in highly purified samples of PA700 and modulator, two multi-protein regulators of the proteasome. We previously argued that this bimodal distribution does not result from the artifactual dissociation of PA700 into subcomplexes, perhaps as a result of exposure of the cell extract to harsh conditions during protein fractionation and purification. However, the results presented here show that the bimodal distribution of p42 and TBP1 exists even after very gentle preparation and minimal treatment of cell extracts. We interpret this finding to indicate that the modulator exists as a distinct complex within cells. Furthermore, these data show that most of the p42 and TBP1 proteins were present in the 26S proteasome. We interpret this finding to indicate that most of the PA700 in these cells is associated with the proteasome. It is unclear, however, whether the modulator is also a component of the 26S proteasome complex identified in these experiments. This issue will be resolved as the biochemical mechanism for the action of the modulator becomes better characterized.

During the preparation of this article we learned of two other independent, unpublished studies which resulted in the identification of p42 and the determination of its primary structure. One study has identified the yeast homolog of p42 as the product of the SUG2 gene. (S.A. Johnston, personal communication and manuscript submitted for publication). Sug2p has been shown to be a component of the 26S proteasome, almost certainly because it is a component of PA700 or the modulator in yeast. These results are in accord with the conclusions reached in the current study. SUG2 was originally identified as a suppressor of a mutation in the GAL4 gene [36]. The GAL4 gene product normally stimulates transcription of galactose-regulated genes. Another suppressor of GAL4 mutants, Sug1p, was originally proposed to be directly involved in transcriptional regulation [37], but now has been clearly identified as a component of PA700 (subunit p45 in mammals) [38]. These findings raise the possibility that PA700 and/or the modulator regulate transcription by mediating the degradation of critical control proteins, a possibility raised originally by Dubiel et al. [31]. The homolog of p42 has also been identified in ground squirrels (M. Andrews, personal communication and manuscript submitted for publication), where it has also been shown to be a subunit of the 26S proteasome. Therefore, p42 seems to be widely distributed and highly conserved.

The AAA protein family contains over 50 members. In addition to p42 and the other five proteins directly linked to the control of proteasome function, the family contains proteins involved in very diverse cellular functions such as vesicle fusion, peroxisome biogenesis, protein sorting in the mitochondrion, mitotic and meiotic spindle function, and transcription [23]. The exact biochemical roles of the AAA proteins in these processes are unknown in most cases. Thus, these diverse cellular functions may utilize a common feature of AAA proteins, such as ATPase activity, in mechanisms specific for a given function. It is also possible, however, that many of these seemingly diverse cellular functions are mediated by AAA proteins through a smaller number of similar biochemical mechanisms, such as ATP-dependent proteolysis. Additional work regarding the structure and function of AAA proteins will provide insight to the molecular basis for the control of these cellular activities.

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