Novel Propeptide Function in 20 S Proteasome Assembly Influences β Subunit Composition*

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The assembly of eukaryotic 20 S proteasomes involves the formation of half-proteasomes where precursor β-type subunits gather in position on an α-subunit ring, followed by the association of two half-proteasomes and β-subunit processing. In vertebrates three additional β-subunits (β1i/LMP2, β2i/MECL1, and β5i/LMP7) can be synthesized and substituted for constitutive homologues (β1/delta, β2/Z, and β5/X) to yield immunoproteasomes, which are important for generating certain antigenic peptides. We have shown previously that when all six β-subunits are present, cooperative assembly mechanisms limit the diversity of proteasome populations. Specifically, LMP7 is incorporated preferentially over X into preproteasomes containing LMP2 and MECL1. We show here that the LMP7 propeptide is responsible for this preferential incorporation, and it also enables LMP7 to incorporate into proteasomes containing delta and Z. In contrast, the X propeptide restricts incorporation to proteasomes with delta and Z. Furthermore, we demonstrate that the LMP7 propeptide can function in trans when expressed on LMP2, and that its NH2-terminal and mid-regions are particularly critical for function. In addition to identifying a novel propeptide function, our results raise the possibility that one consequence of LMP7 incorporation into both immunoproteasomes and delta/Z proteasomes may be to increase the diversity of antigenic peptides that can be generated.

Proteasomes are multisubunit, multicatalytic proteases responsible for the majority of non-lysosomal protein degradation in eukaryotic cells (1). They recognize and degrade ubiquitinated proteins, including those that are misfolded or damaged, as well as other regulatory proteins targeted for rapid turnover (2). Additionally, they are responsible for generating the majority of peptides whose length and COOH-terminal residue are better suited for binding to major histocompatibility complex class I molecules (16), and/or they increase the repertoire of peptides produced from a given antigen, and thus augment an immune response.

In the fully assembled 20 S proteasome, each α- and β-subunit occupies a defined location relative to other subunits (7, 17). Although the mechanism and orchestration of assembly is not well understood, it appears to occur in at least two stages, the first being the formation of a stable preproteasome complex (13–16 S) composed of an intact seven-member α-ring with at least three β subunits (Z/β2, C10-II/β3, and C7-I/β4) (18, 19). Once the β ring is complete, two half-proteasomes dimerize at the β ring interface to form a 20 S complex. In yeast (and probably also in vertebrates (20)), assembly is facilitated by ump1p, a constituent of the preproteasome complex that is degraded once assembly is complete (21). In mammalian cells an added level of complexity results from the presence of IFN-γ-inducible catalytic β subunits. If assembly were random as many as 36 different proteasome subsets could form (22). Instead, the IFN-γ-inducible subunits are incorporated cooperatively resulting predominantly (but not exclusively) in homogeneous subsets of immunoproteasomes and constitutive proteasomes (23). Cooperative assembly appears to involve co-incorporation of LMP2 (β1i) and MECL1 (β2i) into preproteasomes (24) with β3 and β4 (19), thus excluding delta (β1) and Z (β2), with subsequent preferential incorporation of LMP7 (β5i) rather than X (β5) (23). Since LMP2 and MECL1 are adjacent in the β ring their co-incorporation may be a result of physical interaction (25). However, the mature LMP7 subunit does not contact LMP2 or MECL1, either across one β ring or the interface of the two β rings, raising the question of how it is preferentially incorporated.

Each proteolytic β subunit is expressed with an NH2-terminal propeptide that is removed autocatalytically during the final stages of assembly (26, 27). Propeptide removal frees the active site NH2-terminal nucleophile allowing the subunit to become proteolytically active. Thus, one proposed function of the propeptide is to prevent catalytic activity before the active

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site can be sequestered in the proteasome core. The propeptide also protects the NH₂-terminal from acetylation and hence inactivation (28), and may serve as an intramolecular chaperone facilitating subunit folding (29).

The β common propeptide is particularly important for proteasome assembly. In yeast, where there is only one β subunit, deletion of its propeptide disrupts assembly and ultimately viability (26). Interestingly, viscosity is restored when ump1p is deleted, suggesting that an interaction between the β common propeptide and ump1p is normally required for assembly to proceed (21). Similarly, in mammalian cells LMP7 (β5i) is not incorporated into proteasomes without its propeptide (30). Furthermore, a variant of LMP7 (LMP7E1) which differs from the predominant form (LMP7E2) only in its propeptide (31), is not incorporated (32). In the work presented here, we explore the role of β common propeptides in the assembly of mammalian proteasome populations. We define two regions of the LMP7 propeptide critical for efficient incorporation of LMP7, and show that this propeptide can function "in trans" when fused to the mature LMP2 subunit. Most importantly, we provide evidence that differences between the LMP7 (β5i) and X (β5) propeptides influence which population of assembling proteasomes will incorporate each subunit.

EXPERIMENTAL PROCEDURES

Cell Cultures and Antibodies—Lymphoblastoid T2 cells (0.174 × CEIP) (33) obtained from P. Cresswell (Yale University, New Haven, CT) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, t-glutamine, and antibiotics as described (23). TCP21 is a mouse monoclonal antibody that recognizes human C3 and immuno-precipitates both 20 S proteasomes and 13-16 S preproteasomes (34). The hybridoma was obtained from the European Collection of Animal Cell Cultures (Salisbury, Wiltshire, UK). Polyclonal antisera recognizing LMP2 and LMP7 were from rabbits immunized with recombinant mouse subunits (19). Anti-X (PS3250) is a rabbit polyclonal antiserum raised against human X, and was obtained from K. B. Hendil (August Krogh Institute, University of Copenhagen, Copenhagen, Denmark).

DNA Constructs and Transfection—The cDNAs encoding human LMP7 (LMP7E2) and LMP7E1 were expressed using the episomal vector pCEP9 (neomycinr), as described (23). pCEP9 was transfected into T2 cells using this new expression vector. DNA Constructs and Transfection—The cDNAs encoding human LMP7 (LMP7E2) and LMP7E1 were expressed using the episomal vector pCEP9 (neomycinr), as described (23). MCP21 is a mouse monoclonal antibody that recognizes human C3 and immuno-precipitates both 20 S proteasomes and 13-16 S preproteasomes (34). The hybridoma was obtained from the European Collection of Animal Cell Cultures (Salisbury, Wiltshire, UK). Polyclonal antisera recognizing LMP2 and LMP7 were from rabbits immunized with recombinant mouse subunits (19). Anti-X (PS3250) is a rabbit polyclonal antiserum raised against human X, and was obtained from K. B. Hendil (August Krogh Institute, University of Copenhagen, Copenhagen, Denmark).

DNA Constructs and Transfection—The cDNAs encoding human LMP7 (LMP7E2) and LMP7E1 were expressed using the episomal vector pCEP9 (hygromycin) (Invitrogen, Carlsbad, CA), and LMP2 was expressed using pSG5 (Stratagene, La Jolla, CA), as described (23). Subcloned fragments of each cDNA were inserted into pSG5 (neomycinr), as described (23). pCEP9 is a mouse monoclonal antibody that recognizes human C3 and immuno-precipitates both 20 S proteasomes and 13-16 S preproteasomes (34). The hybridoma was obtained from the European Collection of Animal Cell Cultures (Salisbury, Wiltshire, UK). Polyclonal antisera recognizing LMP2 and LMP7 were from rabbits immunized with recombinant mouse subunits (19). Anti-X (PS3250) is a rabbit polyclonal antiserum raised against human X, and was obtained from K. B. Hendil (August Krogh Institute, University of Copenhagen, Copenhagen, Denmark).

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Role of β-Subunit Propeptides in Proteasome Assembly

Fig. 1. Incorporation of LMP7 propeptide deletion mutants into proteasomes. A, proteasomes were immunoprecipitated from untransfected T2 cells (T2), or T2 expressing various forms of LMP7 either alone (lane 1 of each group), or with pro7.LMP2 (lane 2), or LMP2 (lane 3). Proteasome subunits were separated by SDS-PAGE, and LMP7 visualized by immunoblotting with anti-LMP7 antisera. LMP7 propeptide deletion mutants are designated by Δ followed by the number of the first residue following Met in the encoded sequence (upper panel). A nonspecific band typically seen on immunoblots of MCP21 immunoprecipitates is indicated (*). B, His-tagged proteasomes were nickel precipitated from T2 cells co-expressing His-tagged LMP2 (LMP2.His) with various forms of LMP7. Separation and immunoblotting of proteasome subunits was performed as in A. The positions of precursor forms of LMP7 are indicated (arrowheads). Each lane represents material immunoprecipitated or nickel precipitated from 5 × 10⁶ cells.

Incorporation of LMP7 propeptide deletion mutants into proteasomes. A, the NH₂-terminal methionine codon was preserved in all constructs. Each deletion mutant was expressed alone and with LMP2 in T2 cells, which lack endogenous LMP2 and LMP7 (33, 35). Proteasomes were immunoprecipitated from cell lysates using MCP21, a monoclonal antibody that recognizes C3, a proteasome α-type subunit (34). Following immunoprecipitation, proteasomes were denatured and subunits separated by SDS-PAGE. The presence of specific subunits was determined by immunoblotting with subunit-specific antisera. Large deletions of the propeptide (to residue +1, −3, or −35) dramatically reduce LMP7 incorporation. This is apparent when these altered forms of LMP7 are either expressed alone (Fig. 1A, lane 1 of each group), or when co-expressed with LMP2 (lane 3 of each group). In contrast, removal of a smaller region (to −70 or −49) does not prevent incorporation, although the efficiency is reduced compared with full-length LMP7. Deletion of two or four amino acid residues to −69 and −67, respectively, has an effect similar to removal of a single residue (to −70) (data not shown).

The LMP7 subunit is incorporated preferentially over X into proteasomes containing LMP2 and MECL1 (23). It is also incorporated into delta/Z proteasomes when LMP2 is absent. That this latter population might also form in the presence of LMP2 is suggested by pre-clearing experiments using anti-LMP2 (36), and greater overall incorporation of LMP7 when co-expressed with LMP2 (lane 3 of each group). Furthermore, this form of LMP2 is incorporated (data not shown), and replaces the constitutive homologous subunit (delta), as shown previously by Schmidtko et al. (27). These results demonstrate that the LMP7 propeptide can function in trans when expressed on another β subunit. Its inability to function as a separate polypeptide may be due to ineffective competition with the endogenous β5 subunit (X). We have attempted to express LMP2, truncated LMP7, and the LMP7 propeptide together so that a pool of preproteasomes dependent on LMP7 for maturation would be created. However, these experiments have not been successful, at least in part due to difficulties obtaining stable triple transfectants.

The NH₂-terminal Region Is Important for Propeptide Function—The two forms of LMP72 that result from alternative first exon use (LMP7E1 and LMP7E2) encode polypeptides that differ only in the NH₂-terminal two thirds of their propeptides (Fig. 2A), yet LMP7E1 is not incorporated into proteasomes (23, 32). The NH₂-terminal regions of the two functional β5 propeptides (LMP7E2 and X) are homologous, and yet quite different from the same region of LMP7E1 (Fig. 2A). Conse-
The LMP7 Propeptide Facilitates Incorporation of X into Proteasomes—Differential Functional Specifications

The X Propeptide Restricts Incorporation of LMP7 to Primarily Delta-Containing Proteasomes—Since the propeptide of LMP7 appears to be the primary determinant allowing incorporation of β5 into LMP2 and delta-containing proteasomes, it seemed likely that the X propeptide would restrict incorporation to delta-containing proteasomes. To address this, we constructed LMP7 with an X propeptide (proX.LMP7), and ex-
pressed it in the presence or absence of LMP2. When expressed alone proX.LMP7 is incorporated into proteasomes at a level comparable to wild-type LMP7 (Fig. 4, lane 2 versus lane 4). However, unlike full-length LMP7, proX.LMP7 does not enhance LMP2 incorporation (Fig. 4, lane 3 versus lane 5). In addition, proX.LMP7 incorporation is not increased as is typically seen with LMP7 when it is co-expressed with LMP2. This implies that while proX.LMP7 is competent to incorporate into delta-containing proteasomes, it is not capable of efficiently allowing the maturation of LMP2-containing proteasomes. These results suggest that the X propeptide restricts the δ5 subunit to primarily constitutive proteasomes, preventing it from being incorporated into immunoproteasomes.

Quantitative Immunoblotting—To obtain a more quantitative estimate of the effect of the δ5 propeptide on immunoproteasome assembly as judged by LMP2 incorporation, an ECF substrate was used for immunoblotting (see “Experimental Procedures”). LMP2 was co-expressed with His-tagged X or LMP7 containing different δ5 propeptides as indicated. His-tagged proteasomes were isolated by nickel precipitation (3 successive rounds), and remaining proteasomes immunoprecipitated with MCP21. Proteasome subunits were separated and immunoblotted with subunit-specific antisera. Precipitates from 7 × 10^6 cells were used for the X and C3 immunoblots, while 10^6 cells were used for the LMP2 immunoblots.

Fig. 3. The LMP7 propeptide allows X to incorporate into LMP2-containing proteasomes. A, proteasomes were immunoprecipitated from T2 cells expressing LMP2 alone (−), or co-expressing LMP2 with X or pro7.X. Material from 16 × 10^6 cells was separated and immunoblotted with anti-LMP2. B, His-tagged proteasomes were isolated from T2 cells co-expressing LMP2 and X.His (left panel) or LMP2 and pro7.X.His (right panel), by three successive nickel precipitations (lanes 1–3). Remaining material was immunoprecipitated with MCP21 (IP). Proteasome subunits were separated and immunoblotted with subunit-specific antisera. Precipitates from 7 × 10^6 cells were used for the X and C3 immunoblots, while 10^6 cells were used for the LMP2 immunoblots.

The δ5 Propeptide Allows X to Compete Effectively with LMP7 for Incorporation.—To further address the importance of the δ5 propeptide, we expressed His-tagged δ5 subunits in T2 cells overexpressing LMP2 and LMP7. Overexpression of these immunoproteasome subunits leads to virtually complete re-

Fig. 4. The X propeptide on LMP7 reduces LMP2 incorporation and prevents increased LMP7 incorporation. Proteasomes were immunoprecipitated from untransfected T2 cells (−), or T2 cells expressing LMP7 without (+) or with (+) LMP2, and proX.LMP7 without (−) or with (+) LMP2. Proteasome subunits were separated and visualized by immunoblotting with subunit-specific antisera. Material from the first nickel precipitation (Ni) and the immunoprecipitation (IP) is shown. Relative chemiluminescence was quantitated as described under “Experimental Procedures,” and is expressed as the total number of pixels (volume) for each band after background subtraction using an adjacent gel region (bar graph). Starting material was 13 × 10^6 cells for each cell line.

Fig. 5. Quantitation of the δ5 propeptide effect on LMP7 incorporation. LMP2 was co-expressed with His-tagged X or LMP7 containing different δ5 propeptides as indicated. His-tagged proteasomes were isolated by nickel precipitation (3 successive rounds), and remaining proteasomes immunoprecipitated with MCP21. Proteasome subunits were separated, and LMP2 visualized by immunoblotting with anti-LMP2 antisera and ECF substrate. Material from the first nickel precipitation (Ni) and the immunoprecipitation (IP) is shown. Relative chemiluminescence was quantitated as described under “Experimental Procedures,” and is expressed as the total number of pixels (volume) for each band after background subtraction using an adjacent gel region (bar graph). Starting material was 13 × 10^6 cells for each cell line.

DISCUSSION

Our studies indicate that in mammalian cells, where constitutive and inducible catalytic δ5 subunits can be co-expressed, the δ5 propeptide determines which population of assembling proteasomes will incorporate this subunit. The X propeptide restricts δ5 (X) primarily to constitutive proteasomes containing delta (β1) and Z (β2), whereas the LMP7 propeptide facilitates incorporation of δ5i (LMP7) into immunoproteasomes containing LMP2 (β1i) and MECL1 (β2i), but also permits
incorporation into the population containing delta and Z. Thus, the LMP7 propeptide accounts for the contribution of this subunit to cooperative immunoproteasome assembly (23). These results confirm the previously recognized role of the β5 propeptide as an intermolecular chaperone (26), and further demonstrates for the first time that it influences the differential incorporation of homologous β5 subunits into discrete proteasome subsets. Other important functions of the β5 propeptide may include preventing premature proteolytic activity and active site acetylation (28, 38), as well as assisting subunit folding (intramolecular chaperone) (29).

Several observations indicate that the NH$_2$-terminal and mid-regions of the LMP7 propeptide are necessary for complete function. First, deleting the NH$_2$-terminal half of the propeptide (to Ser$^{56}$) abrogates LMP7 incorporation, while removing one to as many as 23 residues (to Ser$^{65}$) has an intermediate effect. Second, in contrast to NH$_2$-terminal deletions, removing 13 residues from the COOH-terminal region (Pro$^{23}$ through Glu$^{11}$) has no effect. Third, replacing the NH$_2$-terminal region of a non-functional propeptide (LMP7e1), with the comparable region from LMP7e2 or X partially restores function. These deletions and switches of the NH$_2$-terminal region did not reveal a domain conferring proteasome subset specificity, as there was no major effect on differential incorporation of LMP7 into immunoproteasomes versus delta-containing proteasomes. In contrast, full propeptide substitutions reversed the specificity of subunit incorporation. Thus, rather than a particular region of the LMP7 propeptide enhancing incorporation into immunoproteasomes, there may be a region of the X propeptide that negatively affects incorporation into immunoproteasomes. Further experiments will be necessary to identify such a region.

*Rhodococcus* proteasome β-subunit propeptides appear to facilitate subunit folding (29), although a similar function has not been demonstrated for eukaryotic proteasome propeptides. Our propeptide deletion experiments do not distinguish between deleterious effects on subunit folding that could indirectly diminish incorporation, versus removal of a region directly involved in incorporation. The observation that truncated forms of LMP7 can be incorporated when the LMP7 propeptide is expressed in trans (fused to LMP2) could indicate that some of the truncated subunits fold appropriately without the influence of an attached full-length propeptide. However, it is also possible that the propeptide of LMP7 attached to LMP2 could interact with truncated forms of LMP7 in trans and facilitate folding. It is worth noting that any propeptide effect on folding is unlikely to be subunit or propeptide-specific, since switching the LMP7 and X propeptides affects which population will accept the subunit, not whether it can be incorporated. Similarly, LMP2 with an LMP7 (27) or delta$^3$ propeptide can be incorporated. Therefore, while we cannot rule out effects of the β5 propeptide on subunit folding, such an effect is not likely to be responsible for differential subunit incorporation.

Accumulating evidence suggests the β5 propeptide and subunit occupy a singular position in proteasome assembly and function (28, 38). For example, in yeast, the β5 propeptide is required for incorporation of this subunit and for viability (26). In mammals, LMP7 (β5i) is also dependent on its propeptide for incorporation, while less is known about the constitutive subunit X (β5). In contrast, β1 and β2 propeptides are not essential for incorporation or viability in yeast, although perturbations in cell growth are observed when these propeptides are deleted, particularly with β2 (28, 38). Truncated forms of LMP2 (β1i) are incorporated into proteasomes, albeit with reduced efficiency$^3$ (25), while the role of the MECL1 (β2i) propeptide has not been investigated. Although the β5 propeptide influences proteasome subset formation as we have shown here, this is not the case with β1. Replacing the LMP2 (β1i) propeptide with the delta (β1) propeptide does not change its dependence on LMP7 for incorporation, suggesting it still incorporates primarily into immunoproteasomes.$^3$ Furthermore, partial deletion, mutation, or complete truncation of the LMP2 (β1i) propeptide does not significantly affect co-incorporation with MECL1 (β2i), suggesting that this results from interactions between the mature subunits rather than being a function of their propeptides (25). Finally, the β5 propeptide is critical for completion of proteasome assembly in yeast, apparently via interaction with ump1p, a key mediator of this process (21).

Recent studies in yeast demonstrate a hierarchy of importance of the catalytic subunits, with β5 maintaining the most crucial proteolytic activity (38, 39). For example, mutation of β5 affects degradation of ubiquitinated substrates and cell growth more profoundly than mutation of either β1 or β2. Interestingly, studies from knockout mice also support a particularly critical role for β5i (LMP7). Major histocompatibility complex class I expression is reduced (~50%) on lymphoid cells from LMP7-deficient (LMP7$^{-/-}$) mice (40), while it is unchanged in cells from LMP2$^{-/-}$ mice (41). Whether these differences are attributable to the absence of LMP7 alone, or are compounded by reduced LMP2 and MECL1 incorporation in LMP7$^{-/-}$ mice (23), is not clear. However, PA28β$^{-/-}$ mice have normal class I expression despite a defect in immunoproteasome assembly (42). Interestingly, while LMP2 and MECL1 are not incorporated into proteasomes in these mice, LMP7 is incorporated, albeit at reduced levels. Likewise, LMP7 is readily incorporated in LMP2$^{-/-}$ mice (23, 41). The presence of LMP7-containing proteasomes in both PA28β$^{-/-}$ and LMP2$^{-/-}$ mice suggests that high level class I expression may be more dependent on the presence of LMP7 than on immunoproteasomes (e.g. LMP2/MECL1/LMP7) per se. This is consistent with previous findings in vitro where expression of LMP7 without LMP2 increases production of peptides with hydrophobic COOH-terminal residues suitable for class I binding (16, 49).

$^3$T. A. Griffin, D. J. Kingsbury, and R. A. Colbert, unpublished observations.
The evolution of a β5 propeptide that enables the formation of delta/βLMP7 as well as LMP2/MECL1/LMP7 proteasomes raises the question of whether the delta/βLMP7 population is immunologically relevant. This subset is most likely to form when there is incomplete replacement of delta/β by LMP2/MECL1, such as in unstimulated dendritic cells (44) and other lymphoid tissues, or with suboptimal induction by IFN-γ. It is plausible that the primary role of LMP7 may be to increase the overall production of peptides that can be presented by class I molecules, and that further digestion by delta/β active sites in addition to LMP2/MECL1, could enhance the overall diversity of peptides available for presentation.

A major challenge will be to determine how differences in the β5 propeptide sequence confer specificity for incorporation into proteasome subsets. Several mechanisms are worth considering. First, the β5 propeptide is large enough (59–72 amino acids) to directly contact β1 or β2 across the ring, so that specificity could be based on differential interaction with LMP2 or MECL1. This would be unlikely to result from β/β1i propeptide differences, as the nature of the LMP2 propeptide seems to have little effect on the requirement for LMP7 for efficient incorporation.5 A second possibility is the existence of two mammalian homologues of yeast ump1p, with one directly assembling constitutive proteasomes and the other immunoproteasomes. We recently identified human and mouse homologues of ump1p by searching EST data bases (20), and find no evidence for a second related protein, making this prospect seem unlikely. A third possibility is that other accessory proteins are involved in immunoproteasome assembly. In this regard, it was recently shown that PA28a and PA28b associate with preimmunoproteasomes, and in the absence of PA28b (PA28b−/−) mice recovery of these complexes is reduced, as is the incorporation of immunoproteasome subunits (42). This raises the possibility that the role of PA28 in proteasome assembly may involve binding to the α-ring and inducing a conformational change that makes preproteasomes more receptive to LMP2 and/or MECL1. It should be noted that these scenarios are not mutually exclusive, nor do they readily explain why the LMP7 propeptide directs more promiscuous incorporation.

In summary, it appears that the two mammalian β5 propeptides are important for influencing the formation of distinct proteasome subsets. It will be critical in future studies to determine the precise function of each of these subsets including LMP7/delta/β, to establish how IFN-γ-inducible subunits influence the immune response.

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