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Intermediates in the formation of mouse 20S proteasomes: implications for the assembly of precursor β subunits

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The assembly of individual proteasome subunits into catalytically active mammalian 20S proteasomes is not well understood. Using subunit-specific antibodies, we characterized both precursor and mature proteasome complexes. Antibodies to PSMA4 (C9) immunoprecipitated complexes composed of α, precursor β and processed β subunits. However, antibodies to PSMA3 (C8) and PSMB9 (LMP2) immunoprecipitated complexes made up of α and precursor β but no processed β subunits. These complexes possess short half-lives, are enzymatically inactive and their molecular weight is ~300 kDa. Radioactivity chases from these complexes into mature, long-lived ~700 kDa proteasomes. Therefore, these structures represent precursor proteasomes and are probably made up of two rings: one containing α subunits and the other, precursor β subunits. The assembly of precursor proteasomes occurs in at least two stages, with precursor β subunits PSMB2 (C7-I), PSMB3 (C10-II), PSMB7 (Z), PSMB9 (LMP2) and PSMB10 (LMP10) being incorporated before others [PSMB1 (C5), PSMB6 (delta), and PSMB8 (LMP7)]. Proteasome maturation (processing of the β subunits and juxtaposition of the two β rings) is accompanied by conformational changes in the (outer) α rings, and may be inefficient. Finally, interferon-γ had no significant effect on the half-lives or total amounts of precursor or mature proteasomes.
Keywords: macromolecular assembly/precursor β subunits/proteasome biogenesis/protein degradation

Introduction

The 26S proteasome-, ubiquitin- and ATP-dependent pathway is important in the non-lysosomal degradation of proteins involved in cell cycle regulation, metabolic adaptation, removal of abnormal proteins, processing of inactive transcription factor precursors and in the degradation of some membrane proteins (Coux *et al.*, 1996). This pathway is also used for the generation of peptides that bind to major histocompatibility complex (MHC)-encoded class I molecules for antigen presentation to $CD8^+$ T lymphocytes (Michalek *et al.*, 1993; Rock *et al.*, 1994; Cerundolo *et al.*, 1997). The 26S complexes (~2000 kDa) are composed of 19S regulatory components attached to both ends of a catalytically active 20S proteasome core (~700 kDa) (Peters *et al.*, 1993; Yoshimura *et al.*, 1993). Besides binding 19S regulator subunits, 20S proteasomes bind to 11S regulators, which are composed of two homologous and interferon (IFN)-γ-inducible subunits, PA28α and PA28β (Dubiel *et al.*, 1992; Ma *et al.*, 1992; Realini *et al.*, 1994; Ahn *et al.*, 1995). PA28 binds to the ends of 20S proteasomes (Gray *et al.*, 1994) and this combination greatly stimulates proteasome-mediated activity against certain substrates (Groetrrup *et al.*, 1995; Ustrell *et al.*, 1995a); such increased proteolysis may be important in the processing and presentation of some antigens (Dick *et al.*, 1996; Groettrup *et al.*, 1996a).

The 20S proteasomes degrade unfolded proteins and peptides in an ATP-independent fashion and are multicatalytic, i.e. a single proteasome molecule contains independent active sites and may possess as many as five to nine different activities (Orlowski *et al.*, 1993; Stein *et al.*, 1996). Proteasomes are cylindrical in shape, with dimensions of ~150 Å in height and 110 Å in diameter. They are composed of four rings, with seven subunits in each ring; the outer rings are made up of α subunits whereas the inner rings are made up of β subunits (Löwe *et al.*, 1995; Groll *et al.*, 1997). The α subunits are important in assembly (Zwickl *et al.*, 1994) and are the elements to which regulators bind (Peters *et al.*, 1993; Yoshimura *et al.*, 1993; Gray *et al.*, 1994). Most β subunits are synthesized as precursors that subsequently undergo processing, and some possess an amino-terminal threonine that is critical for proteolytic activity (see Müller *et al.*, 1995) and that is covalently modified by the proteasome-specific inhibitor, lactacystin (Fenteany *et al.*, 1995). Proteasomes in their simplest form, such as those from *Thermoplasma*, contain only one type of α and one type of β subunit (Löwe *et al.*, 1995). Recently, genes encoding two different α and two different β subunits have been discovered in *Rhodococcus*, but the organization of these subunits in proteasomes is not yet known (Tamura *et al.*, 1995). Yeasts contain seven different α and seven different β proteasome genes, whereas mammals contain seven different α and 10 different β proteasome genes (Monaco and Nandi, 1995). Remarkably, despite the diversity in the numbers of different subunits, the quaternary structure of proteasomes is strongly conserved from *Thermoplasma* to humans (Pühler *et al.*, 1994).

The cytokine IFNγ induces the expression of three proteasome subunits: PSMB8 (LMP7), PSMB9 (LMP2) and PSMB10 (MECL1) [see Table I for the list of proteasome subunits and their corresponding PSM number (Coux *et al.*, 1996)], which replace the constitutive subunits PSMB5 (X/MB1), PSMB6 (Y/delta) and PSMB7 (Z), respectively in different subsets of mammalian proteasomes (Groettrup *et al.*, 1996b; Hisamatsu *et al.*, 1996; Nandi *et al.*, 1996a). There appear to be tissue-specific differences in basal levels of expression between the IFNγ-

Table I. Correspondence between PSM subunits and known proteasome subunits

Subunit	Type	Homolog	Comments
PSMA1	α	C2, LMP13	
PSMA2	α	C3, LMP8	
PSMA3	α	C8, LMP18	
PSMA4	α	C9, LMP14	
PSMA5	α	Zeta, LMP1	
PSMA6	α	Iota, LMP11	
PSMA7	α	XAPC7, C6-I, LMP16	
PSMB1	β	C5, LMP15	
PSMB2	β	C7-I, LMP6	non-processed β subunit
PSMB3	β	C10-II, LMP5	non-processed β subunit
PSMB4	β	N3, LMP3	
PSMB5	β	X, MB1, LMP17	PSMB8 replaceable subunit
PSMB6	β	Y, delta, LMP19	PSMB9 replaceable subunit
PSMB7	β	Z, LMP9	PSMB10 replaceable subunit
PSMB8	β	LMP7	IFNγ inducible
PSMB9	β	LMP2	IFNγ inducible
PSMB10	β	MECL1, LMP10	IFNγ inducible

PSMA2, PSMA1, PSMA4 and PSMB1 correspond to mouse proteasome subunits C3, C2, C9 and C5, respectively (Fruh *et al.*, 1992). Previously, we identified PSMB8, PSMB9 (Nandi *et al.*, 1996b), PSMB4 (Cruz *et al.*, 1997), PSMB6 and PSMA3 (data not shown), using proteasome subunit-specific antibodies. Using antibodies to duck (Grossi de Sa *et al.*, 1988) or human proteasome subunits (D.Nandi, K.Hendil and J.J.Monaco, in preparation) that cross-react with mouse, we identified PSMA5, PSMA6, PSMA7, PSMB2 and PSMB3 as corresponding to mouse Zeta, Iota, XAPC7, C7-I and C10-II, respectively. Furthermore, based on the effects of IFNγ, we suggested that PSMB5, PSMB7 and PSMB10 represent murine homologs of X/MB1, Z and MECL-1, respectively (Nandi *et al.*, 1996a). Recently, we identified directly the precursor and processed forms of PSMB5, PSMB7 and PSMB10 (D.Nandi, M.Cruz and J.J.Monaco, in preparation). Therefore, we have identified all the predominant proteins immunoprecipitated by anti-LMP14 or an antiserum to whole proteasomes (Brown *et al.*, 1991). Our data identifying different proteasome subunits agree well with another study (Groettrup *et al.*, 1996b).

inducible subunits and their constitutive counterparts, with the inducible subunits PSMB8 (LMP7), PSMB9 (LMP2) and PSMB10 (MECL1) being strongly expressed in lymphoid tissues (Stohwasser *et al.*, 1997). PSMB8 (LMP7) and PSMB9 (LMP2) are MHC encoded and play a role in the processing of some antigens (Fehling *et al.*, 1994; Van Kaer *et al.*, 1994). Interestingly, only the three pairs of constitutive and IFNγ-inducible subunits possess the amino-terminal threonine that is critical for proteolytic activity (Seemüller *et al.*, 1995). The two members of each pair are closely related in sequence (Hisamatsu *et al.*, 1996) and probably occupy a single position in the proteasome structure in a mutually exclusive fashion (Kopp *et al.*, 1993, 1995, 1997; Peters *et al.*, 1993). Indeed, PSMB6 (Y/delta) is excluded from PSMB9 (LMP2)-containing proteasomes (Brown *et al.*, 1991, 1993). Although differences in enzymatic activities are observed in proteasomes containing the constitutive or IFNγ-inducible subunits, the nature and magnitude of these changes are still controversial (Driscoll *et al.*, 1993; Gaczynska *et al.*, 1993, 1994, 1996; Aki *et al.*, 1994; Boes *et al.*, 1994; Groettrup *et al.*, 1995; Kuckelkorn *et al.*, 1995; Ustrell *et al.*, 1995a,b; Stohwasser *et al.*, 1996).

Most of the β subunits found in active proteasomes are post-translationally processed by proteolytic removal of amino-terminal sequence from β subunit precursors. Processing has been noted in *Thermoplasma* (Zwickl *et al.*, 1994; Seemüller *et al.*, 1996), *Rhodococcus* (Tamura *et al.*, 1995), yeasts (Chen and Hochstrasser, 1995, 1996) and mammals (Lilley *et al.*, 1990; Seemüller *et al.*, 1995). The assembly of the *Thermoplasma* proteasome has been studied in great detail by expressing wild-type, truncated or mutant α and β subunits in *Escherichia coli* (Zwickl *et al.*, 1994; Seemüller *et al.*, 1996). Interestingly, the *Thermoplasma* precursor β peptide is dispensable and does not appear to play any role in assembly (Zwickl *et al.*, 1994), nor does its length or sequence, with the exception of glycine at the –1 position, appear to influence processing (Seemüller *et al.*, 1996). However, the precursor peptide of a yeast proteasome β subunit, Doa3, is critical for its incorporation into proteasomes and is subunit specific (Chen and Hochstrasser, 1996). The precursor peptide of a mammalian β subunit, PSMB8 (LMP7), is important for the incorporation of this subunit into proteasomes (Cerundolo *et al.*, 1995), but can also substitute for the precursor peptide of PSMB9 (LMP2) at a reduced efficiency (Schmidtke *et al.*, 1996). Recent studies have shown that the processing of at least some precursor β subunits in *Thermoplasma* (Seemüller *et al.*, 1996), yeast (Chen and Hochstrasser, 1996) and mammals (Schmidtke *et al.*, 1996) is autocatalytic.

The existence of precursor proteasomes has been suggested by the following facts: first, precursor and processed PSMB9 (LMP2) segregate into two different complexes (Patel *et al.*, 1994) and, second, one or more 12–16S complexes exist which may contain precursor β subunits (Frentzel *et al.*, 1994; Yang *et al.*, 1995; Svensson *et al.*, 1996). However, the proteasome subunits present in these complexes were not identified. Here we characterize mouse proteasome assembly in detail by using an antibody to PSMA3 (C8), which recognizes most, if not all, precursor proteasomes but not catalytically active proteasomes. We have identified the subunits present in these precursor proteasomes, which appear to be composed only of α and precursor β subunits. Furthermore, some precursor β subunits appear to be incorporated into precursor proteasomes before others. We have also studied the effect of IFNγ on the half-lives and relative amounts of precursor and mature proteasomes and, based on our observations, we propose a model for the assembly of mouse proteasomes.

Results

Characterization of precursor proteasome complexes

Overexpressed, purified GST–PSMB9 (LMP2), GST–PSMA3 (C8) and GST–PSMA4 (C9) fusion proteins were used to immunize rabbits. As shown in Figure 1A, anti-PSMB9 (LMP2), anti-PSMA3 (C8) and anti-PSMA4 (C9) specifically immunoprecipitated complexes of proteins with molecular masses between 35 and 21 kDa, although each of these antibodies specifically recognized single subunits in denatured lysates (data not shown). No higher molecular weight proteins were immunoprecipitated specifically by these antisera. The 60 kDa protein immunoprecipitated by anti-PSMA4 (C9) represents non-specific cross-reactivity because it has also been observed with antisera to non-proteasome proteins (data not shown). The subunit composition of complexes recognized by these

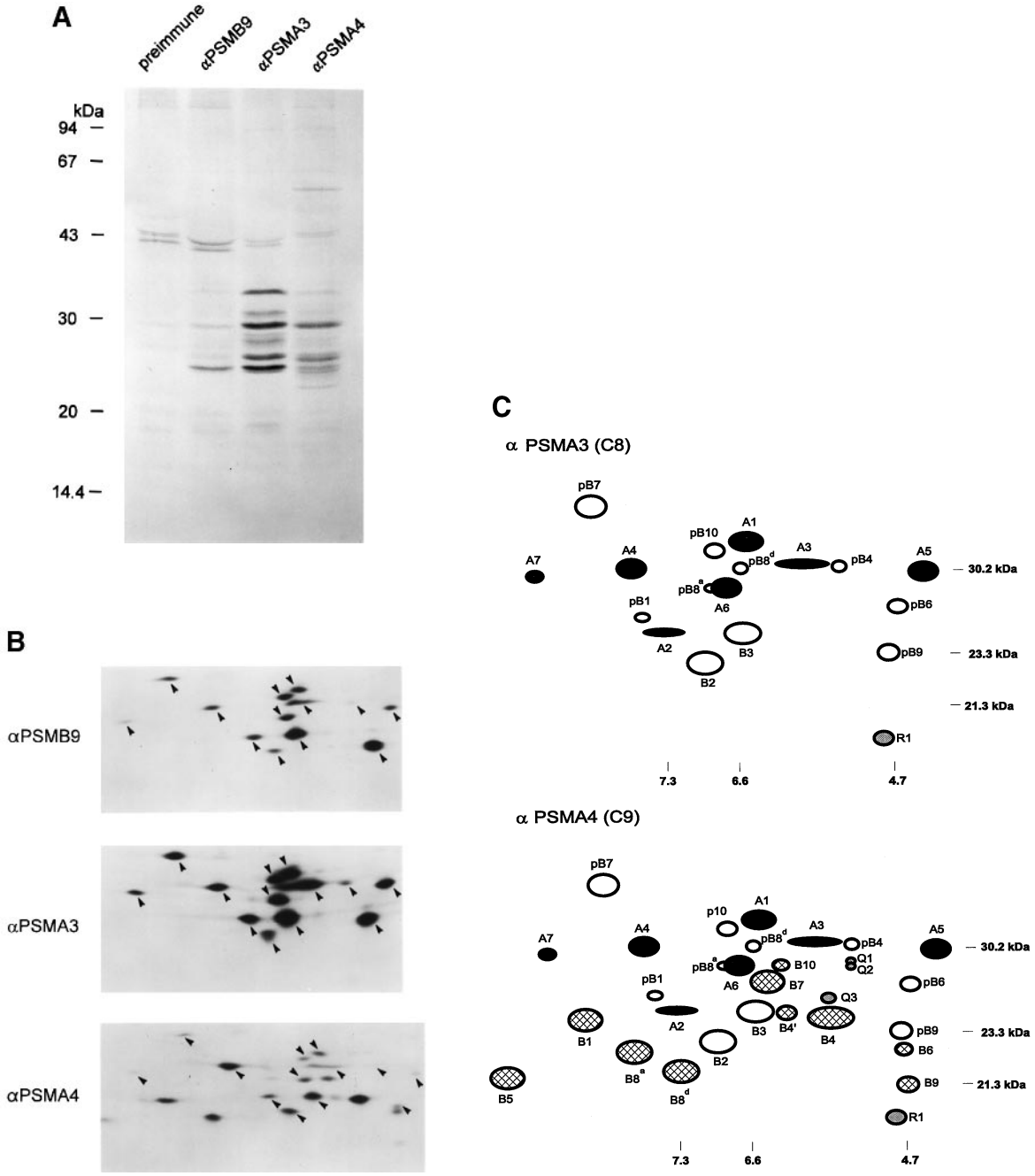


Fig. 1. Anti-PSMB9 (LMP2) and anti-PSMA3 (C8) immunoprecipitate proteasome complexes containing β precursors, whereas anti-PSMA4 (C9) immunoprecipitates proteasome complexes containing both precursor and processed β subunits. H6 cells grown in the presence of IFNγ (30 U/ml) for 4 days were radiolabeled for 4 h with [^{35}S]methionine, solubilized in 0.5% NP-40, immunoprecipitated with the indicated antibodies and analyzed by one-dimensional SDS–PAGE (**A**) or 2D NEPHGE–PAGE (**B**) and fluorography. Proteasome subunits common to the three complexes are marked (B). The subunits that are present in anti-PSMA4 (C9) immunoprecipitates but absent in anti-PSMB9 (LMP2) and anti-PSMA3 (C8) immunoprecipitates are all processed β subunits (see Table I). (**C**) Diagrammatic representation of anti-PSMA3 (C8) and anti-PSMA4 (C9) immunoprecipitates separated by 2D NEPHGE–PAGE. Proteasome subunits are designated by their PSM number, and precursor β subunits are indicated with a 'p' in front of the number. Precursor β subunits are shown as unfilled outlines, processed β subunits are cross-hatched, and α subunits are shown in black. The molecular weights and pIs represent values determined from cloned proteasome subunits. Although precursor PSMB8^{d} (LMP7^{d}) is easily detected in these complexes, it is difficult to detect precursor PSMB8^{a} (LMP7^{a}) because it is partially masked by PSMA6 (Iota). The migration of precursor and processed PSMB8^{d} (LMP7^{d}) is different from that of PSMB8^{a} (LMP7^{a}) due to a polymorphism at amino acid 272 of the molecule (Zanelli *et al.*, 1995; Nandi *et al.*, 1996b). The migration of PSMA3 (C8) fluctuated in different experiments (see (B) and Figure 2A), an observation also reported by others (Aki *et al.*, 1994), and may mask precursor PSMB4 (N3) in some experiments. PSMB4′ is a short-lived form of processed PSMB4 (N3) (Cruz *et al.*, 1997). The intensities of four unidentified proteins (gray), Q1, Q2, Q3 and R1, are low and are detected in only some experiments (see Figure 3A). They may be processing intermediates, chaperones and/or proteins important for proteasome assembly. Q1, Q2 and Q3 are present only in complexes recognized by anti-PSMA4 (C9) or anti-proteasomes; they are also present in cells labeled for a short time and cannot be detected after 1 h of chase (Cruz *et al.*, 1997). R1 is a 17 kDa protein present in complexes recognized by anti-PSMA3 (C8) and anti-PSMA4 (C9) (see Figure 3A); pulse–chase experiments have demonstrated that it is present after short-term labeling of cells and cannot be detected after 3–4 h of chase (data not shown).

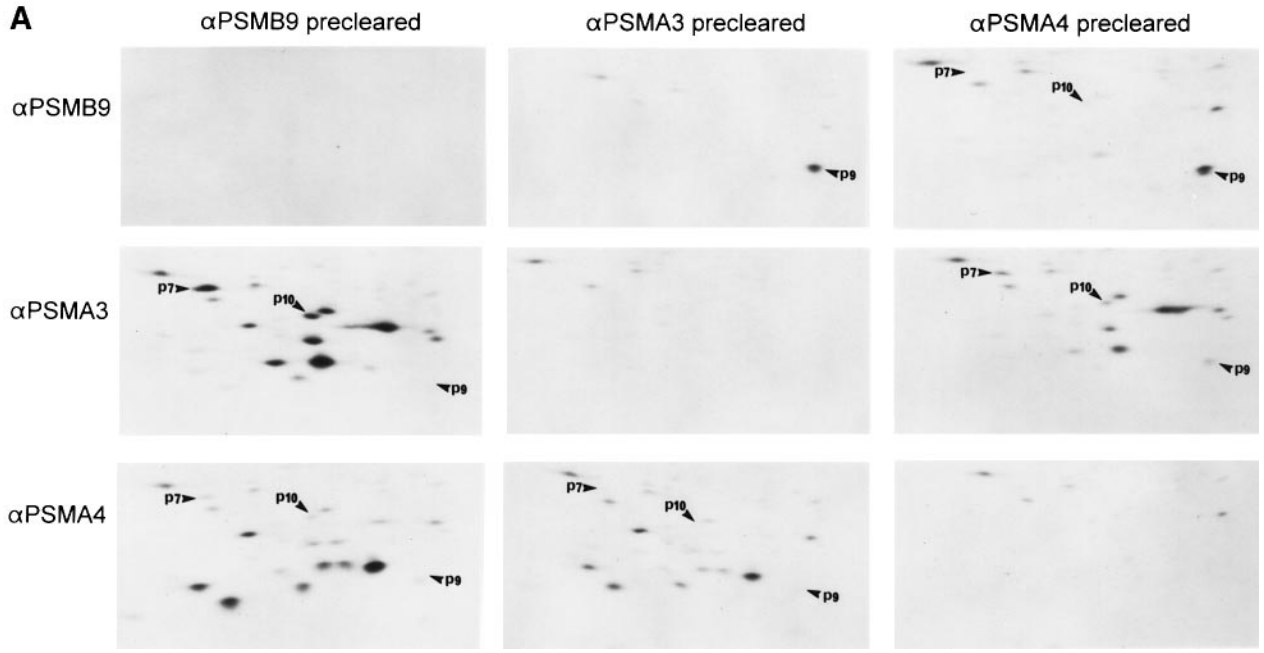


Fig. 2. Pre-clearing with anti-PSMA3 (C8) removes precursor proteasome complexes recognized by anti-PSMB9 (LMP2) and anti-PSMA4 (C9). (**A**) Radiolabeled lysate from H6 cells grown in the presence of IFNγ was split equally into three parts and pre-cleared using excess anti-PSMB9 (LMP2), anti-PSMA3 (C8) or anti-PSMA4 (C9). These were then re-immunoprecipitated with anti-PSMB9 (LMP2), anti-PSMA3 (C8) or anti-PSMA4 (C9) and the complexes subjected to 2D NEPHGE–PAGE analysis and fluorography. (**B**) Venn diagram to illustrate that anti-PSMA3 (C8) and anti-PSMA4 (C9) recognize distinct but overlapping subsets of proteasome complexes. Anti-PSMA4 (C9) (diagonal lines) recognizes some precursor and most, if not all, mature proteasome complexes. Precursor proteasomes recognized by anti-PSMA4 (C9) and anti-PSMB9 (LMP2) (vertical lines) are subsets of those recognized by anti-PSMA3 (C8) (unfilled). Anti-PSMA4 (C9) recognizes a subset of complexes recognized by anti-PSMB9 (LMP2) (cross-hatched overlapped area).

antibodies was studied using two-dimensional non-equilibrium pH gradient electrophoresis–polyacrylamide gel electrophoresis (2D-NEPHGE–PAGE) and fluorography (Figure 1B). Anti-PSMB9 (LMP2) and anti-PSMA3 (C8) each immunoprecipitated 13 proteins, whereas anti-PSMA4 (C9) immunoprecipitated 21 different proteins from NP-40 lysates of H6 cells labeled for 4 h. The 13 proteins immunoprecipitated with anti-PSMB9 (LMP2) and anti-PSMA3 (C8) were identical and were also present in complexes immunoprecipitated with anti-PSMA4 (C9) (Figure 1B; arrows), as judged by mixing the immunoprecipitates (data not shown). Interestingly, the precursors of the IFNγ-inducible subunits, PSMB9 (LMP2) and PSMB10 (MECL1), and the precursor of PSMB7 (Z) whose incorporation is down-regulated by IFNγ (data not shown), were observed in anti-PSMA3 (C8) and anti-PSMB9 (LMP2) complexes. We have identified each of the proteasome subunits separated by 2D-NEPHGE–PAGE (see Table I), and the subunits that were not immunoprecipitated by anti-PSMA3 (C8) and anti-PSMB9 (LMP2) but which were present in anti-PSMA4 (C9) immunoprecipitates all corresponded to processed β subunits (Figure 1C and Table I). These experiments suggested that anti-PSMA3 (C8) and anti-PSMB9 (LMP2) recognized only precursor proteasome complexes, whereas anti-PSMA4 (C9) recognized both precursor and mature proteasome complexes.

To study the relationships between the complexes recognized by anti-PSMB9 (LMP2), anti-PSMA3 (C8) and anti-PSMA4 (C9), we performed pre-clearing experiments. Radiolabeled lysate from H6 cells induced with IFNγ was split into three equal parts and pre-cleared extensively using excess anti-PSMB9 (LMP2), anti-PSMA3 (C8) and anti-PSMA4 (C9). Pre-cleared lysates were split again into three equal parts and immunoprecipitated using the three different antibodies. As seen in Figure 2A, pre-clearing with all three antisera was complete, as re-immunoprecipitation with the same antibodies failed to reveal labeled complexes. Anti-PSMB9 (LMP2) also failed to immunoprecipitate any complexes from lysates pre-cleared with anti-PSMA3 (C8), although free precursor PSMB9 (LMP2) was recognized. However, anti-PSMA3 (C8) immunoprecipitated complexes from anti-PSMB9 (LMP2)-pre-cleared lysates, but these complexes lacked precursor PSMB9 (LMP2), suggesting that precursor PSMB9 (LMP2) is not present in all the complexes

recognized by anti-PSMA3 (C8). These results indicate that the precursor PSMB9 (LMP2)-containing complexes recognized by anti-PSMB9 (LMP2) represent a subset of complexes recognized by anti-PSMA3 (C8). Anti-PSMA4 (C9) immunoprecipitated proteasome complexes from anti-PSMA3 (C8)-pre-cleared extracts, but these complexes lack any radiolabeled β precursors, including pPSMB7 (Z), pPSMB9 (LMP2) and pPSMB10 (MECL1). Therefore, anti-PSMA3 (C8) appears to recognize most or all of the proteasome complexes containing precursor β subunits. Anti-PSMA3 (C8) immunoprecipitated complexes containing precursor β subunits from lysates pre-cleared with anti-PSMA4 (C9), although the amounts of radioactivity immunoprecipitated by anti-PSMA3 (C8) from anti-PSMA4 (C9)-pre-cleared lysates were less than from virgin lysates. There was also some loss in the amounts of radioactivity immunoprecipitated by anti-PSMA4 (C9) from anti-PSMA3 (C8)-pre-cleared lysates, indicating that some, but not all, precursor proteasome complexes recognized by anti-PSMA3 (C8) are also recognized by anti-PSMA4 (C9). Similarly, anti-PSMB9 (LMP2) immunoprecipitated some complexes from lysates pre-cleared with anti-PSMA4 (C9), although the amounts of these complexes were lower than that immunoprecipitated from lysates pre-cleared with normal rabbit serum (data not shown). Therefore, anti-PSMA4 (C9) recognizes some precursor proteasome complexes that are also recognized by anti-PSMB9 (LMP2). These results are summarized in the Venn diagram (Figure 2B). Anti-PSMA3 (C8) and anti-PSMA4 (C9) recognize discrete but overlapping subsets of proteasomes: anti-PSMA4 (C9) recognizes proteasome complexes containing both precursor and processed β subunits, whereas anti-PSMA3 (C8) recognizes complexes containing only precursor β subunits. The complexes recognized by anti-PSMB9 (LMP2) and the precursor complexes recognized by anti-PSMA4 (C9) are subsets of complexes recognized by anti-PSMA3 (C8). These results suggest that precursor proteasomes are heterogenous, similar to mature proteasome subsets (Brown *et al.*, 1993).

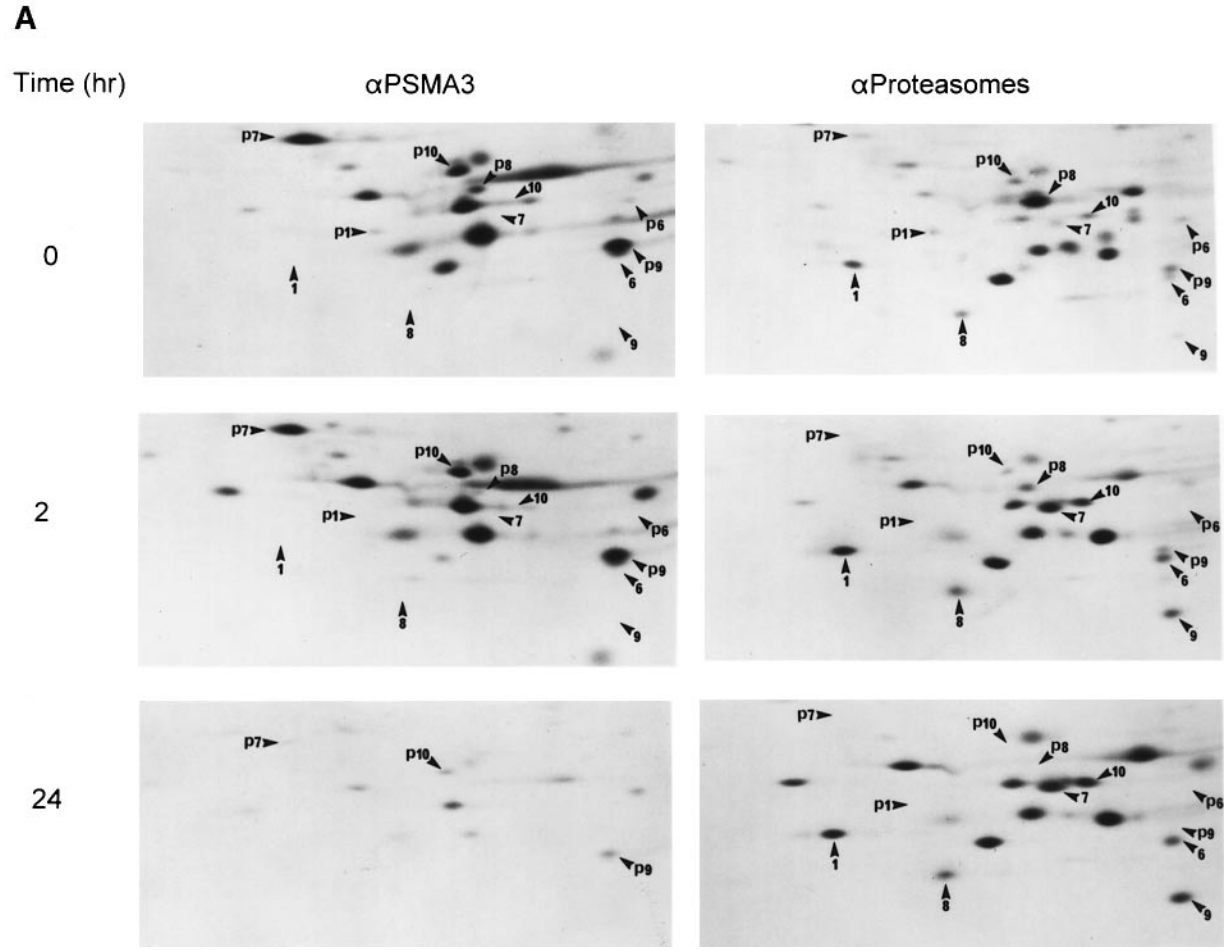
Next, we performed pulse–chase experiments using P388D1 cells stimulated with IFNγ for 4 days (Figure 3A). Cells were labeled for 45 min, washed extensively and cultured in media containing excess cold (non-radioactive) methionine. At various time intervals, cells were harvested, solubilized in 0.5% NP-40 and immunoprecipitated using anti-PSMA3 (C8) or polyclonal antiserum to whole proteasomes. Precursor β subunits pPSMB1 (C5), pPSMB6 (Y/delta), pPSMB7 (Z), pPSMB8 (LMP7), pPSMB9 (LMP2) and pPSMB10 (MECL1) were present in complexes recognized by both antisera at 0 h of chase. However, after 2 h of chase, pPSMB1 (C5), pPSMB6 (Y/delta) and pPSMB8 (LMP7) were no longer present or were present in reduced amounts, but pPSMB7 (Z), pPSMB9 (LMP2) and pPSMB10 (MECL1) were still present. After 24 h of chase, only α and processed β subunits were associated with complexes recognized by antiserum to whole proteasomes and there was no noticeable loss of radioactivity, consistent with the reported long half-lives of proteasomes (Hendil, 1988; Tanaka and Ichihara, 1989; Cuervo *et al.*, 1995). However, by 24 h, >90% of the complexes recognized by anti-PSMA3 (C8) disappeared. Therefore, by 24 h almost all precursor proteasomes are converted to mature proteasomes because the intensity of processed β subunits increased, as judged by immunoprecipitation with anti-proteasomes, whereas the amounts of complexes immunoprecipitated by anti-PSMA3 (C8) dramatically decreased.

The observation that some precursor β subunits are present for longer time periods than others in these complexes suggested two scenarios: (i) some precursor β subunits are processed more slowly than others, and/or (ii) some precursor β subunits are incorporated before others. We have not been able to document any major differences in the rate of formation for any processed β subunits, as judged by immunoprecipitation with anti-proteasomes (see Figure 3A). Indeed, if the first hypothesis were true, we would expect the intensities of processed PSMB1 (C5), PSMB6 (Y/delta) and PSMB8 (LMP7) to be greater than those of PSMB7 (Z), PSMB9 (LMP2) and PSMB10 (MECL1) in complexes recognized by anti-proteasomes after 2 h of chase; however, this was not observed (Figure 3A). We suggest that the epitopes recognized by anti-PSMB9 and anti-PSMA3 are lost shortly after the incorporation of precursor β subunits that are incorporated late, which would explain the short-lived presence of some β precursors in precursor proteasome complexes. Therefore, the assembly of precursor proteasomes occurs in at least two stages: the precursor β subunits that are assembled earlier are long-lived [PSMB2 (C7-I), PSMB3 (C10-II), pPSMB7 (Z), pPSMB9 (LMP2) and pPSMB10 (MECL1)], whereas the last precursor β subunits to be assembled [pPSMB5 (C5), pPSMB6 (Y/delta) and pPSMB8 (LMP7)] are present for only a short time in these precursor proteasome complexes. Further maturation of complete precursor proteasomes is rapid and results in a loss of precursor proteasome-specific epitopes recognized by the antisera.

A second series of pulse–chase experiments was performed to estimate more accurately the half-lives of complexes recognized by anti-PSMA3 (C8) and anti-PSMA4 (C9), and to study the effect of IFNγ on their half-lives. We used H6 cells for this study because the three IFNγ-inducible proteasome subunits, PSMB8 (LMP7), PSMB9 (LMP2) and PSMB10 (MECL1), are absent in unstimulated H6 cells but are greatly up-regulated in the presence of IFNγ (Nandi *et al.*, 1996a). As seen in Figure 3B, the amount of complexes recognized by anti-PSMA3 (C8) was reduced dramatically after 4–8 h of chase. On the other hand, complexes recognized by anti-PSMA4 (C9) were observed until 72 h of chase. Therefore, some radioactivity, but not all, from precursor proteasomes recognized by anti-PSMA3 (C8) chases into mature and long-lived proteasomes recognized by anti-PSMA4 (C9). The mean half-lives of complexes recognized by anti-PSMA3 (C8) and anti-PSMA4 (C9) quantified by phosphorimager analysis were 5.0 and 41.0 h respectively without IFNγ, and 5.8 and 42.7 h respectively in the presence of IFNγ. Therefore, complexes recognized by anti-PSMA3 (C8) possess half-lives that are ~8-fold shorter than those recognized by anti-PSMA4 (C9), and IFNγ has no effect on their half-lives, at least in H6 cells.

Precursor proteasomes are catalytically inactive and their molecular weight is ~300 kDa

We performed sucrose gradient centrifugation experiments to study the molecular weight of complexes recognized



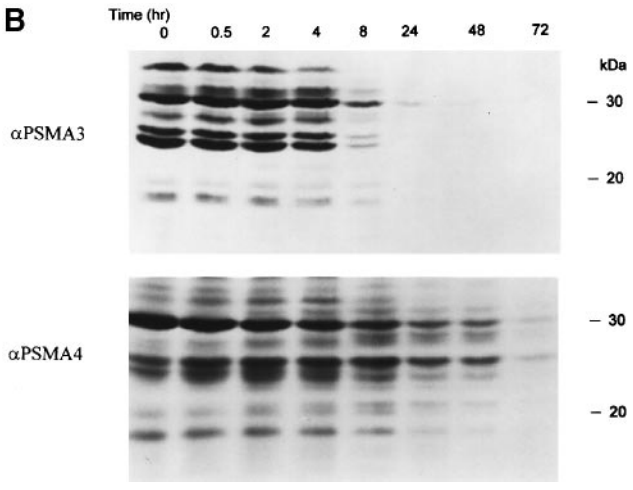


Fig. 3. The half-life of complexes recognized by anti-PSMA3 (C8) is shorter than that of those recognized by anti-PSMA4 (C9); some precursor β subunits, PSMB1 (C5), PSMB6 (Y/delta) and PSMB8 (LMP7), are incorporated later into these complexes than other precursor β subunits, PSMB2 (C7-I), PSMB3 (C10-II), PSMB7 (Z), PSMB9 (LMP2) and PSMB10 (MECL1). (**A**) P388D1 cells grown in the presence of IFNγ were radiolabeled with [^{35}S]methionine for 45 min, washed and grown in media containing 2 mM cold methionine. Cells were harvested at various time intervals, solubilized in 0.5% NP-40 and immunoprecipitated with excess anti-PSMA3 or antiserum to whole proteasomes, followed by 2D NEPHGE–PAGE analysis and fluorography. Proteasome β subunits are designated by their PSM number and precursor β subunits are indicated with a 'p' in front of their number. (**B**) H6 cells were grown in the absence or presence of IFNγ (30 U/ml) for 4 days, radiolabeled for 45 min, washed, split equally into 8–10 aliquots and grown in media containing 2 mM cold methionine. Cells were harvested at various time intervals, washed, solubilized in 0.5% NP-40 and immunoprecipitated with excess anti-PSMA3 (C8) or anti-PSMA4 (C9) followed by one-dimensional SDS–PAGE analysis. The amount of complexes was quantified by phosphorimager analysis and the natural log of the percentage of remaining complexes was plotted versus time [0, 0.5, 2. 4, 8 and 24 h for complexes recognized with anti-PSMA3 (C8) and 0, 8, 24, 48 and 72 h for complexes recognized by anti-PSMA4 (C9)]. The half-lives of complexes were calculated by dividing the natural log of 2 by the slope of the line (*K* decay). The half-lives of complexes recognized by anti-PSMA3 (C8) and anti-PSMA4 (C9), in the absence of IFNγ, were 5.04 ± 0.84 h and 41.01 ± 3.1 h, respectively. In the presence of IFNγ, the half-lives of complexes recognized by anti-PSMA3 (C8) and anti-PSMA4 (C9) were 5.88 ± 0.72 h and 42.78 ± 4.92 h, respectively. The data reported are the mean of three independent experiments.

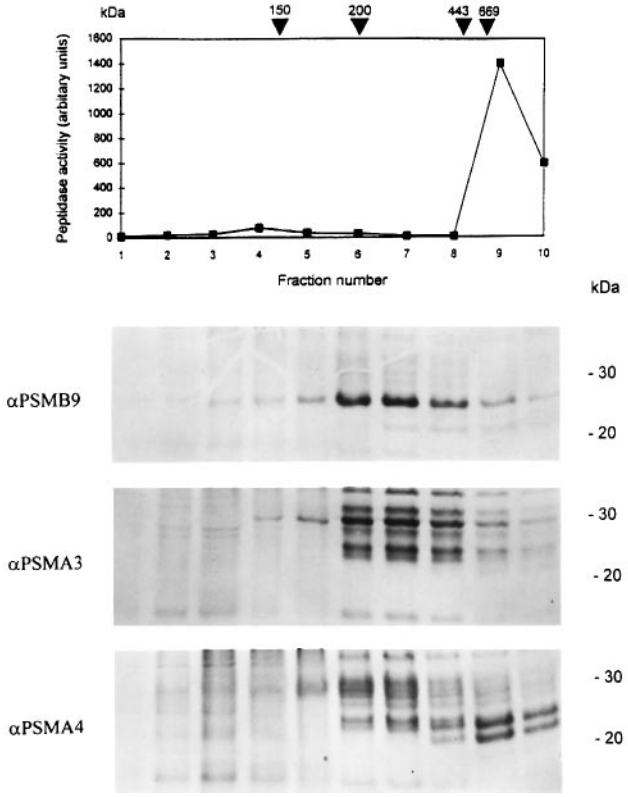


Fig. 4. Complexes recognized by anti-PSMB9 (LMP2) and anti-PSMA3 (C8) are proteolytically inactive and their molecular weight is smaller than that of mature proteasomes. Anti-PSMA4 (C9) recognizes precursor and mature proteasome complexes. Radiolabeled H6 cells grown in the presence of IFNγ were Dounce homogenized and centrifuged to remove cellular debris. The labeled supernatant was layered onto a 10–40% sucrose gradient and centrifuged at 285 000 *g* for 16 h. Fractions (1 ml each) were collected, and portions of each fraction were assayed for peptidase activity and simultaneously immunoprecipitated with anti-PSMB9 (LMP2), anti-PSMA3 (C8) and anti-PSMA4 (C9), followed by one-dimensional SDS–PAGE and fluorography. The molecular weight markers used in the sucrose density gradient centrifugation were: alcohol dehydrogenase (150 kDa), β amylase (200 kDa), apoferritin (443 kDa) and thyroglobulin (669 kDa).

by anti-PSMB9 (LMP2), anti-PSMA3 (C8) and anti-PSMA4 (C9). Radiolabeled H6 homogenate was layered on 10–40% sucrose density gradients and centrifuged for 16 h. Fractions were collected, and peptidase assays and immunoprecipitations with anti-PSMB9 (LMP2), anti-PSMA3 (C8) and anti-PSMA4 (C9) were performed followed by one-dimensional SDS–PAGE and fluorography. As seen in Figure 4, one major peak of Suc-LLVY-MCA-specific peptidase activity was observed at ~700 kDa. Most of the precursor complexes immunoprecipitated by anti-PSMB9 (LMP2) and anti-PSMA3 (C8) were observed in fraction 7, suggesting that precursor proteasomes possessed a molecular weight between 200 and 443 kDa. Gel filtration studies confirmed the molecular weight of precursor proteasomes to be ~300 kDa (data not shown, also see Figure 5B). Interestingly, anti-PSMA4 (C9) immunoprecipitated complexes present in two peaks: fraction 7 and fraction 9. The predominant bands in fraction 7 were higher in molecular weight, consistent with bands of unprocessed precursor β subunits, whereas the predominant bands in fraction 9 were in the lower molecular weight range, consistent with bands of processed β subunits. Hence, anti-PSMA4 (C9) recognized precursor proteasome complexes in fraction 7 and mature proteasomes in fraction 9. The molecular weight analysis of precursor proteasomes strongly suggested that they are composed of two rings: one containing all the α subunits and the other containing some β precursors.

Immunoprecipitates of each fraction with anti-PSMB9 (LMP2) and anti-PSMA3 (C8) failed to demonstrate peptidase activity in any of the fractions; however, a peak of peptidase activity was present in anti-PSMA4 (C9) immunoprecipitates of fraction 9 (data not shown), indicating that proteasomes were responsible for this activity. The fact that precursor proteasomes are catalytically inactive is consistent with previous reports (Frentzel *et al.*, 1994; Yang *et al.*, 1995) and the prediction that only proteasomes containing processed β subunits are catalytically active due to the presence of the important amino-terminal threonine (Seemüller *et al.*, 1995). Therefore, the reported proteolytic activity of precursor proteasomes (Svensson *et al.*, 1996) is likely to be due to contaminating protease(s).

Precursor proteasomes are found in high amounts in H6 cells but not in spleen and liver

Proteasomes are abundant in cells, representing 0.5–1% of total cellular proteins (Hendil, 1988; Tanaka and Ichihara, 1989). The experiments described thus far analyzed newly synthesized precursor and mature proteasomes; therefore, we felt it important to compare their steady-state levels. Homogenates of H6 cells grown in the presence of IFNγ were separated by sucrose density ultracentrifugation and the presence of individual subunits monitored by SDS–PAGE and immunoblotting. The Suc-LLVY-MCA peptidase activity in individual fractions was similar to that shown in Figure 4; fraction 9, presumably containing mature proteasomes, displayed maximum activity (data not shown). As shown in Figure 5A, PSMA4 (C9) was detected in fractions 6–10. However, precursor PSMB8 (LMP7) was present only in fraction 7 whereas processed PSMB8 (LMP7) was detected in fractions 7–10, with the highest amount present in fraction 9. Similarly, precursor PSMB9 (LMP2) peaked in fraction 7 whereas the processed form peaked in fraction 9. Therefore, precursor proteasomes migrated predominantly in fraction 7 whereas fraction 9 contained mature proteasomes, similar to the results in Figure 4. The fact that precursor β subunits could be detected from H6 homogenates in fraction 7 suggested that precursor proteasomes are present in large amounts in steady-state conditions. Also, the observation that the amount of precursor PSMB9 (LMP2) relative to its processed form was greater than that of precursor PSMB8 (LMP7) relative to its processed form suggested that precursor LMP2 was present in greater amounts than precursor LMP7. This observation is consistent with the long half-life of precursor PSMB9 (LMP2) relative to precursor PSMB8 (LMP7) in precursor proteasomes, as suggested by pulse–chase experiments (Figure 3A). Therefore, it is possible that in steady-state conditions precursor proteasomes contain more precursor β subunits that are incorporated earlier, e.g. PSMB9 (LMP2), than precursor β subunits that are incorporated later, e.g. PSMB8 (LMP7).

To quantitate precursor and mature proteasomes, equal amounts of protein from liver, spleen, or uninduced or

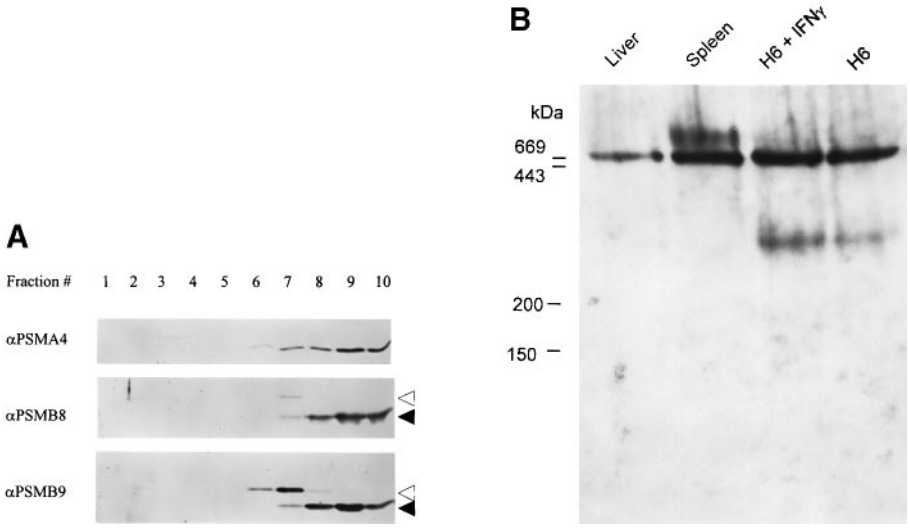


Fig. 5. Precursor proteasomes are present in high amounts in H6 cells but not in spleen and liver. (**A**) Homogenates of H6 cells grown in the presence of IFNγ (30U/ml) were separated by sucrose density ultracentrifugation. Fractions were collected and the presence of PSMA4 (C9), PSMB8 (LMP7) and PSMB9 (LMP2) detected by SDS–PAGE and immunoblotting. The positions of precursor (△) and processed (▲) β subunits are indicated. (**B**) H6 cells, grown in the absence or presence of IFNγ (30 U/ml) for 4 days, splenic and liver tissues were homogenized and cellular debris removed by centrifugation. Equal amounts of protein (60 μg) were loaded on 4.5% native gels and run at 10 mA until the dye front reached the bottom of the gel. Proteins were transferred onto Immobilon-P membrane, probed using anti-PSMA4 (C9) followed by goat anti-rabbit Ig conjugated to peroxidase, then detected using chemiluminescence. Precursor proteasomes and mature proteasomes were quantified using the same procedure, but different amounts of protein were loaded and ^{125}I-labeled donkey anti-rabbit Ig was used in the final step. The blots were quantified by phosphorimager analysis and the pixel values were plotted against the various amounts of proteins used. Points in the linear range of detection were selected, and the differences in the slopes of precursor and mature proteasomes were used to quantify the relative amounts (Harrer *et al.*, 1995). The ratio of proteasomes to precursor proteasomes was 4.01 ± 1.00 for H6 cells grown in the absence of IFNγ and 2.87 ± 0.81 in H6 cells grown in the presence of IFNγ. The data represent three different protein preparations and six independent experiments.

IFNγ-induced H6 homogenates were separated by native gel electrophoresis and electroblotted. Western analysis was performed using anti-PSMA4 (C9) and visualized by chemiluminescence (Figure 5B). Two bands of reactivity were observed in H6 cells. One migrated at ~700 kDa, presumably corresponding to mature proteasomes, and the other at ~300 kDa, presumably corresponding to precursor proteasomes. Liver and splenic tissue showed only the mature proteasome band. In splenic tissue, a minor amount of reactivity was observed with a band that had a molecular weight higher than that expected for mature proteasomes; however, this band was not reproduced in two other experiments. 26S proteasomes were not identified in our Western blots, perhaps due to the low amounts of these complexes in the absence of ATP in our buffers (Orino *et al.*, 1991). The relative amounts of proteasomes and precursor proteasomes were determined using quantitative Westerns (Harrer *et al.*, 1995). Proteasomes were 4.01- and 2.8-fold more abundant than precursor proteasomes in H6 cells grown in the absence and presence of IFNγ, respectively. Precursor proteasomes appear, therefore, to be present in substantial amounts in cell lines. The fact that precursor proteasomes are 3- to 4-fold less abundant than mature proteasomes, despite the fact that the half-lives of precursor proteasomes are ~8-fold shorter than mature proteasomes in H6 cells, suggests that conversion of precursor to mature proteasomes may not be a very efficient process.

Discussion

Complexes recognized by anti-PSMA3 (C8) and anti-PSMB9 (LMP2) are precursor proteasomes

Several lines of evidence suggest that the complexes recognized by anti-PSMA3 (C8) and anti-PSMB9 (LMP2) are precursor proteasomes. First, these antisera immunoprecipitate complexes containing α subunits and precursor β subunits, but not processed β subunits. Second, complexes recognized by anti-PSMA3 (C8) and anti-PSMB9 (LMP2) do not possess peptidase activity. Third, the complexes recognized by anti-PSMA3 (C8) have very short half-lives, ~8-fold shorter than those recognized by anti-PSMA4 (C9). Some radioactivity from precursor proteasomes recognized by anti-PSMA3 (C8) chases into mature and long-lived complexes recognized by anti-PSMA4 (C9). Fourth, the molecular weight of complexes recognized by anti-PSMA3 (C8) is ~300 kDa, suggesting that they are composed of only two rings (half-proteasomes), with the α subunits constituting one ring and some or all of the β precursors constituting the other. These observations are consistent with previous reports (Frentzel *et al.*, 1994; Yang *et al.*, 1995) that mammalian proteasome β subunits are incorporated into precursor proteasomes before giving rise to functional 20S proteasomes. Our study is unique, in several different aspects, compared with previous observations. First, we used anti-PSMA3 (C8), which recognizes exclusively most, if not all, precursor proteasomes. It is surprising that anti-PSMA3 (C8) fails to react with mature, proteolytically active proteasomes, although the α type PSMA3 (C8) subunit is present in mature proteasomes. Moreover, after disruption of mature proteasomes into individual subunits by SDS treatment followed by diluting the SDS under conditions that do not allow spontaneous proteasome assembly, anti-PSMA3 (C8) recognized PSMA3 (C8) present in these mature proteasomes (data not shown). These results suggest that anti-PSMA3 (C8) recognizes a conformational-dependent epitope(s) present only in precursor proteasomes. Importantly, these results suggest

that proteasome maturation, i.e. the proteolytic processing of proteasome β subunits and the juxtaposition of two precursor proteasome particles along their β subunit faces, results in conformational changes in an α subunit [PSMA3 (C8)]. This may represent a mechanism by which competition between precursor and mature proteasomes for regulatory subunits that bind to the outer α rings is avoided.

Second, precursor proteasomes are heterogenous, as demonstrated by pre-clearing experiments (Figure 2). Third, we identified the subunits that are present for short or long periods in precursor proteasomes (Figure 3A). The steady-state levels in precursor proteasomes of two precursor β subunits, PSMB8 (LMP7) and PSMB9 (LMP2), agree very well with our predictions (Figure 5A). Fourth, although IFNγ alters the subunit composition of proteasomes, no significant effect is observed on the half-lives and steady-state levels of precursor and mature 20S proteasomes in IFNγ-treated cells. Finally, we suggest that proteasome biogenesis is inefficient because the majority of precursor proteasomes are not converted to mature and long-lived 20S proteasomes in H6 cells.

Differential incorporation of precursor β subunits during proteasome biogenesis

We found that the half-life of some precursor β subunits is short in complexes recognized by four antibodies to different proteasome subunits [anti-PSMA3 (C8), anti-PSMA4 (C9), anti-PSMB9 (LMP2) and anti-proteasomes]. Detecting precursor PSMB4 (N3) is difficult because it is often masked by the migration of PSMA3 (C8) and it is present for a short time in precursor complexes (Cruz *et al.*, 1997). It is very difficult to detect precursor PSMB5 (X/MB1) in complexes immunoprecipitated with anti-PSMA3 (C8) and anti-PSMA4 (C9), and it is possible that its association with these complexes is too short-lived to be visualized. Thus, precursor β subunits PSMB1 (C5), PSMB4 (N3), PSMB5 (X/MB1), PSMB6 (Y/delta) and PSMB8 (LMP7) are present for a shorter time, whereas the remaining precursor β subunits, PSMB7 (Z), PSMB9 (LMP2) and PSMB10 (MECL1), and the non-processed β subunits, PSMB2 (C7-I) and PSMB3 (C10-II), together with all the seven α subunits, are present for longer time intervals in precursor proteasomes. Also, the amount of precursors PSMB5 (X/MB1) and PSMB6 (Y/delta) relative to their processed forms is low, whereas the relative amount of precursors PSMB7 (Z) and PSMB10 (MECL) to their processed forms is high in H6 lysates separated by sucrose density centrifugation and immunoblotted with subunit-specific antisera (data not shown). These data, together with the results in Figure 5A, suggest that of the 10 mammalian β subunits, five precursor β subunits [PSMB7 (Z), PSMB9 (LMP2), PSMB10 (MECL1) and non-processed PSMB3 (C10-II) and PSMB2 (C7-I)] are incorporated at an earlier stage to form incomplete precursor proteasomes (consisting of a ring of seven α and another ring of some precursor β subunits). According to the subunit organization of yeast proteasomes (Groll *et al.*, 1997), it would appear that the precursor β subunits that are incorporated earlier [PSMB2 (C7-I), PSMB3 (C10-II), PSMB7 (Z) or PSMB10 (MECL1) and PSMB9 (LMP2)] are localized next to one another, assuming that the IFNγ-inducible subunits occupy the exact positions of their constitutive counterparts. Therefore, it is likely that incomplete precursor proteasomes consist of a ring of all α subunits and half a ring of precursor β subunits that are adjacent to one another. Further assembly involves the incorporation of the remaining subunits [PSMB1 (C5), PSMB4 (N3), PSMB5 (X/MB1) or PSMB8 (LMP7) and PSMB6 (Y/delta)] into appropriate locations to form complete precursor proteasomes (consisting of a ring of seven α and another ring of seven precursor β subunits).

Thermoplasma α subunits are essential for proteasome assembly and proper folding of β subunits (Zwickl *et al.*, 1994; Seemüller *et al.*, 1996). When expressed in *E.coli*, they spontaneously form a ring consisting of seven subunits, suggesting that they have a strong affinity for each other (Zwickl *et al.*, 1994). It is possible that this feature is conserved in eukaryotes, because anti-PSMA3 (C8) and anti-PSMB9 (LMP2) appear to immunoprecipitate all of the α subunits. As shown in our model for the assembly of proteasomes (Figure 6A), the seven eukaryotic α subunits, similarly to *Thermoplasma*, may form a ring that initiates the assembly of precursor β subunits. This is followed by the incorporation of precursor β subunits PSMB2 (C7-I), PSMB3 (C10-II), PSMB7 (Z) or PSMB10 (MECL1) and PSMB9 (LMP2) to form incomplete precursor proteasomes. Subsequently, other precursor β subunits, notably PSMB1 (C5), PSMB4 (N3), PSMB5 (X/MB1) or PSMB8 (LMP7) and PSMB6 (Y/delta), bind incomplete precursor proteasomes to generate complete precursor proteasomes. Two complete precursor proteasomes dimerize either before or shortly after processing of their β subunits to produce catalytically active 20S proteasomes. The observations that the half-lives of precursor β subunits that are incorporated later into precursor proteasomes are short (Figure 3A) and their amounts are low in steady-state levels (Figure 5A) supports the hypothesis that the transition from complete precursor proteasomes to mature proteasomes is rapid. Due to the loss of the anti-PSMA3 (C8) conformational epitopes in the later stages of proteasome maturation, we cannot determine the order of dimerization and processing. The suggestion that the two halves of proteasomes associate during assembly before processing of β subunits derives from two observations. First, mutations in the yeast β subunit, Pre1, are rescued by compensatory mutations in another subunit, Doa3, which are presumed to contact each other on opposite β rings (Chen and Hochstrasser, 1996). Second, purified *Thermoplasma* α and precursor β subunits assemble into proteasome-like particles *in vitro*, and only a subpopulation of these contain processed β subunits (Seemüller *et al.*, 1996). Therefore, most probably two complete mouse precursor proteasomes dimerize followed by the autocatalytic processing of β subunits, resulting in the formation of catalytically active mature proteasomes. Recent evidence suggests that the processing of a mammalian β subunit, PSMB9 (LMP2), occurs in two steps: first, the prosequence length is reduced to about eight amino acid residues, followed by the removal of the residual prosequence, resulting in exposure of the amino-terminal threonine residue (Schmidtke *et al.*, 1996). Other cellular processes may also be important in proteasome biogenesis: mouse proteasome formation requires constant protein synthesis, as suggested by the ability of cycloheximide to slow proteasome formation (Frentzel *et al.*,

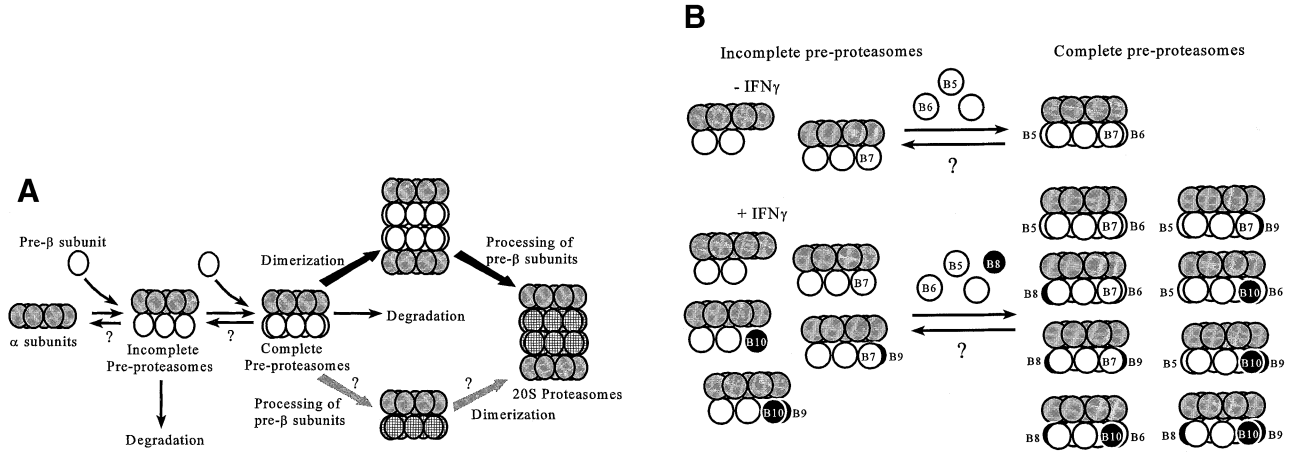


Fig. 6. A model for the assembly of mouse proteasomes. The incorporation of IFNγ-inducible subunits into complete precursor proteasomes results in eight different complexes. (**A**) Proteasome assembly may be initiated by the formation of a ring containing all seven α subunits as reported in *Thermoplasma* (Zwickl *et al.*, 1994). PSMB2 (C7-I), PSMB3 (C10-II), PSMB7 (Z) or PSMB10 (MECL1), and PSMB9 (LMP2) precursor β subunits are incorporated first to form incomplete precursor proteasomes, which is followed by the incorporation of PSMB1 (C5), PSMB4 (N3), PSMB5 (X/MB1) or PSMB8 (LMP7), and PSMB6 (Y/delta) to form complete precursor proteasomes. Most likely, these dimerize followed by the autocatalytic processing of the β subunits, resulting in the formation of catalytically active proteasomes. However, we cannot rule out the possibility that processing of some β subunits occurs before dimerization. Although processed β subunits incorporated into mature proteasomes cannot be exchanged (Aki *et al.*, 1994), it is not clear if this is also true for precursor β subunits present in precursor proteasomes. Precursor proteasomes that are not converted to mature 20S proteasomes are probably targeted for degradation. α subunits are shaded in gray, precursor β subunits are colorless and processed β subunits are hatched. (**B**) In the absence of IFNγ, one type of complete precursor proteasome is possible, whereas in the presence of the IFNγ-inducible subunits, eight different precursor proteasomes are possible. PSMB7 (Z) or PSMB10 (MECL1) and PSMB9 (LMP2) are present in incomplete precursor proteasomes, and PSMB5 (X/MB1) or PSMB8 (LMP7) and PSMB6 (Y/delta) are incorporated at a later stage to form complete precursor proteasomes. The arrangement of the β subunits shown is based on the crystal structure of the yeast proteasome (Groll *et al.*, 1997).

1994), and studies with okadaic acid have suggested a role for dephosphorylation in this process (Yang *et al.* 1995).

The fact that seven different α and 10 different β subunits incorporate into proteasome complexes complicates the assembly of mammalian proteasomes. Electron microscopy and image analysis of mammalian proteasomes have suggested that the subunits are organized precisely within proteasome complexes and that the IFNγ-inducible subunits occupy the exact positions occupied by the constitutive subunits that they replace (Kopp *et al.*, 1993, 1995, 1997; Peters *et al.*, 1993). It is interesting to note the order of incorporation of the six catalytically important mammalian subunits: PSMB9 (LMP2), PSMB10 (MECL1) and PSMB7 (Z) precursors are present for a long time, whereas the PSMB6 (Y/delta), PSMB8 (LMP7), PSMB5 (X/MB1) precursors are probably present for a short time in precursor proteasomes. The effect of IFNγ on precursor proteasomes is summarized in our model (Figure 6B). In the absence of IFNγ, incomplete precursor proteasomes containing precursor PSMB7 (Z) are first formed and PSMB5 (X/MB1) and PSMB6 (Y/delta) precursors are incorporated later to form a single type of complete precursor proteasome. In the presence of IFNγ, PSMB8 (LMP7), PSMB9 (LMP2) and PSMB10 (MECL1) are produced, and incomplete precursor proteasomes contain PSMB9 (LMP2) and PSMB10 (MECL1) precursors, with some complexes also containing precursor PSMB7 (Z). It is interesting that PSMB10 (MECL1) and its constitutive counterpart, PSMB7 (Z), are both incorporated earlier in the assembly process, but probably into distinct particles, since they are presumed to occupy the same location within any given particle. PSMB6 is incorporated into precursor proteasomes lacking PSMB9 (LMP2) at a later stage, together with PSMB5 (X/MB1) or PSMB8 (LMP7) to form as many as eight different types of complete precursor proteasome complexes. These may dimerize at random, resulting in up to 36 different proteasome complexes (Nandi *et al.*, 1996a). IFNγ induces subunit replacement during the assembly of proteasomes (Aki *et al.*, 1994) and, according to our model (Figure 6B), the early incorporation of the IFNγ-inducible subunits, PSMB9 (LMP2) and PSMB10 (MECL), will result in the majority of precursor proteasomes containing these subunits at the expense of their constitutive counterparts, PSMB6 (Y/delta) and PSMB7 (Z), in cells lines or tissues strongly expressing the IFNγ-inducible subunits. Although eight different precursor proteasome complexes are theoretically possible, the incorporation of PSMB9 (LMP2) and PSMB10 (MECL) may facilitate the incorporation of PSMB8 (LMP7) or impede the incorporation of PSMB5 (X/MB1) to form complete precursor proteasomes containing primarily IFNγ-indicible subunits. Indeed, there is some evidence to suggest that the incorporation of one subunit affects the incorporation of another (Gaczynska *et al.*, 1996; unpublished observations). This mechanism may explain the reduced amounts of PSMB5 (X/MB1), PSMB6 (Y/delta) and PSMB7 (Z) in 20S proteasomes isolated from cells stimulated with IFNγ (Groettrup *et al.*, 1996b) or tissues containing high levels of the IFNγ-inducible subunits (Stohwasser *et al.*, 1997).

Proteasome biogenesis may be inefficient in rapidly dividing cells

The half-life of complexes recognized by anti-PSMA3 (C8) is 5- to 14-fold longer than that of unincorporated precursor β subunits PSMB1 (C5), PSMB6 (Y/delta) and PSMB8 (LMP7) (data not shown) and is ~10-fold longer than the half-life of precursor PSMB4 (N3) (Thompson

and Rivett, 1996). Also, the steady-state levels of unincorporated or free α and precursor β subunits are probably very low because they are not detected by Western analysis of H6 homogenates separated by sucrose density ultracentrifugation (Figure 5A). These results are consistent with the notion that subunits that are not incorporated into complexes are degraded rapidly (Olson and Dice, 1989). The half-life of complete precursor proteasomes may be shorter than the overall half-life of complexes recognized by anti-PSMA3 (C8) ($t_{0.5}$ = ~5 h), as indicated by the short-lived presence of some precursor β subunits. The short half-life of complete precursor proteasomes recognized by anti-PSMA3 (C8) is at least in part a result of conversion of precursor proteasomes into mature proteasomes. However, as observed in Figure 3B, the majority of precursor proteasomes recognized by anti-PSMA3 (C8) are not converted to mature and long-lived proteasomes recognized by anti-PSMA4 (C9) and probably are targeted for degradation. The half-life of precursor proteasomes recognized by anti-PSMA3 (C8) ($t_{0.5}$ = ~5 h) may be somewhat longer than that observed by another group (Yang *et al.*, 1995), whereas the half-life of proteasomes in H6 cells is shorter than that reported for HeLa cells (Hendil, 1988). These discrepancies could be due to the use of different cell lines and antibodies that may recognize different proteasome subsets. Proteasomes are thought to be degraded in lysosomes (Cuervo *et al.*, 1995) and it will be interesting to determine whether this is also true of precursor proteasomes.

Precursor proteasomes are present in high amounts in rapidly growing H6 cells, only 3- to 4-fold less abundant than proteasomes, although they could not be detected in quiescent tissue, e.g. spleen and liver. However, the half-lives of liver proteasomes are between 8 and 16 days (Tanaka and Ichihara, 1989; Cuervo *et al.*, 1995) compared with ~2 days in H6 cells. Therefore, normal cells containing proteasomes with long half-lives probably produce low levels of precursor proteasomes that could not be detected at the level of sensitivity of our Western experiments. As suggested previously, it is possible that rapidly growing cells turn over proteasomes faster than quiescent cells (Tanaka, 1994; Ichihara and Tanaka, 1995), resulting in increased synthesis of precursor proteasomes. The high levels of precursor proteasomes present in rapidly growing cells may result in part from the inability of all complete precursor proteasomes to form catalytically active proteasomes, as has been suggested for the formation of *Thermoplasma* proteasomes *in vitro* (Seemüller *et al.*, 1996).

In summary, we have identified the precursor β subunits that are incorporated earlier or later during the assembly of mouse proteasomes. We have also provided a model on how the early incorporation of two of the IFNγ-inducible subunits, PSMB9 (LMP2 and PSMB10 (MECL1), may lead to the biased generation of 20S proteasomes containing the IFNγ-inducible subunits at the expense of their constitutive counterparts. Further work is required to investigate whether the incorporation of a particular precursor β subunit enhances or impedes the incorporation and processing of other β subunits, and whether differential processing of β subunits occurs during proteasome maturation (Thompson and Rivett, 1996). The precursor proteasome-specific anti-PSMA3 (C8) reagent described here should prove to be useful in addressing these issues in future studies.

Materials and methods

Cell culture

P388D1 (macrophage, *H-2^d*) and H6 cells (hepatoma, *H-2^a*) were cultured in RPMI 1640 media supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine at 37°C in the presence of 5% CO_2, with or without 30 U/ml of IFNγ (Boehringer Mannheim) for 4 days.

Antisera production

The generation of antisera to PSMB8 (LMP7) and PSMB9 (LMP2) has been described previously (Nandi *et al.*, 1996b). Mouse *PSMA3 (C8)* (residues 1–255) (E.Woodward and J.J. Monaco, in preparation) and rat *PSMA4 (C9)* (residues 25–261) (Kumatori *et al.*, 1990) cDNAs were cloned into PGEX vectors (Pharmacia, NJ). Bacteria containing *PGEX-2-PSMA4 (C9)* or *PGEX-3-PSMA3 (C8)* were induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the resulting GST fusion proteins were purified on glutathione–agarose beads (Frangioni and Neel, 1993). New Zealand White rabbits were immunized with purified GST–PSMA4 (C9) fusion protein in Freund's adjuvant (Sigma), or GST–PSMA3 (C8) in Titermax (Vaxcel). Sera were collected 1 week after boosts and titered before use.

Metabolic labeling, immunoprecipitations and electrophoresis

Cells were washed and labeled with 0.5–1 mCi of [^{35}S]methionine/cysteine (Amersham or Dupont-NEN) at 37°C for 45 min to 4 h, as indicated, in RPMI medium deficient in methionine and cysteine, with 10% dialyzed FCS, 2 mM glutathione, penicillin (100 U/ml) and streptomycin (100 μg/ml). After labeling, cells were washed in phosphate-buffered saline (PBS) containing 2 mM methionine and 0.1% sodium azide and solubilized in 0.5% NP-40 in Tris-buffered saline for 30 min at 4°C. Insoluble material was removed by centrifugation at 12 000 *g* for 10 min. Lysates were pre-cleared with normal rabbit serum and incubated with the respective antisera for 30 min at 4°C, followed by the addition of 150 μl of 10% *Staphylococcus aureus* suspension (Calbiochem, CA). Immune complexes were washed and resuspended in either SDS sample buffer or NEPHGE sample buffer (Brown *et al.*, 1993). For one-dimensional SDS–PAGE, samples were boiled and loaded on 11% gels. 2D-NEPHGE–PAGE and fluorography were performed as previously described (Brown *et al.*, 1993).

Sucrose gradient fractions and peptidase assay

H6 cells were Dounce homogenized in buffer A (20 mM Tris–HCl, pH 7, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM β-mercaptoethanol and 1% glycerol) followed by removal of insoluble debris by centrifugation at 16 000 *g* for 10 min. The supernatant was layered on top of a 10–40% linear sucrose gradient containing 20 mM Tris–HCl, pH 7, 1 mM $MgCl_2$, 0.1 mM EDTA, 1 mM β-mercaptoethanol and 1% glycerol and centrifuged at 285 000 *g* for 16 h at 2°C using a Beckman SW40 Ti rotor. Fractions (1 ml) were collected and used for immunoprecipitations or peptidase assays. Peptidase assays were performed as follows: 5 μl from each fraction were incubated in buffer containing 50 mM Tris–HCl, pH 8, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT) and 300 μM Suc-LLVY-MCA (Sigma), a fluorogenic peptide known to be cleaved by proteasomes, in a total volume of 50 μl. After incubation at 37°C for 45 min, the reaction was quenched with 150 μl of cold 100% ethanol and fluorescence measured in a Cytofluor 2350 apparatus (Millipore).

Native gel electrophoresis and immunoblotting

Cells or tissue were Dounce homogenized in buffer A and centrifuged to remove insoluble debris. The supernatant was removed and glycerol added to a final concentration of 10%. Protein concentration was determined using a protein assay kit (Bio-Rad) and various amounts of protein were mixed with 2× native gel buffer and loaded on 4.5% native gels, as described in the non-denatured protein molecular weight marker kit (Sigma). Gels were run at 10 mA at 4°C until the dye front reached the bottom of the gel. Proteins were transferred onto Immobilon-P membranes (Millipore). Western analysis was performed using anti-PSMA4 (C9), anti-PSMB8 (LMP7) or anti-PSMB9 (LMP2) (1:1000 dilution) and chemiluminescence using ECL (Amersham). For quantitative Westerns, ^{125}I-labeled donkey anti-rabbit Ig (Amersham) was

used as secondary antibody and the bands were quantified using a phosphorimager (Molecular Dynamics).

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