In Vivo Assembly of the Proteasomal Complexes, Implications for Antigen Processing*

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The multicatalytic and multisubunit proteasomal complexes have been implicated in the processing of antigens to peptides presented by class I major histocompatibility complex molecules. Two structural complexes of this proteasome, 20 S and 26 S proteasomes, have been isolated from cells. By analyzing in vivo assembly of the proteasomal complexes we show that the 20 S proteasomal complexes are irreversibly assembled via 15 S assembly intermediates containing unprocessed β-type subunits. The 20 S proteasomes further associate reversibly with proteasome activators PA28 or pre-existing ATPase complexes to form 26 S proteasomal complexes. Our findings that not all of the 20 S proteasomal complexes are assembled into 26 S proteasomal complexes within cells and that all of PA28 and ATPase complexes are associated with 20 S proteasomes strongly suggest that all proteasomal complexes coexist with cells. We further demonstrate that 26 S proteasomal complexes are predominantly present in the cytoplasm and a significant portion of the 20 S proteasomal complexes is associated with the endoplasmic reticulum membrane. Taken together, our findings suggest that depending upon their associated regulatory components, 26 S and 20 S-PA28 proteasomal complexes serve different housekeeping functions within the cells, while they degrade antigens in a cooperative manner in antigen processing.

Cytotoxic T-lymphocytes recognize antigenic peptides of 8–10 amino acid residues presented by class I major histocompatibility complex (MHC)1 molecules (1). This trimeric complex of class I MHC molecule is assembled in the endoplasmic reticulum from class I heavy chain, β2-microglobulin, and antigenic peptide imported from the cytoplasm through a peptide transporter (2–4). Because antigen exogenously introduced into the cytoplasm needs to be ubiquitinated before T-cell epitopes are generated (5), it seems that antigenic peptide generation follows a ubiquitin-mediated protein degradation pathway. It has been shown that ubiquitinated proteins are degraded by the 26 S multicatalytic and multisubunit proteasome complex, or 26 S proteasome (6) and that inhibitors of the proteasomal complexes block the generation of peptides presented on class I MHC molecules (7).

The 26 S proteasome consists of an “ATPase complex” (8) or “19 S complex” (9) and the 20 S proteasome which is believed to be the catalytic core of the 26 S proteasome (10–12). The 20 S proteasome is a highly conserved structure of 7-fold symmetry in all eucaryotic cells and the archaebacterium Thermoplasma acidophilum (10–12). The x-ray crystallographic analysis of the 20 S proteasome from the archaebacterium T. acidophilum has recently been reported (13). The 20 S proteasome of eucaryotes consists of a family of 14 different subunits of molecular masses between 20 and 35 kDa (14, 15). The subunits can be classified into seven different but homologous α-type or β-type subunits according to their homology with the α- and β-type subunits of the T. acidophilum proteasome (16, 17). In addition, three additional β-type subunits, LMP2, LMP7, and MECL1, are up-regulated by interferon-γ (IFN-γ) (15, 18–24). The MHC-encoded and IFN-γ-inducible LMP2 and LMP7 (23) are found to displace two constitutively expressed and highly homologous housekeeping β-type subunits 2 and 10, respectively, from the proteasomal complexes (23, 24), thus changing the subunit composition without altering the number of subunits per complex. Although incorporation of LMP2 and LMP7 is not an essential prerequisite for peptide generation (25, 26), the enzymatic activities of proteasomal complexes are thereby altered in such a way that preferred peptidic substrates for peptide transporters and class I molecules are generated (27–31). Further circumstantial evidence for the involvement of proteasomal complexes in antigen processing came from studies of a family of homologous proteasome activators PA28 (32–35) which consists of at least two IFN-γ-inducible homologues. It has been shown that in vitro binding of PA28 to 20 S proteasomes activates distinct peptidase activities of 20 S proteasomes (32–34), indicating that peptide products appropriate for antigen presentation may be preferentially generated.

Both 20 S and 26 S proteasomes can be isolated from cells (11, 12, 14, 18), but it has not been clearly established whether both complexes coexist in vivo. We have therefore studied the individual steps leading to the formation of 26 S proteasomal complexes. We show that β-type subunit precursors assemble into distinct assembly intermediates prior to processing, whereby the amino-terminal sequences play a crucial role. The assembly of 20 S proteasomes is completed with the association of additional subunits to the assembly intermediate during and/or after β-type subunits are processed, whereas the assembly of 26 S proteasomal complexes occurs in a single step from the pre-existing ATPase complexes and 20 S proteasomes. Furthermore, we present the first in vivo observation that IFN-γ-inducible proteasome activators PA28 directly bind to 20 S proteasomes and that binding of PA28 does not interfere with the assembly of 26 S proteasomal complexes. Although the assembly of 20 S proteasomes is irreversible, assembly of the 26 S proteasomal complexes, possibly mediated by subunit phos-

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1 The abbreviations used are: MHC, major histocompatibility complex; IFN-γ, interferon-γ; LMP, low molecular weight polypeptide.
2 Y. Yang et al., unpublished data.
phorylation, is reversible. Our demonstration that 20 S and 26 S proteasomal complexes localize in different subcellular compartments further suggests the differential functions of 20 S and 26 S proteasome complexes in antigen processing.

**MATERIALS AND METHODS**

Cell Cultures and Antisera—The murine lymphoma RMA cells (3) were grown in RPMI 1640 medium. For transactivator-containing HtTA cells (24, 36), 500 μg/ml G418 and 0.1 μg/ml tetraacycline (Sigma) were included in Dulbecco's modified Eagle's medium (BioWhittaker) supplemented with 10% fetal calf serum. For IFN-γ induction, cells were treated with 500 units/ml of recombinant mouse IFN-γ (Boehringer Mannheim) for 48 h. The anti-C9, anti-LMP2, and anti-proteasome antisera were raised in rabbits against recombinant murine C9, murine LMP2, and purified bovine 20 S proteasome as described (22, 24). The anti-PA28 antisera was raised against a synthetic peptide corresponding to the carboxy-terminal 20 amino acid residues of human PA28 (34). A rabbit antisera against ATPase complexes was kindly provided by M. Rechsteiner.

DNA Constructs and Transfection—The cDNAs coding for hLMP7-E1, hLMP7-E2, hLMP2, and mLMP2 were subcloned from pCMU11 (22) into puroH10–1 (36). mLMP7 was subcloned from pUHG10–3 (24). In the vector containing a chimeric eukaryotic translation initiation sequence was introduced in front of all start codons. The cDNA coding for PA28 was isolated from a cDNA library of HeLa cells by polymerase chain reaction using specific primers (34) and was cloned into puroH10–3. The LMP or PA28 cDNA constructs were transfected together with a plasmid conferring resistance against G418 into HtTA cells by the calcium phosphate method. After 16 h, cells were washed in phosphate-buffered saline and incubated in Dulbecco's modified Eagle's medium, 10% fetal calf serum for an additional 24 h. Okubai (Sigma) was then added to a final concentration of 1 μM. Untransfected cells died within the next 24 h. The surviving cells were labeled for immunoprecipitation as described below. For studies on PA28, from several stable clones a single stable transfecant was selected for further investigation.

Metabolic Labeling and Immunoprecipitation—Metabolic labeling of cells was carried out as described (23) with the following modifications. Pulse labeling media contained 0.3 μCi of Tran35S-label mixture (ICN) per ml of methionine-deficient RPMI or Dulbecco's modified Eagle's medium. Cells were labeled for 0.5, 1, or 4 h followed by chase periods of different lengths of time in the presence of normal culture medium. Immunoprecipitation, SDS-polyacrylamide gel electrophoresis, first-dimension nonequilibrium pH gradient gel electrophoresis (using Ampholines pH 3.5–10), and fluorography were carried out as described (23).

Sucrose Gradient Fractionation—RMA or stable PA28-expressing HtTA transfected cells were homogenized in the isotonic sucrose solution described in Ref. 23. For isolation of 20 S complexes, ATP in homogenates was depleted by including 100 units/ml hexokinase and 10 mM glucose in the isotonic solution. For isolation of the 26 S complexes, the isotonic sucrose solution was supplemented with 5 mM MgCl₂ and 2 mM ATP and the resultant homogenates were layered on top of a 10–40% linear sucrose gradient containing 25 mM Hepes, 1 mM EDTA, 5 mM MgCl₂, 2 mM ATP, pH 7.5. The gradient volume was 12 ml. Samples were centrifuged for 4 h at 25,000 rpm using a Beckman SW27 rotor and 0.5 (Fig. 2) or 0.6 ml (Figs. 4–6) per fraction was collected and used for immunoprecipitation.

Cellular Fractionation—RMA cells were labeled for 30 min followed by a chase of 4 h and homogenized as described (23). The homogenates were first spun for 30 min at 13,000 rpm to collect the nuclei. Cytosolic and crude microsomal fractions were separated by spinning at 100,000 × g for 30 min. Proteasomal complexes were immunoprecipitated from each fraction and analyzed by SDS-gel electrophoresis.

In Vivo Phosphorylation—RMA cells were labeled in phosphate-free Dulbecco's modified Eagle's medium supplemented with dextralized serum containing [32P]orthophosphate (Amersham) (0.5 μCi/ml) for 4 h. After adding 1 μM okadaic acid and 0.1 μM calyculin A (Calbiochem) cells were cultured for an additional 30 min prior to lysis in 1% Nonidet P-40 lysis buffer containing phosphatase inhibitors (1 μM okadaic acid, 0.1 μM calyculin A, 50 mM NaF, 30 mM pyrophosphate, and 0.1 mM Na₃VO₄) followed by immunoprecipitation.

**RESULTS**

**Precursor Processing Is a Requirement for Incorporation of β-Type Subunits—**The human LMP7-gene codes for two alternatively spliced mRNAs, LMP7-E1 and LMP7-E2, which differ only in their amino-terminal prosequence (22). However, only LMP7-E2 is expressed as a protein in vivo (22), which might reflect the lack of appropriate translation signals upstream from the start codon in LMP7-E1. We therefore examined whether LMP7-E1, expressed as a LMP7 precursor protein, could be incorporated into proteasomal complexes by transient transfection of HeLa cells with LMP7-E1 cDNA (Fig. 1). We had previously reported that in LMP7-E1-transfected HeLa cells the incorporation of LMP7 into proteasomal complexes was slightly increased (22). However, these initial experiments were difficult to interpret due to low expression levels and low transfection efficiencies of LMP7 as well as the fact that HeLa cells express relatively high levels of endogenous LMP7. By increasing expression levels and transfection efficiencies as described under “Materials and Methods” and by using a subclone of HeLa cells, HTA, which expresses almost no endogenous LMP7 (24), we observed that the precursor proteins of all transfected-LMP7 constructs were expressed at high levels (lanes 1, 3, and 5 of Fig. 1) comparable to the expression level of endogenous LMP7 induced by IFN-γ (lane 8).

When we immunoprecipitated the proteasomal complexes, protein bands corresponding to the processed forms of LMP7 were present only in proteasomal complexes from cells transfected with either human LMP7-E2 (Fig. 1, lane 4) or mouse LMP7 (lane 2) but not human LMP7-E1 (lane 6). No processed LMP7-E1, which should be identical to processed transfected- or IFN-γ-induced endogenous LMP7-E1 (lane 6), was detected in over-exposed fluorograms either (not shown); thus, it seems that only LMP7-E2 gives rise to a functional protein. We conclude that the amino-terminal prosequence is crucially involved in the mechanism by which β-type subunits are assembled into the proteasomes. Our finding in conjunction with the observations that the prosequences differ significantly among different β-type subunits and that the sequences surrounding the cleavage site (17, 24) are highly conserved suggest that the prosequences may play a subunit-specific role in the initial folding and/or assembly of β-type subunits (see below), whereas the cleavage is performed by a specific protease.

20 S Proteasomes Assembly—via 15 S Assembly Intermediates Containing β-Type Subunit Precursors—To examine whether precursor processing of the β-type subunit occurs before or after the assembly of proteasomal complexes, we combined pulse-chase labeling with sucrose gradient fractionation of homogenates prepared from RMA cells constitutively expressing both LMP2 and LMP7. Proteasomal complexes contained in such fractions were immunoprecipitated using an antiserum specific for the α-type subunit C9 (22) and an antiserum to the proteasome (not shown). As shown in Fig. 2A, immediately after labeling, anti-C9 antiserum immunoprecipitated both...
free non-assembled subunit C9 (fractions 1–3) and two additional distinct complexes, one with a 15 S sedimentation coefficient (fractions 4–7) and the other with a 20 S sedimentation coefficient (fractions 8–11). After a 2-h chase, both the free C9 subunit and the 15 S complexes were no longer detectable and all the label was chased to the 20 S complexes in fractions 8–11 (Fig. 2B). A similar finding was also obtained by using an anti-proteasome antiserum in the same experiments (not shown). We conclude that the 15 S complexes are the assembly intermediates of the 20 S complexes. Interestingly, when an anti-LMP2 antiserum (22) was used in the same experiments, only the free LMP precursor protein and 15 S complexes were detected in pulse-labeled samples (not shown). The observation that even after longer chase periods (2–18 h) no 15 S and 20 S complexes were detected suggests that after completion of the 20 S complex assembly, LMP2 is no longer recognized by the anti-LMP2 antiserum. Since the antisera was raised against recombinant LMP2 protein, the inaccessibility of anti-LMP2 antibodies to LMP2 epitopes is likely due to the addition of processing forms are only present in the 20 S proteasomes (Fig. 3B). Since β-type subunit precursors are only present in the 15 S assembly intermediates, we conclude that β-type subunits assemble into 15 S complexes prior to their processing.

The almost identical subunit composition and half-life of both the 15 S and 20 S assembly intermediates recognized by anti-C9 and anti-LMP2 antisera as well as similar complexes observed by Frentzel et al. (37) and Patel et al. (38) indicates a time-limited step at this point of proteasomal complex assembly. Our data further show that 20 S proteasome assembly is an irreversible process, because free processed β-type subunits could not be detected within the cells (24). In addition, our findings that no β-type subunit precursors were detected in 20 S complexes and no intermediate assembly complexes of αβ rings (see below) were detected suggest that the completion of 20 S proteasomal complex assembly, i.e. proteasome cleavage and dimerization of the two αβ rings, occurs rather rapidly. We conclude that due to this irreversible process, homologous subunit exchanges can only take place during assembly of the 15 S complexes, as we have indeed observed for LMP subunits (24). Interestingly, although both LMP2 and subunit 2 are present in 20 S proteasomes of RMA cells, indicating a mixed proteasome population, only the LMP2 precursor is detected in the 15 S assembly intermediates immunoprecipitated by either anti-C9 or anti-LMP2 antisera. This suggests that the order of

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assembly with other subunits is different for LMP2 than for subunit 2.

The 26 S Proteasomes Are Formed by Assembly of the Pre-existing ATPase Complexes and 20 S Proteasomes—To address the question whether the 26 S proteasomes assemble via 20 S proteasomes or via individual proteasome subunit associates with ATPase complexes, the assembly of the 26 S complexes were monitored as in Fig. 2, except that 2 mM ATP and 5 mM MgCl₂ were included in cell homogenization isosonic solution and sucrose gradients. Subunits smaller than 32 kDa belong to the 20 S proteasomes (see Fig. 2), subunits larger than this molecular mass are part of the ATPase complexes (see Fig. 5). The sedimentation coefficient for the complexes at fraction 11 was approximately 26 S. Some of the proteasomal subunits as well as their precursors are indicated. The 14C-methylated protein markers (M) are myosin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.3 kDa). E, the enzymatic activities of the different proteasomal complexes present in each sucrose gradient fractions were assayed for hydrolysis of the fluorogenic peptidic substrate succinyl-LLVY-AMC.

Fig. 4. Assembly of 26 S proteasomal complexes. RMA cells were labeled for 30 min (A) followed by 2 h chase (B), 6 h chase (C), or 12 h chase (D). Fractionation of RMA cell homogenates was performed as in the legend to Fig. 2, except that 2 mM ATP and 5 mM MgCl₂ were included in cell homogenization isosonic solution and sucrose gradients. Subunits smaller than 32 kDa belong to the 20 S proteasomes (see Fig. 2), subunits larger than this molecular mass are part of the ATPase complexes (see Fig. 5). The sedimentation coefficient for the complexes at fraction 11 was approximately 26 S. Some of the proteasomal subunits as well as their precursors are indicated. The 14C-methylated protein markers (M) are myosin (200 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.3 kDa).

Regulation of Proteasome Assembly

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fractions for hydrolysis of the succinyl-LLVY-AMC, a fluorogenic peptidic substrate commonly used for detection of proteasome activity (11, 12). The enzymatic activities were only detected in fractions containing the 20 S and 26 S complexes, whereas no enzymatic activity was observed for the fractions containing free subunits or 15 S assembly intermediates (Fig. 4E). This result indicates that only after cleavage of the β-type subunit prosequences are proteasomal complexes enzymatically active.

Proteasome Activators PA28 Bind to 20 S Proteasomes in Vivo—Contrary to previous findings that purified 20 S proteasomes are rather enzymatically inactive regardless whether their housekeeping subunits are displaced by MHC-encoded LMPs or not (11), 3 our data that fractions containing 20 S or 26 S proteasomes have higher enzymatic activities (Fig. 4E) suggest that additional components are required for purified 20 S proteasomes to be enzymatically active. Support for this view came from in vitro studies on PA28 (18, 32–35). It has been shown that purified PA28 is associated with purified 20 S proteasomes in vitro (32–34) and that PA28 stimulates purified 20 S proteasomes to cleave after hydrophobic or positive charge residues at the carboxyl terminus of fluorogenic peptidic substrates (32–34). We, therefore, examined whether in vivo binding of IFNγ-inducible PA28 to 20 S proteasomes occurs and whether the ATPase complex is displaced from 26 S proteasomes by PA28 in vivo. Homogenates prepared from stable PA28-expressing HTa transfectant cells were fractionated in 10–40% sucrose gradients and the resulting fractions were analyzed by immunoprecipitations for the presence of PA28 and 20 S as well as 26 S proteasomes using antisera specific to proteasomes or PA28. As shown in Fig. 6, 20 S and 26 S proteasomes were detected at fractions 8–9 and 10–12, respectively, whereas PA28 was mainly present at 20 S proteasome-containing fraction 9, indicating that PA28 binds to 20 S proteasomes. Since the intensities of 20 S and 26 S proteasomes at respective fractions were not changed regardless of the expression of PA28 (data not shown), PA28 appears to be associated with free 20 S proteasomes. This is the first demonstration that PA28 and 20 S proteasomes associate in vivo.

Subcellular Localization of 15 S Assembly Intermediates, 20 S and 26 S Proteasomes—The observation that distinct proteasomal complexes coexist within the cell raised the question whether they would be distributed within different subcellular compartments. We therefore analyzed the intracellular distributions of the 15 S, 20 S, and 26 S complexes by subcellular
fractionation of pulse-chase-labeled RMA cell homogenates followed by immunoprecipitation with anti-C9 or anti-LMP2 antisera. As shown in Fig. 7, 20 S proteasomes were equally distributed among the nuclear, cytosolic, and microsomal fractions. By contrast, 26 S proteasomal complexes were predominantly present in the cytosolic fraction, very little in the nuclear fraction, and were completely absent from the microsomal fraction (compare to Fig. 48 for the presence of ATPase complexes). Similarly, the majority of the 15 S assembly intermediates were only detected from the cytosol by both antisera but were absent from nuclear and microsomal fractions. These findings suggest that the 15 S and 20 S complexes assemble in the cytoplasm, whereas 20 S complexes further associate with the ATPase complexes to form the 26 S complexes. Consistent with the immunocytochemical and immunoelectron microscopic studies that up to 14% of proteasomes are close or actually on the endoplasmic reticulum (11), we found that a significant proportion of 20 S proteasomal complexes not only remain in free forms but also have different cellular localizations than the 26 S proteasomal complexes.

Regulation of Proteasomal Complex Assembly—A comparison of the subunit compositions of the two assembly intermediates and the mature 20 S complexes shown in Fig. 3 suggested that some of the different subunits were in fact different modifications of the same subunit. For instance, the charge of subunit C3 (lower left) was different between the “anti-LMP2 intermediate” and the “anti-C9 intermediate.” Likewise subunit 17 (upper right; Ref. 23) appeared as a streak. A possible explanation for these observations is post-translational modification due to phosphorylation (40–42). Indeed, we found that two subunits of the 20 S complex and two subunits of the ATPase complex were predominantly phosphorylated as shown by in vivo $^{32}$P labeling followed by immunoprecipitation (Fig. 8A). According to their electrophoretic mobility the two phosphorylated ATPase complex subunits correspond to subunits S6 and S10 (43) and the two phosphorylated 20 S subunits correspond to subunits 17 and 18 (23). The extent of in vivo phosphorylation of both ATPase complex subunits was diminished in the presence of okadaic acid and calyculin A, inhibitors of serine/threonine phosphatases, whereas the extent of phosphorylation for subunit 18 of the 20 S complex was not affected and was increased for subunit 17 in the presence of okadaic acid and calyculin A. Thus, different kinases and phosphatases seem to be responsible for phosphorylation of the ATPase complexes compared to the 20 S complexes.

Since no phosphorylated subunits were detected in 15 S assembly intermediates by in vivo $^{32}$P labeling followed by immunoprecipitation with anti-LMP2 antisera (data not shown), it is unlikely that phosphorylation plays a role in assembly of the 15 S assembly intermediates. We therefore studied whether phosphorylation and dephosphorylation would have an effect on the assembly of the 20 S and 26 S proteasomal complexes. RMA cells were either treated in vivo with the serine/threonine phosphatase inhibitors okadaic acid and calyculin A (Fig. 8B) or with the kinase inhibitor staurosporine (Fig. 8D). In addition, we analyzed the effect of cycloheximide (Fig. 8C) on the proteasomal complex assembly. As shown in Fig. 8C, the cycloheximide treatment resulted in drastically reduced assembly of the 20 S proteasome. Since under this condition the subunit composition of anti-C9 immunoprecipitates (only subunit 7, 23) coprecipitated significantly
with the subunit C9) is simpler than 15 S assembly intermediates, we conclude that cycloheximide blocked assembly at a very early stage prior to 15 S complex formation, presumably due to the lack of newly synthesized subunits. By contrast, treatment by okadaic acid and calyculin A did not inhibit the assembly of the 15 S complexes but resulted in decreased processing of these assembly intermediates as shown by immunoprecipitation of the 15 S complexes with anti-LMP2 antiserum (Fig. 8B). Similarly, a decreased rate of 20 S complex formation was also observed, when anti-C9 antiserum was used for immunoprecipitation (data not shown). Furthermore, when the kinase inhibitor staurosporine was used, the anti-C9 antiserum no longer coprecipitated the ATPase complexes with the 20 S complexes (Fig. 8D, right panel). We ruled out that this effect was due to an increased turnover of 26 S proteasomes, since after a prolonged chase period of several days no noticeable difference in the half-life of 20 S proteasomes between staurosporine-treated and untreated cells was observed (Fig. 8D). Consistent with the observation that in vitro the 26 S proteasome can be reversibly disassembled into the ATPase complex and 20 S complex by removal of ATP/Mg (11, 12), we found that upon removal of staurosporine 26 S proteasomes were again detectable.2 This effect of staurosporine indicates that kinases play a role in the assembly and disassembly of the 26 S complexes. Since staurosporine treatment gives a similar result in vivo, we propose that the role of ATP in 26 S complex assembly is dependent on an ATP-dependent protein kinase. It is interesting to note that PA28 is phosphorylated (42), suggesting that phosphorylation may account for the association with the 20 S proteasomal complexes both for the ATPase complex and PA28.

**DISCUSSION**

Based on studies of the assembly and structure of Thermoplasma proteasomes (44, 13) and our findings presented in this paper, a hypothetical model for the in vivo assembly of 26 S complexes is proposed in Fig. 9. Eucaryotic β-type subunits assemble on a ring-like structural backbone composed of seven α-type subunits. Thus, seven α-type subunits first associate among each other to form a ring-like structural backbone composed of seven α-type subunits. Thus, seven α-type subunits first associate among each other to form a ring-like structural backbone composed of seven α-type subunits. Then each α-type subunit occupies a particular position within the ring-like structure and each β-type subunit precursor also assembles in a given order by binding to the corresponding α-type subunits and each other. This process may lead to proteasomal complexes differing in the content of their exchangeable subunit homologues, e.g. LMP2 and LMP7. The β-type subunit proteasomes are immediately cleaved off either before or during the dimerization of the two αβ-type subunits. During assembly of proteasomal intermediate complexes, the proteasomes of β-type subunits might play a role in either the protein folding or the proteasome assembly process or by inactivating the enzymatic activity of the catalytic sites created during assembly. Thus, seven α-type and seven β-type subunits each form a ring-like structure of 7-fold symmetry which further assembles into a dimer consisting of a four-ring cylindrical structure in the order αβαβ, i.e. the 20 S complexes. The 20 S proteasomal complexes are then capped end-on to regulatory components such as the PA28 or the ATPase complexes, thus forming the 26 S complexes. Association with these regulatory complexes appears to be reversible and probably regulated by phosphorylation.

**Fig. 9. Proposed assembly pathway of the proteasomal complexes.** Seven α-type subunits first associate among each other to form a ring-like structural backbone of 7-fold symmetry in which seven β-type subunits are allowed to assemble. Thus, seven α-type and seven β-type subunits each form a ring-like structure of 7-fold symmetry which further assembles into a dimer consisting of a four-ring cylindrical structure in the order αβαβ, i.e. the 20 S complexes. The 20 S proteasomal complexes are then capped end-on to regulatory components such as the PA28 or the ATPase complexes, thus forming the 26 S complexes. Association with these regulatory complexes appears to be reversible and probably regulated by phosphorylation.
20 S complexes, differing with regard to their subunit composition, are also reflected in the subsets of 26 S complexes and that both 20 S-PA28 and 26 S complexes are enzymatically active suggest that both 20 S-PA28 and 26 S complexes may be involved in antigen processing. Our observations that peptide loading onto class I MHC molecules is interfered with by okadaic acid/calyculin A treatment (46), which inhibits the in vivo assembly of 20 S proteasomal complexes, and that upon expression of PA28 the intracellular transport rate of class I MHC molecules increases 25% in conjunction with our finding that IFN-γ-inducible PA28 directly binds to 20 S proteasomes without interfering with the assembly of 26 S proteasomal complexes strongly suggest that 20 S-PA28 proteasomal complexes play a direct role in antigen processing. Moreover, our finding that at least two PA28 homologs are up-regulated by IFN-γ further supports the above hypothesis. It is interesting to note that isolated 20 S proteasomes of T. acidophilum have been shown to cleave small protein substrates into peptides with a length distribution centering around 8 amino acids (45). Thus, it is reasonable to suggest that IFN-γ-inducible LMP and PA28 serve to optimize antigen processing in proteasome-mediated protein degradation.

With regard to antigen processing for MHC class I antigen presentation, several parallel pathways could coexist. 26 S proteasomal complexes could be responsible for antigenic peptide generation from ubiquitinated antigens, whereas 20 S or 20 S-PA28 proteasomal complexes could digest either partially unfolded antigens or newly synthesized abnormal polypeptides to antigenic peptides. This notion is consistent with the findings that 26 S proteasomal complexes but not 20 S or 20 S-PA28 proteasomal complexes are responsible for the degradation of ubiquitinated proteins and that distinct peptidase activities of 20 S proteasomes can be activated by IFN-γ-inducible PA28. Alternatively, antigen degradation could occur in several successive steps conducted by various proteasomal complexes. 26 S proteasomal complexes could be the protease to degrade whole antigen into degradation intermediates and 20 S-PA28 complexes could function in further processing of such degradation intermediates into peptides of 8–10 amino acid residues suitable for class I loading. Based on our findings that 26 S proteasomal complexes are predominantly present in the cytoplasm and a significant portion of the 20 S proteasomal complexes is associated with endoplasmic reticulum membrane, it can be envisaged that after ubiquitinated proteins are fragmented by the 26 S proteasomal complexes, final trimming to the size of peptides required for transport and class I binding is performed by the 20 S-PA28 complexes in close spatial vicinity to the peptide transporters which are located in the endoplasmic reticulum membrane. The direct association of 20 S proteasomal complexes with transporter-associated with antigen processing, our finding that no MHC class I peptide loading occurs if the ubiquitination pathway is blocked (5) strongly supports the latter hypothesis.

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