β2 Subunit Propeptides Influence Cooperative Proteasome Assembly*

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Mita De‡, Krupakar Jayarapu‡, Laura Elenich§, John J. Monaco§, Robert A. Colbert‡, and Thomas A. Griffin‡‡

From the ‡William S. Rowe Division of Rheumatology, Cincinnati Children’s Hospital Medical Center, Cincinnati, Ohio 45229 and the §Department of Molecular Genetics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267

Vertebrate proteasomes are structurally heterogeneous, consisting of both “constitutive” (or “standard”) proteasomes and “immunoproteasomes.” Constitutive proteasomes contain three ubiquitously expressed catalytic subunits, Delta (β1), Z (β2), and X (β5), whereas immunoproteasomes contain three interferon-γ-inducible catalytic subunits, LMP2 (β1i), MECL (β2i), and LMP7 (β5i). We recently have demonstrated that proteasome assembly is biased to promote immunoproteasome homogeneity when both types of catalytic subunits are expressed in the same cell. This cooperative assembly is due in part to differences between the LMP7 (β5i) and X (β5) propeptides. In the current study we demonstrate that differences between the MECL (β2i) and Z (β2) propeptides also influence cooperative assembly. Specifically, replacing the MECL propeptide with that of Z enables MECL incorporation into otherwise constitutive (ΔLMP2/i) proteasomes and facilitates X incorporation into otherwise immunoproteasomes (MECL/i/LMP2i). We also show, using MECL-Δ/ mice, that LMP2 incorporation does not require MECL, in contrast with previous suggestions that their incorporation is mutually dependent. These results enable us to refine our model for cooperative proteasome assembly by determining which combinations of inducible and constitutive subunits are favored over others, and we propose a mechanism for how propeptides mediate cooperative assembly.

Eukaryotic proteasomes are an integral component of ubiquitin-mediated protein degradation, which plays a major role in the turnover of cytoplasmic and nuclear proteins (1–5). By virtue of their role in protein metabolism, proteasomes are involved in a number of cellular processes, including cell cycle control, cellular stress responses, intracellular signaling, and major histocompatibility complex class I antigen processing (6). Immunoproteasomes are a specialized subset of vertebrate proteasomes that contain three interferon-γ-inducible catalytic subunits, LMP2 (β1i), MECL (β2i), and LMP7 (β5i) (7, 8). Immunoproteasomes are thought to possess enhanced capability for generating major histocompatibility complex class I-binding peptides with basic or hydrophobic C termini as compared with constitutive proteasomes, which contain three constitutively synthesized catalytic subunits, Delta (β1), Z (β2), and X (β5) (9–14).

The 20 S catalytic proteasome core is comprised of 28 subunits arranged in four stacked seven-member rings (15–17). Each outer ring contains seven different non-catalytic α-type subunits, α1–α7, and each inner ring contains seven different β-type subunits, β1–β7 (18), at least three of which are catalytic (β1 or β1i, β2 or β2i, and β5 or β5i). The N-terminal proteolytic active sites are on the inner surface of the β rings, whereas the C termini of β subunits are on the outer surface of proteasomes (19–21), enabling us to use C-terminal “epitope tags” to immunoprecipitate and track specific subunits because these tags do not appear to interfere with proteasome structure or catalytic activity. For example, attaching green fluorescent protein to the C terminus of LMP2 did not interfere with LMP2 incorporation or proteolytic function (22).

Proteasome assembly is a slow process that involves detectable intermediate complexes with half-lives of several hours (such as 5–6 h in H6 cells) (27). These “preproteasome” intermediates contain one complete α ring, an incomplete and variable complement of unprocessed β subunits, and an assembly chaperone, “proteassemblin” (23–28). Completion of assembly involves completion of the β ring, juxtaposition of two preproteasomes at the β ring interface, autolysis of β subunit N-terminal propeptides, and degradation of proteassemblin (29, 30). The β5 subunits (LMP7 and X) are incorporated relatively late and are absent from most preproteasomes (27). Interestingly, immunoproteasome assembly proceeds in a different order from constitutive proteasome assembly with LMP2 (β1i) being an early component of pre-immunoproteasomes, whereas its homologue, Delta (β1), is a late component of constitutive preproteasomes (27).

An important question is what happens to proteasome diversity when both types of catalytic subunits are expressed in the same cell, which is the case in cells of the immune system and other cells under the influence of interferon-γ. Originally it was thought that subunits were incorporated according to mass action, whereas we have recently demonstrated that immunoproteasome homogeneity is favored even in the presence of constitutive subunits (31). Manifestations of this cooperative assembly process include the observations that LMP2 is required for efficient MECL incorporation and that LMP7 is incorporated preferentially over X into proteasomes containing LMP2 and MECL (31, 32). This latter effect is dependent on differences between the LMP7 and X propeptides, as demonstrated by “propeptide switch” experiments in which the LMP7 propeptide facilitates X incorporation into LMP2/MECL” proteasomes, whereas the X propeptide inhibits LMP7 incorpora-
tion into these same proteasomes (33).

In the current study, we demonstrate that differences between the MECL (β2i) and Z (β2) propeptides also influence cooperative proteasome assembly. Specifically, MECL with a Z propeptide is more efficiently incorporated into otherwise constitutive (Δ2 i/Δ2 f) proteasomes, and X is more efficiently incorporated into otherwise immunoproteasomes containing LMP2 and MECL when MECL is expressed with a Z propeptide. We also used cells from MECLΔ Z mice to establish that LMP2 does not require MECL for efficient incorporation into proteasomes, in contrast with MECL requiring LMP2 (31, 32). Our results enable us to refine our model for propeptide-mediated cooperative proteasome assembly, in which certain combinations of inducible and constitutive subunits are favored over others. Particularly inhibited are combinations of the X subunit with any of the inducible subunits.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs**—The cDNAs encoding human MECL and Z were expressed in T2 cells using an episomal vector, pCEP4, which confers hygromycin resistance (Invitrogen). Propeptide switches were carried out using KpnI restriction sites that were engineered at the site of propeptide cleavage between Gly−1 and Thr−1 (GGTACC). These restriction sites were introduced using the Altered Sites II in vitro mutagenesis system (Promega, Madison, WI). An internal KpnI site in the MECL cDNA also was removed using this system. C-terminal Myc and FLAG epitope tags were incorporated by PCR amplification, in which sequences encoding the tags were included in the 3′ primers. Human MECL cDNA was provided by J. Monaco (University of Cincinnati College of Medicine, Cincinnati, OH). Human Z cDNA was provided by K. Tanaka (The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). cDNAs encoding human Delta, LMP2, and LMP7 were expressed in T2 cells using a stably integrated vector, pSG5 (Stratagene, La Jolla, CA), which was co-transfected with pSV2Neo (Clontech, Palo Alto, CA), which confers G418 resistance. Human Delta cDNA was generated by PCR amplification of human C1R cell cDNA using primers made from known terminal sequences of Delta. Human LMP2 and LMP7 cDNAs have been described previously (31). FLAG epitope tags were attached to the C termini of Delta and LMP2 by PCR amplification. The fidelity of PCR-generated constructs was confirmed by direct sequencing.

**Antibodies**—Polyclonal antisera recognizing LMP2, LMP7, MECL, Delta, Z, and C8 were obtained from rabbits immunized with recombinant mouse subunits. Anti-proteasembolin is a rabbit polyclonal antiserum against recombinant human proteasemblin. Anti-X (P93230) is a rabbit polyclonal antiserum raised against human X that was obtained from K. B. Hendil (August Krogh Institute, University of Copenhagen, Copenhagen, Denmark). Anti-N3, obtained from K. B. Hendil, is a mouse monoclonal antibody that recognizes human N3 (34). Anti-Myc and anti-FLAG are mouse monoclonal antibodies that respectively recognize a Myc epitope, EQKLISEEDL (Invitrogen), or a FLAG epitope, DYGKD (Sigma).

**Cell Culture and Transfection**—Lymphoblastoid T2 cells (35) obtained from P. Cresswell (Yale University School of Medicine, New Haven, CT) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, t-glutamine, and antibiotics (R10) as described (31). Episomal expression vectors (pC64F) containing MECL or Z constructs were transfected by electroporation (250 V and 500 microfarads) in
were anti-FLAG, anti-Myc, and anti-LMP7. Immunoprecipitates from 8/H11003 mice were generated by targeted disruption of the mouse MECL gene.1 MECL-deficient lane each for Immunology, Basel, Switzerland (36). LMP2 (Calbiochem). T2-derived stable transfectant cell lines were generated grown in R10 for 48 h before the addition of hygromycin at 360 units/ml MECL/H9262 serum-free RPMI at 2.5 – Cells were lysed with 1% Nonidet P-40 in 20 m M Tris, pH 7.6, with either G418 for pSV2.Neo and/or hygromycin for pCEP4. Selected cells were maintained in RPMI with 5% bovine serum (R5) and their associated complexes were immunoprecipitated from post-nuclear supernatants with anti-FLAG-M2-Sepharose (Sigma), sub-jected to 12.5% SDS-PAGE, and transferred by electroblotting onto polyvinylidene difluoride paper at 500 mA for 60 min. Co-precipitating proteins were detected using specific antisera (anti-LMP2, anti-LMP7, anti-Z, anti-Delta, anti-X, anti-N3, and anti-proteassemblin) or mono-clonal antibodies (anti-FLAG and anti-Myc) as primary antibodies, alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies, and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma) for color development.

Anti-FLAG Immunoprecipitation, Gel Electrophoresis, and Immunoblotting—Cells were lysed with 1% Nonidet P-40 in 20 mM Tris, pH 7.6, 10 mM EDTA, and 100 mM NaCl. FLAG-tagged proteasome subunits and their associated complexes were immunoprecipitated from post-nuclear supernatants with anti-FLAG-M2-Sepharose (Sigma), subjected to 12.5% SDS-PAGE, and transferred by electroblotting onto polyvinylidene difluoride paper at 500 mA for 60 min. Co-precipitating proteins were detected using specific antisera (anti-LMP2, anti-LMP7, anti-Z, anti-Delta, anti-X, anti-N3, and anti-proteassemblin) or monoclonal antibodies (anti-FLAG and anti-Myc) as primary antibodies, alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies, and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma) for color development.

RESULTS

The Z Propeptide Enables MECL Incorporation into Proteasomes That Contain Delta and X and Facilitates Incorporation of X into MECL/LMP7+ Proteasomes—The inducible protea-some subunit MECL (β2i) is co-incorporated efficiently into proteasomes only in the presence of LMP2 (β1i), which is also an inducible subunit (31, 32). Conversely, Z (β2), the constitutive homologue of MECL, is incorporated readily in the absence of LMP2. We tested whether this difference is because of propeptide differences by replacing the MECL propeptide with the Z propeptide (ppZ.MECL). We attached C-terminal FLAG tags to MECL and ppZ.MECL and expressed these recombinant proteins in T2 cells, which lack both LMP2 and LMP7 because of a homozygous deletion that encompasses both of

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these genes. Thus, Delta is the only β1 subunit and X is the only β5 subunit expressed in these recipient cells. Analysis of FLAG-tagged immunoprecipitated complexes from these cells demonstrated that MECL.FLAG accumulated in an unprocessed form that was not associated with other preproteasome subunits (pre-X, pre-Delta, or pre-N3) and thus did not appear to be incorporated into preproteasomes (Fig. 1A). Conversely, ppZ.MECL.FLAG was efficiently processed and incorporated into Delta \(^{-}/\)X\(^{-}/\)proteasomes, as demonstrated by co-precipitation of predominantly processed MECL.FLAG with other processed β subunits that included Delta (β1), X (β5), and N3 (β3). The lack of associated proteins with unprocessed MECL.FLAG also was demonstrated by labeling cellular proteins with \(^{[35}S\)methionine/cysteine and immunoprecipitating labeled MECL.FLAG with anti-FLAG-Sepharose. In this experiment, no other labeled proteins co-precipitated with MECL.FLAG (data not shown).

We have shown that preproteasomes containing LMP2 (β1i) and MECL (β3i) accumulate in the absence of LMP7 (β5i) because of the relatively inefficient incorporation of X (β5), which is the constitutive homologue of LMP7, into these otherwise immunoproteasomes (31). We tested whether the MECL propeptide contributed to this effect by co-expressing MECL.FLAG or ppZ.MECL.FLAG with LMP2 in T2 cells. Analysis of FLAG-tagged immunoprecipitated complexes from these cells demonstrated that MECL.FLAG accumulated in an unprocessed form that surprisingly co-precipitated a minimal amount of unprocessed LMP2 (Fig. 1B). Thus, most of the unprocessed MECL.FLAG in these cells appeared not to be incorporated into preproteasomes. Conversely, ppZ.MECL.FLAG was processed efficiently and co-precipitated with processed LMP2, demonstrating increased assembly efficiency of LMP2\(^{-}/\)MECL\(^{-}/\)X\(^{-}/\)proteasomes when the MECL propeptide is replaced by the Z propeptide. We could not directly demonstrate increased incorporation of X into LMP2\(^{-}/\)proteasomes because more efficient assembly of Delta\(^{-}/\)MECL\(^{-}/\)X\(^{-}/\)proteasomes also occurred in these cells, as it did in T2 cells transfected with ppZ.MECL.FLAG (data not shown). We speculate that the poor incorporation of pre-MECL.FLAG into preproteasomes even in the presence of LMP2 is because of competition with a large pool of endogenous MECL, as suggested by Groettrup et al. (32), who found that overexpression of MECL mRNA in T2 cells with or without LMP2 and LMP7 does not increase the amount of MECL present in the 20 S proteasomes. The finding that the Z propeptide, when attached to MECL, facilitated assembly of LMP2\(^{-}/\)MECL\(^{-}/\)X\(^{-}/\)proteasomes was confirmed by co-expressing MECL-Myc or ppZ.MECL-Myc with LMP2.FLAG in T2 cells, where we found minimal incorporation of MECL-Myc into complexes containing LMP2.FLAG. However, attaching the Z propeptide to MECL-Myc significantly increased the amount of processed MECL-Myc relative to pre-MECL-Myc that co-precipitated with LMP2.FLAG (Fig. 1C). The presence of ppZ.MECL-Myc did not reduce significantly the accumulation of pre-LMP2.FLAG that is observed in the absence of LMP7 because of the relatively large pool of endogenous MECL in these cells.

We have shown that in the presence of both LMP2 and LMP7, MECL is incorporated predominantly into proteasomes that also contain both LMP2 and LMP7, which are referred to as immunoproteasomes (31). Because ppZ.MECL is incorporated more efficiently than MECL into Delta\(^{-}/\)X\(^{-}/\)proteasomes in the absence of LMP7, we assessed whether this still occurred in the presence of both LMP2 and LMP7, which simulates the situation in which all six catalytic subunits are expressed in the same cell. We expressed MECL.FLAG or ppZ.MECL.FLAG with LMP2 and LMP7 in T2 cells and found that co-precipitation of X with FLAG-tagged MECL was detectable only when the Z propeptide was attached to MECL, demonstrating that ppZ.MECL is more efficiently incorporated than MECL into X-containing proteasomes, even in the presence of LMP7 (Fig. 1D). Conversely, the absence of co-precipitating X with MECL.FLAG confirms that incorporation of MECL and X into the same proteasome occurs inefficiently when all of the inducible and constitutive catalytic subunits are present. We speculate that the amount of LMP7 co-precipitating with ppZ.MECL.FLAG was not significantly reduced because there still is relatively much more LMP7 in these proteasomes compared with X. Co-precipitation with MECL.FLAG of relatively more Delta than X indicates that Delta\(^{-}/\)MECL\(^{-}/\)LMP7\(^{-}/\)proteasomes can be assembled effectively when all six catalytic subunits are present. Conversely, in the absence of LMP7, Delta\(^{-}/\)MECL\(^{-}/\)X\(^{-}/\)proteasomes are assembled inefficiently, as demonstrated by the minimal amount of Delta and X that co-precipitates with MECL.FLAG in that situation (Fig. 1A).

The MECL Propeptide Does Not Prevent Incorporation of Z into Constitutive Proteasomes, but It Stabilizes Z\(^{-}/\)LMP2\(^{-}/\)Preproteasomes in the Absence of LMP7—Because the Z propeptide enabled incorporation of MECL (β2i) into otherwise constitutive (Δ\(^{-}/\)X\(^{-}/\)) proteasomes, we tested whether the MECL propeptide inhibited incorporation of Z (β2) into constitutive proteasomes by expressing Z.FLAG or ppMECL.Z.FLAG in T2 cells. Analysis of FLAG-tagged immunoprecipitated complexes from these cells showed that attaching the MECL propeptide to Z had a minimal effect on Z incorporation and processing, as demonstrated by a small increase in the amount of pre-Z.FLAG relative to the amount of processed Z.FLAG, and had negligible effects on the amounts of co-precipitating Delta, X, and N3 (Fig.
Because the Z propeptide facilitated assembly of MECL\(^{-/-}\)/LMP2\(^{-/-}\)/X\(^{-/-}\) proteasomes in the absence of LMP7 (Fig. 1, B and C), we also investigated whether the MECL propeptide, when attached to Z, facilitated the assembly of Z\(^{+}/\)LMP2\(^{-/-}\)/X\(^{-/-}\) proteasomes by expressing Z.FLAG or ppMECL.Z.FLAG with LMP2 in T2 cells. Analysis of FLAG-tagged immunoprecipitated complexes from these cells revealed that the MECL propeptide, when attached to Z, increased the content of Z\(^{+}/\)LMP2\(^{-/-}\)/X\(^{-/-}\) proteasomes, as demonstrated by increased pre-Z.FLAG, pre-LMP2, and proteassemblin, but it did not facilitate the assembly of Z\(^{+}/\)LMP2\(^{-/-}\)/X\(^{-/-}\) proteasomes, as demonstrated by similar minimal amounts of mature Z.FLAG and LMP2 (Fig. 2B). This result was confirmed by expressing Z.Myc or ppMECL.Z.Myc with LMP2.FLAG in T2 cells, where there was a greater amount of unprocessed ppMECL.Z.Myc in association with LMP2.FLAG as compared with Z.Myc, demonstrating the increased steady state level of Z\(^{+}/\)LMP2\(^{-/-}\)/X\(^{-/-}\) preproteasomes in the absence of LMP7 when the MECL propeptide is attached to Z (Fig. 2C). The increase in Z\(^{+}/\)LMP2\(^{-/-}\)/X\(^{-/-}\) preproteasomes is because of increased stability (i.e. decreased maturation and/or degradation) of preproteasomes rather than increased formation, as we observed no difference in the rate of incorporation of Z.Myc versus ppMECL.Z.Myc into FLAG-tagged LMP2\(^{-/-}\) preproteasomes in pulse-chase experiments (data not shown). Finally, we assessed whether the MECL propeptide attached to Z had an effect on Z incorporation into LMP2\(^{-/-}\)/LMP7\(^{-/-}\) proteasomes by expressing Z.Myc or ppMECL-Z.Myc with LMP2.FLAG and LMP7 in T2 cells. Analysis of FLAG-tagged immunoprecipitated complexes indicated that the MECL propeptide minimally increased the incorporation of Z into these proteasomes, as demonstrated by the slightly greater amount of Z.Myc relative to co-precipitating LMP2.FLAG and LMP7 (Fig. 2D).

Efficient Assembly of LMP2-containing Proteasomes Does Not Require MECL—The recent availability of MECL\(^{-/-}\) mice provided the opportunity to investigate the role of MECL in immunoproteasome assembly using MECL-deficient cells. We derived ConA-stimulated splenic T-cell lymphoblasts (ConA blasts) from mice deficient in each of the immunosubunits and immunoblotted cell lysates to assess the steady state levels of each immunosubunit and its precursors (Fig. 3A). Wild-type ConA blasts express predominantly immunosubunits, although basal expression of constitutive homologues in these cells can compensate when an immunosubunit is absent (31). Absence of MECL did not affect significantly the level of LMP2, and it did not result in an accumulation of precursor LMP2, unlike what is observed in the absence of LMP7, where LMP2\(^{+}/\)MECL\(^{+}\) preproteasomes accumulate because of inefficient incorporation of X (31). The absence of MECL also did not affect the levels of LMP7 and pre-LMP7, similar to what is observed in the absence of LMP2, indicating that LMP7 can be incorporated efficiently with any combination of β1 and β2 subunits. We crossed MECL\(^{-/-}\) with LMP7\(^{-/-}\) mice to generate mice deficient in both of these immunosubunits, from which we derived ConA blasts and immunoblotted cell lysates for LMP2 (Fig. 3B). Absence of LMP7 resulted in accumulation of pre-LMP2, whether MECL was present or absent, although the absence of MECL appeared to decrease the ratio of pre-LMP2 to mature LMP2. This result suggests that although the presence of MECL may reduce the incorporation of X into otherwise
immunoproteasomes, it is not fully responsible for this effect. Conclusions based on steady state subunit levels were supported by pulse-chase metabolic labeling of ConA blasts from wild-type, MECL-1 \(^{-/-}\), MECL-1 \(^{-/-}\)/LMP7 \(^{-/-}\), and LMP7 \(^{-/-}\) mice. Cells were pulsed with \(^{35}\)S]methionine/cysteine for 45 min and then chased for 0 or 4 h, which under normal circumstances readily labels preproteasome subunits with the 45-min pulse and then chases most of the label out of the preproteasomes and into the 20 S proteasomes with the 4-h chase (29).

We immunoprecipitated preproteasomes from pulse-chase-labeled cell lysates using the preproteasome-specific anti-C8 antibody, and we visualized labeled preproteasome subunits by two-dimensional gel electrophoresis and autoradiography (Fig. 4). After the 4-h chase, very little label remained in the preproteasomes in either wild-type or MECL-deficient cells, whereas most of the label was not chased out of the preproteasomes in either LMP7-deficient cells or cells deficient in both LMP7 and MECL. Thus, the inefficient assembly of proteasomes containing LMP2 that is observed in the absence of LMP7 is largely independent of the presence of MECL.

**DISCUSSION**

We demonstrate that differences between the MECL (\(\beta2i\)) and Z (\(\beta2\)) propeptides influence proteasome assembly at two levels, \(\beta2\) subunit incorporation into preproteasomes and the assembly of catalytically active 20 S proteasomes. The influence on subunit incorporation is demonstrated by the ability of the Z propeptide to enhance MECL incorporation into Delta \(\times\) proteasomes (Fig. 1A), whereas the effect on 20 S proteasome assembly is demonstrated by the enhanced assembly of LMP2-/MECL \(^{-/-}\) proteasomes in the absence of LMP7 (Fig. 1B). Taken together with our previous work, our present findings allow us to refine our model for cooperative assembly and propose a mechanism for propeptide involvement. Fig. 5 shows eight pathways that lead to combinations of catalytic subunits in a proteasome \(\beta\) ring and indicates which pathways are inhibited by cooperative assembly. Pathway 1 leads to homogeneous immunoproteasomes and is favored over pathway 2, in which X substitutes for LMP7, as demonstrated by the accumulation of MECL-/-LMP7-/- preproteasomes in cells expressing LMP2 but not LMP7 (Figs. 1B, 1C, 3A, and 4) (31). The influence of propeptides is demonstrated by the observation that pathway 2 occurs more efficiently when the MECL propeptide is replaced by the Z propeptide (Fig. 1, B and C). Pathway 3 that leads to proteasomes containing the constitutive subunit Z in combination with LMP2 and LMP7 is not inhibited by cooperative assembly, as demonstrated both by efficient incorporation of Z into proteasomes containing LMP2.FLAG and LMP7 (Fig. 2D) and by normal levels of LMP2 and LMP7 in ConA blasts from MECL-/- mice where Z is the only available \(\beta2\) subunit (Fig. 3A). Pathway 3 is favored over pathway 4, in which X substitutes for LMP7, as demonstrated by accumulation of Z-/LMP2-/- preproteasomes both in T2 cells expressing LMP2.FLAG and Z.Myc (Fig. 2C) and in ConA blasts from...
MECL−/LMP7−/− mice (Fig. 3B). Pathway 5 leads to proteasomes containing the constitutive subunit Delta in combination with MECL and LMP7. This pathway is inhibited to some degree by cooperative assembly, as demonstrated by accumulation of MECL preproteasomes in ConA blasts from LMP2−/− mice (Fig. 3A). Pathway 5 does occur when all six subunits are present, as demonstrated by the presence of Delta but not X in MECL−/− proteasomes when LMP7 is present (Fig. 1D); also demonstrated is the preference for pathway 5 over pathway 6, in which X substitutes for LMP7. The influence of propeptides on cooperative assembly again is demonstrated by the observation that pathway 6, formed through the incorporation of MECL into Delta−/X− proteasomes, occurs more efficiently when the MECL propeptide is replaced by the Z propeptide (Fig. 1A). Pathway 7 that leads to proteasomes containing the inducible subunit Z in combination with Z and Delta is not inhibited by cooperative assembly, as demonstrated by normal levels of LMP7 in ConA blasts from either MECL−/− or LMP2−/− mice (Fig. 3A). Finally, pathway 8 leads to homogeneous constitutive proteasomes is favored by cooperative assembly, as demonstrated by efficient incorporation of Delta and X into Z− proteasomes in the absence of LMP2 and LMP7 (Fig. 2A).

We speculate that propeptides influence cooperative assembly via differential propeptide-protein interactions and propose the following hypothetical mechanism based on our data. MECL (β2i) normally requires LMP2 (β1i) for efficient co-incorporation into preproteasomes, which occurs relatively early during assembly, noting that LMP2 is incorporated earlier than its homologue Delta (β1). Conversely, Z (β2) appears to be incorporated into preproteasomes before Delta and thus is incorporated without an adjoining β1 subunit. Replacing the MECL propeptide with the Z propeptide obviates the need of MECL for LMP2, and we suggest that the Z propeptide enables MECL to be incorporated without an adjoining β1 subunit. We further suggest that the X propeptide may inhibit X incorporation into preproteasomes that already contain a β1 subunit. Thus, X has a more difficult time being incorporated into LMP2− proteasomes than Delta− proteasomes because LMP2 is incorporated earlier than Delta. Thus, if the Z propeptide enhances incorporation of MECL into preproteasomes without a β1 subunit, it could enhance X incorporation. This mechanism could explain not only the enhanced assembly of Delta−/ MECL−/X− proteasomes but also the increased incorporation of X into LMP2−/MECL− proteasomes where the incorporation of LMP2 and MECL are no longer linked because of the presence of the Z propeptide. To identify the propeptide-protein interactions that mediate these effects, we currently are determining functionally important propeptide residues. Analysis of the amino acid sequences of the human MECL and Z propeptidases reveals a high degree of homology (Fig. 6), yet there are many of the residues that are conserved among these β2 propeptides are the same residues that are identical between the human Z and MECL propeptidases. Conservation of these residues in non-vertebrates that lack immunosubunits suggests that they may be important for propeptide functions other than cooperative assembly. Interestingly, residues 17 to 14 of the mammalian propeptidases represent an insertion as compared with non-vertebrates. The uniqueness of this segment to mammalian propeptidases and its difference between the Z and MECL propeptidases suggests that these residues may play a role in cooperative assembly.

In summary, differences between the MECL and Z propeptidases influence cooperative proteasome assembly, with the Z propeptide able to overcome inhibition of pathways that lead to co-incorporation of MECL and X. It is unclear why the assembly of certain combinations of catalytic subunits is inhibited by cooperative assembly, but understanding the mechanisms of cooperativity will provide tools to investigate this question and advance our understanding of proteasome function in cell biology and immune responses.

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REFERENCES
1. Rock, K. L., Gramm, C., Bochtiker, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. (1994) Cell 78, 761–771
2. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) Annu. Rev. Biochem. 66, 801–847
3. Hilt, W. and Wolf, D. H. (1996) Trends Biochem. Sci. 21, 96–102
4. Baumeister, W., Walz, J., Zuhl, F., and Seemuller, E. (1998) Cell 92, 367–380
5. Hochstrasser, M. (1995) Curr. Opin. Cell Biol. 7, 215–223
6. Diczfalusy, E., Gaczynska, M., Schuette, M., Grama, C. F., Fenton, G., Goldberg, A. L., and Rock, K. L. (1997) J. Biol. Chem. 272, 13437–13445
7. Akiyama, K., Kagawa, S., Tamura, T., Shimbara, N., Takashima, M., Kristensen, P., Hendil, K. B., Tanaka, K., and Ichihara, A. (1994) FEBS Lett. 343, 85–88
8. Monaco, J. J., and Nandi, D. (1995) Annu. Rev. Genet. 29, 729–754
9. Grottrup, M., Soza, A., Kuckelkorn, U., and Kloetzel, P. M. (1996) Immunol. Today 17, 429–435
10. Rock, K. L., and Goldberg, A. L. (1999) Annu. Rev. Immunol. 17, 739–779
11. Driscoll, J., Brown, M. G., Finley, D., and Monaco, J. J. (1993) Nature 367, 262–264
12. Gaczynska, M., Rock, K. L., Spies, T., and Goldberg, A. L. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9213–9217
13. Boes, B., Hengel, H., Ruppert, T., Mullhaup, G., Kossinowski, U. H., and Kloetzel, P. M. (1994) J. Exp. Med. 179, 901–909
14. Ehring, B., Meyer, T. H., Eckerstor, C., Lottspeich, F., and Tampe, R. (1996) Eur. J. Biochem. 235, 404–415
15. Groll, M., Ditzel, I., Lowe, J., Stock, D., Bochtler, M., Bartunik, H. D., and Huber, R. (1997) Nature 383, 463–471
16. Kopp, F., Hendil, K. B., Dahlmann, B., Kristensen, P., Sobek, A., and Uerkvitz, W. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 2939–2944
17. Dahlmann, B., Kopp, F., Kristensen, P., and Hendil, K. B. (1999) Arch. Biochem. Biophys. 363, 296–300
18. Kopp, F., Dahlmann, B., and Hendil, K. B. (1993) J. Mol. Biol. 229, 14–19
19. Baumeister, W., Cejka, Z., Kania, M., and Seemuller, E. (1997) Biol. Chem. 378, 121–130
20. Schindtke, G., Kraft, R., Kostka, S., Henklein, P., Frommel, C., Lowe, J., Huber, R., Kloetzel, P. M., and Schmidt, M. (1997) Mol. Cell Biol. Res. Commun. 3, 212–217
21. Schindtke, G., Kraft, R., Kostka, S., Henklein, P., Frommel, C., Lowe, J., Huber, R., Kloetzel, P. M., and Schmidt, M. (1996) EMBO J. 15, 6887–6898
22. Schmidtke, G., Paudolf-Hurt, B., Seelig, A., and Kloetzel, P. M. (1994) J. Mol. Biol. 236, 975–981
23. Yang, Y., Fruh, K., Ahn, K., and Peterson, P. A. (1995) J. Biol. Chem. 270, 27687–27694
24. Nandi, D., Woodward, E., Ginsburg, D. B., and Monaco, J. A. (1997) EMBO J. 16, 5363–5375
25. Schindtke, G., Schmidt, M., and Kloetzel, P. M. (1997) J. Biol. Chem. 272, 95–106
26. Chen, P., and Hochstrasser, M. (1996) Cell 86, 961–972
27. Mayr, J., Seemuller, E., Muller, S. A., Engel, A., and Baumeister, W. (1998) J. Struct. Biol. 124, 179–188
28. Grottrup, M., Stanéda, S., Stohwasser, R., and Kloetzel, P. M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 8970–8975
29. Kingsbury, D. J., Griffin, T. A., and Colbert, R. A. (2000) J. Mol. Biol. 296, 212–217
30. Schmidtke, G., Kraft, R., Kostka, S., Henklein, P., Frommel, C., Lowe, J., Huber, R., Kloetzel, P. M., and Schmidt, M. (1996) EMBO J. 15, 6887–6898
31. Freiheit, S., Paudolf-Hurt, B., Seelig, A., and Kloetzel, P. M. (1994) J. Mol. Biol. 236, 975–981
32. Yang, Y., Fruh, K., Ahn, K., and Peterson, P. A. (1995) J. Biol. Chem. 270, 27687–27694
33. Nandi, D., Woodward, E., Ginsburg, D. B., and Monaco, J. A. (1997) EMBO J. 16, 5363–5375
34. Schindtke, G., Schmidt, M., and Kloetzel, P. M. (1997) J. Biol. Chem. 272, 95–106
