Simultaneous binding of PA28 and PA700 activators to 20 S proteasomes

Klavs B. HENDIL^{*1}, Selina KHAN^{*} and Keiji TANAKA[†]

^{*}August Krogh Institute, University of Copenhagen, Universitetsparken 13, DK-2100 Copenhagen O, Denmark, and [†]The Tokyo Metropolitan Institute of Medical Science, 18-22 Honkomagome 3-chome, Bunkyo-ku, Tokyo 113-0021, Japan

Two activators, named PA700 and PA28, are known to bind to 20 S proteasomes, forming two different complexes. The PA700–proteasome complex, also known as the 26 S proteasome, can degrade intact proteins, whereas complexes with PA28 can degrade only peptides. Monoclonal antibodies to 20 S proteasomes or the p45 ATPase subunit (Trip1, Sug1) of PA700 precipitated the same set of proteins from HeLa extracts, including six different ATPase subunits of PA700. This shows that p45 is not present in other protein complexes and suggests that all 26 S proteasome particles contain the same set of ATPase subunits. Interferons α and γ had no effect on the composition of

the 26 S proteasome, except for the replacement of subunits delta, MB1 and Z with Lmp2, Lmp7 and MECL1 respectively. Surprisingly, antibodies to PA28 precipitated p42, a component of PA700. Conversely, anti-p45 antibodies precipitated not only 26 S proteasomes but also PA28 α , β and γ , indicating that 20 S proteasomes can simultaneously bind both PA700 and PA28. PA28 $\alpha\beta$ is known to be involved in antigen presentation. Conceivably, intact substrate proteins are recognized by PA700 and fed into proteasomes whose cleavage specificity is optimized for antigen presentation on MHC class I by PA28 and three interferon inducible proteasome subunits.

INTRODUCTION

Energy-dependent proteolysis in eukaryotic cells is mediated by proteasomes, large proteinase complexes found in nuclei and cytoplasm. Proteasomes are found as 20 S or 26 S particles. The 20 S proteasomes consist of two copies of each of 14 different subunits. The subunits are arranged in four stacked rings to make a barrel-shaped structure. In turn, 26 S proteasomes are formed by the association of ATPase complexes to each end of the 20 S proteasome barrel. The ATPase complexes are called PA700 or AC (ATPase complex), have a molecular mass of approx. 700 kDa and contain more than 15 different subunits (reviewed in [1]). Most proteins are conjugated to ubiquitin before they are degraded by the 26 S proteasome. PA700 recognizes ubiquitinated proteins and is thought to unfold them, so that they can be cleaved in the interior cavity of the proteasome, where the proteolytically active sites are localized [2].

Another proteasome regulator, PA28 or REG, is ring-shaped and also associates with the ends of the barrel-shaped 20 S proteasome [3]. PA28, composed of α and β subunits, is induced by interferons and changes the cleavage specificity of proteasomes (reviewed in [4–6]). A closely related protein, the Ki antigen or PA28 γ , has recently been described [6–10]. 20 S proteasomes or proteasomes associated with PA28 can only degrade peptides [11,12] in contrast with the 26 S proteasome, which can also degrade intact proteins.

Protein degradation is involved in regulation of metabolism, gene transcription and cell division. It is also implicated in the presentation of protein antigens on MHC class I: such antigens are degraded in the cytoplasm before the fragments are transported into the endoplasmic reticulum through the transporter associated with antigen presentation (TAP) peptide pump, assembled with MHC class I molecules and transported to the cell surface, where they can be recognized by T lymphocytes (reviewed in [4,5]).

Type I (α and β) and in particular type II (γ) interferons, which stimulate immune responses, not only induce the synthesis of PA28 $\alpha\beta$: they also cause the replacement of three enzymically active subunits of the proteasome with three others [1]. In

addition, interferons induce the synthesis of the TAP1/TAP2 peptide transporter and MHC molecules in the endoplasmic reticulum [13].

In the present study we have investigated the effects of interferons on the composition of the 26 S proteasome. The results show that interferons α and γ do not induce new subunits in the 26 S proteasome besides those already known. However, we detected ternary complexes consisting of proteasomes with both PA28 and PA700. The existence of such complexes suggests that PA28 might influence the degradation of intact proteins.

PA700 contains six different ATPase subunits [1] and it has been debated whether they reside in disparate subpopulations of 26 S proteasomes. However, our results also suggest that all 26 S proteasome particles contain the same set of ATPases.

EXPERIMENTAL

Buffer A is 25 mM Tris/HCl (pH 7.6)/1 mM dithiothreitol/2 mM ATP/2 mM MgCl₂/5 mM KCl/17% (v/v) glycerol; buffer B is buffer A without glycerol and ATP but with 200 mM NaCl. cDNA for p42 [14], p45 [15], MSS1 [16], TBP-1 [17] or TBP7 [17,18] was ligated into the pET16b vector (Novagen, Madison, WI, U.S.A.). Recombinant proteins were induced in transformed BL21(DE3) cells with isopropyl β -D-thiogalactoside and purified on Ni²⁺-NTA-agarose (Quiagen, Chatsworth, CA, U.S.A.) in accordance with the manufacturer's instructions. Monoclonal antibodies against recombinant proteins were prepared by standard methods. Isotypes were determined as described [19]. Antibodies against PA28 [6], S4 subunits [20] and 20 S proteasomes [19,21] are described elsewhere. Antibodies against p42 were biotinylated [22] with biotinamidocaproate *N*-hydroxysuccinimide ester (Sigma, St. Louis, MO, U.S.A.). Peroxidase-conjugated streptavidin and antibodies against mouse or rabbit immunoglobulins were obtained from Dako (Copenhagen, Denmark).

HeLa cells were grown in Eagle's minimal essential medium with 5% (v/v) newborn-calf serum. For treatment with interferons, 5×10^6 HeLa cells in 175 cm² culture flasks (Nunc) with

Abbreviation used: TAP, transporter associated with antigen presentation.

¹ To whom correspondence should be addressed (e-mail KBHendil@aki.ku.dk).

Figure 1 Composition of 20 S proteasome and 26 S proteasome changed by interferons

HeLa cells were incubated without additions (a), with interferon α (b) or with interferon γ (c), and labelled with [3 H]leucine. Proteasomes (20 S and 26 S) were precipitated from cell lysates with an antibody against proteasomes, and analysed by two-dimensional PAGE. The acidic end is to the left. The interferon-inducible subunits Lmp2, Lmp7 and MECL1 are marked, as are their housekeeping counterparts δ , Z and MB1. The arrows in (c) indicate PA28 β and PA28 α subunits, identified below. On the original films, three closely spaced subunits can be discerned in the large spot, marked with an arrowhead (a).

25 ml of medium were incubated with 50 i.u./ml interferon γ (human, recombinant; Boehringer Mannheim) or 2000 i.u./ml interferon α_{2a} (Roferon A injection substance; Roche). Media were changed once a day for 3 days. On the third day after inoculation the cells were labelled by incubation for 6 h in a methionine-free medium with [35 S]methionine (Pro-Mix; Amersham; approx. 370 kBq/ml) and 5% (v/v) undialysed calf serum and then left overnight in unlabelled, complete medium. Alternatively, the cells were incubated for 3 days in medium with 0.1 mM [3 H]leucine (370 kBq/ml) with or without interferon. In both protocols the cells from each flask were harvested with trypsin, washed in saline and homogenized by sonication for 5 s in 4 vol. of buffer A with 40 mM creatine phosphate and creatine kinase (Boehringer; 0.2 mg/ml). After centrifugation (12000 g for 3 min) the supernatant was incubated at 37 °C for 30 min, pipetted up and down a few times and centrifuged again. The final supernatant was tumbled for 60 min at room temperature

Figure 2 Specificity of antibodies against p45

HeLa cell proteins were separated by SDS/PAGE and either stained with Coomassie Blue (a) or probed by immunoblotting with antibody p45-110 (b), where only one protein reacts. The positions of molecular mass markers (in kDa) are indicated at the left. 26 S proteasomes from 35 S-labelled HeLa cells were also precipitated with an antibody against proteasomes subunit HN3, separated by two-dimensional electrophoresis, and blotted on nitrocellulose. Proteins were detected by autoradiography (c) and p45 by immunoblotting with antibody p45-110 (d). The heavy chains from immunoglobulin, used to precipitate the 26 S proteasome, stain faintly as a curtain above p45. The position of p45 is marked by an arrowhead in (c). Several other subunits were also identified with subunit-specific antibodies against proteasome subunits [21] or the ATPase subunits of PA700. The ATPase subunits are: 1, TBP-1; 2, TBP-7; 3, S4; 4, MSS1; 5, p42. The 20 S proteasome subunits are: 6, zeta; 7, delta; 8, C8; 9, N3; 10, Z; 11, C10; 12, C2; 13, iota; 14, C7; 15, C3; 16, C9; 17, C5; 18, MB1; 19, XAPC-7. With a shorter exposure, three subunits were resolved in the spot with TBP-1 and TBP-7 (compare with Figure 1). The two subunits labelled by arrows (most probably p112 and p97 [1]) were present in small amounts and were not visible on all two-dimensional PAGE gels.

with the appropriate antibody adsorbed on 20 μ l of Protein G-Sepharose (GammaBind G Sepharose; Pharmacia). The Sepharose beads were then washed four times by centrifugation in 15 ml of ice-cold buffer A and once in 15 ml of 25 mM ammonium acetate, pH 7.5. The final pellets were suspended in 3 ml of distilled water and freeze-dried to prepare salt-free samples. Precipitated proteins were resolved by two-dimensional PAGE [19] followed by fluorography with preflashed XAR-5 film. Alternatively, the proteins were blotted on nitrocellulose sheets and detected by direct autoradiography with BioMax MS films (Eastman-Kodak) and by immunoblotting [19].

For gel-filtration analysis, HeLa cells were treated as in the labelling experiments but without isotopes. Immediately after homogenization, extracts in buffer A were centrifuged at 12000 g for 3 min and filtered through a 0.45 μ m filter (Millipore type SJHV004NS) before 50 μ l of filtrate was injected into a Superose 6 column (Superose 6 PC 3.2/30; Pharmacia) and eluted at 40 μ l/min with the same buffer as used for homogenization. Fractions of 80 μ l were collected after 19 min. Samples of 10 μ l were analysed by non-denaturing PAGE [19] followed by im-

munoblotting. Homogenates in buffer B were aged overnight at 6 °C before they were similarly analysed.

RESULTS

HeLa cells were treated with either interferon α or interferon γ before proteasomes were precipitated from the cell extracts with a monoclonal antibody to HN3, a proteasome β -type subunit. The subunit is localized in the middle of the proteasome barrel [23], so the antibody cannot interfere sterically with the binding of the proteasome regulators PA28 [3] and PA700 [24] to the ends of the proteasome barrel. The antibody therefore precipitates free 20 S proteasomes, 26 S proteasomes and proteasomes in complex with PA28. Figure 1 shows that interferon α induces a partial, and interferon γ an almost complete, replacement of proteasome subunit delta with Lmp2, and of MB1 with Lmp7. Subunit MECL1, with high similarity to subunit Z, was induced with interferon γ but not with interferon α . In addition two spots, later identified as PA28 α and PA28 β , were induced by both interferons. However, the other proteins in the precipitates were identical in control cells and in cells treated with interferons,

so no other subunit of either proteasome or PA700 regulator was induced by interferons.

To be able to analyse cellular complexes of PA700, we generated monoclonal antibodies against recombinant protein of the ATPase subunits p42, p45, TPB-1, TBP-7 and MSS1. Most antibodies reacted well with antigens on blots but none of them bound 26 S proteasomes in solution, except for 3 out of 57 antibodies to p45, which could precipitate proteins from HeLa extracts. Of these three antibodies, p45-32, p45-49 and p45-110, the first was an IgG2a isotype, whereas the latter two were of the IgG2b isotype. They reacted avidly with recombinant p45 in ELISA but not at all with several structurally related (recombinant) ATPases from PA700, namely p42, TBP-1, TBP-7 or MSS1 [1] (results not shown). The same series of proteins were precipitated from HeLa extracts by the three antibodies (results not shown). Only one of the precipitating antibodies, p45-110, could also detect p45 in blots, where it reacted with only one protein both in blots with HeLa cell bulk protein and in 26 S proteasomes (Figure 2), indicating that the antibodies are highly specific. The other ATPase subunits of PA700 were detected with antibodies in similar blots, as indicated on Figure 2.

It is generally believed that proteasomes bind either to PA28

Figure 3 Gel filtration of HeLa cell extracts

Protein complexes from HeLa cell extracts were separated first by gel filtration on Superose 6 and then by non-denaturing gel electrophoresis and immunoblotting. The column was calibrated with Blue Dextran (V_0), α_2 -macroglobulin (725 kDa), ferritin (440 kDa) and mouse immunoglobulin G (160 kDa). (a, c, e) Cell extracts were in a stabilizing buffer with glycerol and ATP. (b, d, f) Cell extracts were prepared in the presence of salts to promote the dissociation of proteasome complexes. Blots were probed with antibodies against proteasome subunit C2 (a, b; antibody MCP20), p45 (c, d; antibody p45-110) or PA28 β (e, f). Qualitatively identical results were obtained with and without incubation of cells with interferon γ .

Figure 4 Co-precipitation of PA28 and PA700

HeLa cells were treated with interferon γ and homogenized. Proteins were then precipitated with antibodies against 20 S proteasomes (lanes 1 and 4; antibody MCP444), PA28 β (lane 5) or p45 (lane 2; antibody p45-110). Alternatively, proteins were mock-precipitated with an irrelevant antibody (lanes 3 and 6; antibody AM2 [48]). Precipitates were then analysed by SDS/PAGE and blotting. Lanes 1, 2 and 3 were probed with an antibody against PA28 β followed by peroxidase-conjugated antibody against rabbit immunoglobulin (arrow). Lanes 4, 5 and 6 were probed with biotinylated antibody against p45 (antibody p45-110) followed by peroxidase-conjugated streptavidin (arrowhead). The positions of protein standards (in kDa) are indicated at the left.

or to PA700, but not to both at the same time [4,6,25,26]. We analysed HeLa cells extracts by fast gel filtration followed by non-denaturing PAGE and immunoblotting. Cell extracts were prepared either in a stabilizing buffer or in a buffer without ATP and glycerol but with salts, to dissociate the 26 S proteasome and PA28–proteasome complexes.

Figure 3(b) shows that free proteasomes were eluted as expected for a protein of approx. 700 kDa. In contrast, in the

presence of ATP (Figure 3a) most proteasomes were present as complexes with other proteins, so that they were eluted earlier from the column. The complex eluted in fractions 4–6 (Figure 3a), and having a low electrophoretic mobility, was identified as the 26 S proteasome by blotting with an antibody against p45, a component of PA700 and therefore of the 26 S proteasome [1] (Figure 3c). In the presence of salts, PA700 dissociated from the 26 S proteasome and was eluted later during gel filtration (Figure 3d). Figure 3(c) confirms [26] that no free PA700 was present in the cell extracts. We interpret the presence of two bands in the lanes with 26 S proteasome (Figure 3c) as being due to partial disintegration of the 26 S proteasome during electrophoresis, giving rise to a mixture of 26 S proteasome and free PA700. p45 was not detected in any other proteins (Figures 3c and 3d).

Blotting with antibodies against PA28 β showed that PA28 dissociates from proteasomes in the presence of salt (Figure 3f) but under stabilizing conditions is found in complexes with proteasomes (compare Figures 3a and 3e). No free PA28 was present in the ATP- and glycerol-stabilized extracts, in agreement with other studies [26,27]. Interestingly, one of the complexes with low electrophoretic mobility reacted with antibodies against both proteasomes, p45 and PA28 (Figures 3a, 3c and 3e), suggesting the presence of a complex containing all three components.

However, the alternative interpretation, that proteasomes, p45 and PA28 form disparate complexes with similar masses and electrophoretic mobilities, could not be ruled out. Protein complexes were therefore precipitated with antibodies against either PA28 β or the PA700 subunit p45. Subsequent SDS/PAGE and immunoblotting showed (Figure 4) that antibodies against PA28 co-precipitated p42, a component of PA700. Conversely, antibodies against p45, another component of PA700, also precipitated PA28, so PA28 and PA700 must be present in the same complex. Proteins precipitated with antibodies against proteasome subunit HN3 or against p45 were also analysed by two-dimensional PAGE. Figures 5(a) and 5(b) show that precipitates obtained with either antibody gave identical patterns in two-dimensional gels. Precipitates from interferon-induced cells made

Figure 5 Composition of 26 S proteasome complexes

26 S proteasomes and proteasome complexes were precipitated from [35 S]methionine-labelled HeLa cell extracts either with an antibody against 20 S proteasomes (anti-HN3) (a) or an antibody against p45 (p45-49) (b–f). (a–c) Autoradiograms; (d–f) blots incubated with antibodies against PA28 α , PA28 β or PA28 γ respectively. Cells used in (c), (d) and (e) were incubated with interferon γ . The circle in (a) indicates the position of PA28 γ , which is detectable by blotting (f) but visible only in overexposed autoradiograms. Subunits induced by interferon γ are marked in (c). α and β denotes the PA28 subunits. A few irrelevant protein impurities were present in (c).

with p45 antibody contained the three interferon-induced proteasome subunits, Lmp2, Lmp7 and MECL1, so these are all found in the 26 S proteasomes. Interestingly, PA28 α and PA28 β were also precipitated (Figures 5c, 5d and 5e). Similarly, PA28 γ , which is down-regulated by interferon [6], was detected in precipitates with antibody against p45 (Figure 5f) but decreased to undetectable levels after incubation of the cells with interferon. The results show that the cell extracts contained a complex consisting of proteasomes, PA28 and PA700.

The association of PA28 $\alpha\beta$ and PA700 does not depend on interferon: PA28 α and β were both detectable in precipitates made with anti-p45 antibody from cells that were not incubated with interferon (results not shown).

DISCUSSION

Mammalian p45 (Trip1) or its yeast homologue Sug1 is an RNA-stimulated ATPase [28]. It is an integral component of the 26 S proteasome [1] and has been thought also to be present in a number of other protein complexes [29–35]. However, our gel-filtration analysis and the identity of two-dimensional gel patterns of precipitated proteins obtained with antibodies to proteasomes and to p45 show that in human cells, as in yeast [36,37], p45 is present only in PA700 and the 26 S proteasome. In addition, the six putative ATPases [1] in the 26 S particle seem to coexist in the same particle. This is shown by the precipitation (Figure 5b) of all six ATPases (identified in Figure 2c) by an antibody against one of them, p45.

Conjugation of ubiquitin to proteins is generally necessary for their recognition and degradation by the 26 S proteasome. Accordingly, ubiquitin seems to be involved in the production of MHC-presented fragments of some [38–41] but perhaps not all antigens [42].

Curiously, PA28 is important for the presentation of at least some antigens [43], although intact antigenic proteins cannot be degraded by the proteasome–PA28 complex [11,12]. It has therefore been suggested that antigens are initially cleaved by the 26 S proteasome and that the fragments are subsequently pruned by PA28–proteasome complexes to fit with the specificities of the TAP transporters and MHC class I. Such a mechanism is not excluded, but many protein fragments generated by proteasomes already have the optimal length for binding to MHC class I [44].

PA28 promotes the formation of antigenic peptides by proteasomes [45] and increases antigen presentation by intact cells [43]. The detection of simultaneous binding of PA28 and PA700 to the same proteasome suggests that intact proteins, usually after ubiquitination, are recognized by the PA700 regulator, unfolded, and fed into the proteasome cylinder for cleavage. The PA28 complex at the other end of the same proteasome might then [46], together with the interferon-induced subunits in the proteasome itself, change the cleavage specificity to provide a spectrum of peptides that are more efficiently presented on MHC than the fragments generated by the 26 S proteasome alone.

PA28 γ was also detected in complexes with proteasomes and PA700. Like the interferon-inducible PA28 $\alpha\beta$ regulator, PA28 γ can influence the cleavage specificity of proteasomes [10]. In contrast with PA28 $\alpha\beta$, which is found in both nuclei and cytoplasm, PA28 γ is confined to cell nuclei [47]. Phylogenetically, PA28 γ antedates the development of an adaptive immune system [9] and is therefore most probably important for a type of proteolytic protein processing that has no relation to immunity.

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