

Two abundant proteasome subtypes that uniquely process some antigens presented by HLA class I molecules

Benoît Guillaume^{a,b}, Jacques Chapiro^{a,b}, Vincent Stroobant^{a,b}, Didier Colau^{a,b}, Benoît Van Holle^{a,b}, Grégory Parvizi^{a,b}, Marie-Pierre Bousquet-Dubouch^{c,d}, Ivan Théate^{a,b}, Nicolas Parmentier^{a,b}, and Benoît J. Van den Eynde^{a,b,1}

^aLudwig Institute for Cancer Research, Brussels Branch, 1200 Brussels, Belgium; ^bde Duve Institute, Université Catholique de Louvain, 1200 Brussels, Belgium; ^cInstitut de Pharmacologie et de Biologie Structurale, Centre National de la Recherche Scientifique, 31077 Toulouse, France; and ^dUniversité de Toulouse, Université Paul Sabatier, 31077 Toulouse, France

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Most antigenic peptides presented by MHC class I molecules result from the degradation of intracellular proteins by the proteasome. In lymphoid tissues and cells exposed to IFN γ , the standard proteasome is replaced by the immunoproteasome, in which all of the standard catalytic subunits β 1, β 2, and β 5 are replaced by their inducible counterparts β 1i, β 2i, and β 5i, which have different cleavage specificities. The immunoproteasome thereby shapes the repertoire of antigenic peptides. The existence of additional forms of proteasomes bearing a mixed assortment of standard and inducible catalytic subunits has been suggested. Using a new set of unique subunit-specific antibodies, we have now isolated, quantified, and characterized human proteasomes that are intermediate between the standard proteasome and the immunoproteasome. They contain only one (β 5i) or two (β 1i and β 5i) of the three inducible catalytic subunits of the immunoproteasome. These intermediate proteasomes represent between one-third and one-half of the proteasome content of human liver, colon, small intestine, and kidney. They are also present in human tumor cells and dendritic cells. We identified two tumor antigens of clinical interest that are processed exclusively either by intermediate proteasomes β 5i (MAGE-A3_{271–279}) or by intermediate proteasomes β 1i- β 5i (MAGE-A10_{254–262}). The existence of these intermediate proteasomes broadens the repertoire of antigens presented to CD8 T cells and implies that the antigens presented by a given cell depend on their proteasome content.

antigen processing | antigenic peptide | immunoproteasome | tumor antigen | MAGE

Most antigenic peptides presented by MHC class I molecules are produced through degradation of intracellular proteins by the proteasome, a large protease complex responsible for most of the nonlysosomal protein degradation in eukaryotic cells (1). As a major actor of ubiquitin-mediated protein degradation, the proteasome is also involved in many cellular processes, including cell cycle control and cell signaling. The 20S core of the proteasome has the shape of a barrel made of 28 subunits assembled in four stacked rings (2). The two outer rings are identical and contain seven different α subunits, whereas the two identical inner rings contain seven different β subunits, three of which exert catalytic activities, namely β 1, β 2, and β 5. Each of these subunits was ascribed a typical activity profile on the basis of studies of the activity of yeast mutant proteasomes on small fluorogenic substrates: subunit β 1 is considered to cleave mostly after acidic residues (caspase-like activity), subunit β 2 after basic amino acids (trypsin-like activity), and subunit β 5 after hydrophobic residues (chymotrypsin-like activity) (3).

In some lymphoid tissues and in cells exposed to IFN γ , the standard catalytic subunits are replaced by their inducible counterparts β 1i (LMP2), β 2i (MECL1), and β 5i (LMP7), which are preferentially incorporated into proteasome particles, leading to the formation of immunoproteasomes (1). The activity of immunoproteasomes on fluorogenic substrates is different from that of

the standard proteasome, with a lower caspase-like activity and higher chymotrypsin- and trypsin-like activities. The processing and presentation of a number of antigenic peptides is thereby favored in cells harboring immunoproteasomes, and this contributes to shape the repertoire of immunodominant epitopes (4). However, a number of other antigens, mainly derived from self or tumoral proteins, are produced less efficiently by the immunoproteasome (5). In many cases, these processing differences result from the occurrence of a prominent internal cleavage by one proteasome type, which destroys the antigenic peptide (6). Because the immunoproteasome is abundant in dendritic cells, those differences have implications for the induction of immune responses against such epitopes (7–9). Recently, the immunoproteasome was shown to contribute to the induction of inflammatory cytokines, and its selective inhibition attenuates inflammation in a model of rheumatoid arthritis (10). The immunoproteasome also plays a key role in the degradation of oxidized proteins, thereby maintaining protein homeostasis (11, 12). The thymic cortex harbors an alternative proteasome form containing a thymus-specific β 5 subunit called β 5t, which associates with β 1i and β 2i to form thymoproteasomes (13).

The assembly of vertebrate proteasomes is a highly ordered process involving the building of half-proteasomes containing one α ring and one β ring in which catalytic β subunits are maintained inactive by the presence of an N-terminal propeptide that masks the catalytic N-terminal threonine of the mature subunit (14). The α ring serves as a scaffold for the assembly of β subunits, which are incorporated in a defined order dictated by the mutual interaction of their propeptides. Incorporation of the last β subunit, β 7, triggers the dimerization of two half-proteasomes through the intercalation of its C-terminal tail between β 1 and β 2 of the opposing β ring. This is orchestrated by a chaperone named proteasembilin or POMP. During dimerization, the catalytic subunits are activated by autocatalytic removal of the propeptides, and proteasembilin is degraded, as the first substrate of the newly matured proteasome. The assembly of immunoproteasomes was studied using cells transfected with various constructs encoding catalytic subunits and was found to be cooperative: subunits β 1i, β 2i, and β 5i interact with each other to favor their common incorporation into homogeneous immunoproteasome particles containing all three subunits, even in cells coexpressing immuno-

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¹To whom correspondence should be addressed. E-mail: Benoit.Vandeneeynde@bru.licr.org.

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and standard catalytic subunits (15). This is favored by a quicker incorporation of $\beta 1i$ over $\beta 1$, which then favors further assembly of $\beta 2i$ and $\beta 5i$ (15–18). Indeed, incorporation of $\beta 2i$ is dependent on that of $\beta 1i$, whereas $\beta 5i$ is needed for removal of the propeptide of $\beta 1i$ and $\beta 2i$. $\beta 5i$ was the only subunit able to incorporate into proteasomes independently from the other immunosubunits. These effects, which are dictated by the propeptide sequence of each subunit, theoretically allow for the existence of intermediate proteasomes containing $\beta 5i$ without $\beta 2i$ (18). Intermediate proteasomes were observed in transfected cells and were suggested to partly account for the heterogeneity of proteasome preparations isolated from rat muscle, which displayed varying physicochemical and functional properties and contained varying compositions in catalytic β subunits (18–23). Such heterogeneity was also observed in rodent heart (20, 24) and in human colon and liver (25). Posttranslational modifications, such as phosphorylation, acetylation, or methylation, were suggested to also contribute to this heterogeneity (20, 22, 26, 27). The lack of catalytic subunit-specific antibodies recognizing native proteasome particles has prevented the isolation of intermediate proteasomes so that it proved difficult to confirm their existence in normal tissues and study their actual composition in catalytic subunits. Here we produced a set of unique subunit-specific antibodies that allowed us to isolate intermediate proteasomes, confirm and quantify their presence in normal tissues, determine their composition stoichiometry in β subunits, and study their function.

Results

We produced antibodies able to immunoprecipitate human proteasomes containing subunit $\beta 1i$, $\beta 2i$, $\beta 5i$, or $\beta 5$ by immunizing rabbits with small peptides corresponding to the C terminus of each subunit and selected for their lack of homology with the other subunits, their lack of secondary structure, and their accessibility as predicted from the structure of bovine proteasome (Fig. S1) (28). From one of these rabbits, we also obtained a monoclonal antibody against subunit $\beta 5$ (29). When tested by immunoblot on purified standard or immunoproteasomes or on proteasomes isolated from cells transfected with $\beta 1i$ and/or $\beta 5i$, these antibodies each stained a unique band of the expected size only in the relevant samples (Fig. S2A). The specificity of the four antibodies was further established by immunoblotting after 2D electrophoresis and identification of the spots by mass spectrometry (Figs. S3 and S4). Each antibody stained only two spots corresponding to two isoforms of the relevant subunit. Additional data further illustrating the high specificity of these antibodies are provided in the *SI Materials and Methods*. Importantly, all these antibodies specifically recognized not only denatured proteasome subunits, but also native proteasome particles, as indicated by a sandwich ELISA (Fig. S2B). This was not true for commercial antibodies that we used for comparison, which recognized denatured proteasome subunits but not native particles (anti- $\beta 1$ shown on Fig. S2B).

We analyzed the presence of inducible subunits $\beta 1i$, $\beta 2i$, and $\beta 5i$ in total proteasomes immunoprecipitated from a series of human tumor lines (Fig. 1A). Eight of 16 tumor lines showed the presence of $\beta 1i$ and $\beta 5i$ in their proteasomes but the absence of $\beta 2i$. This lack of $\beta 2i$ excluded the presence of immunoproteasome and suggested the presence of intermediate proteasome forms containing only $\beta 1i$ and/or $\beta 5i$. To characterize those intermediate proteasomes, we depleted melanoma EB81-MEL cell lysates of $\beta 5i$ -containing proteasomes using anti- $\beta 5i$ -coated beads. We immunoprecipitated all remaining proteasomes with an anti- $\alpha 2$ antibody and analyzed their subunit content (Fig. 1B). We detected $\beta 5$ but not $\beta 5i$, indicating that the depletion was complete. We also did not detect any $\beta 1i$. This result excluded the presence of intermediate proteasomes bearing $\beta 1i$ as the only inducible subunit. We then returned to the melanoma lysate, which we depleted of $\beta 5$ -containing proteasomes. The remaining proteasomes contained $\beta 1i$, $\beta 5i$, and $\beta 1$ (Fig. 1B). They corresponded to inter-

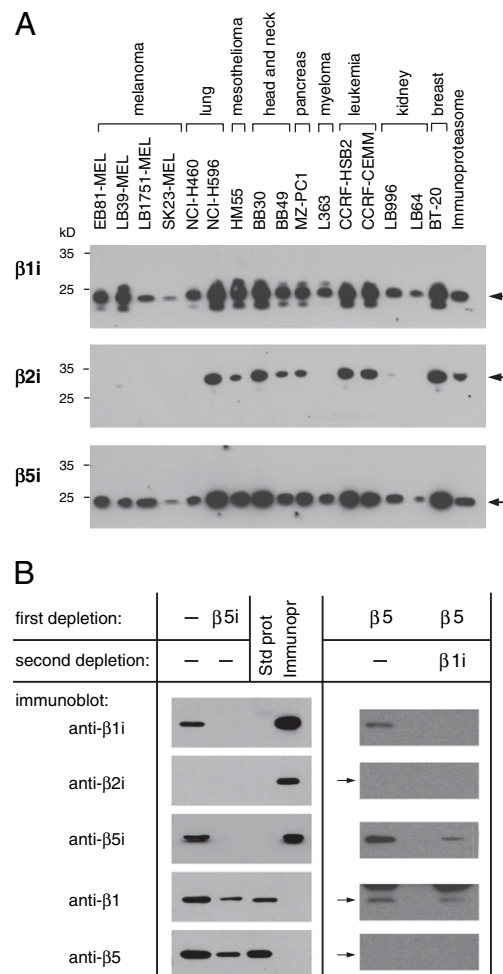


Fig. 1. Identification of intermediate proteasomes in human cancer cell lines. (A) Immunoblots performed with total proteasomes isolated by immunoprecipitation with anti- $\alpha 2$ mAb MCP21 (39) from tumor lines not exposed to IFN γ . Subunits were detected using the rabbit antibodies characterized in Figs. S2–S4. (B) Characterization of intermediate proteasomes from melanoma line EB81-MEL. Cell lysates were depleted of $\beta 5i$ -containing or $\beta 5$ -containing proteasomes before immunoprecipitation with mAb MCP21 of all of the remaining proteasomes and their analysis by immunoblot. Where indicated, a second depletion with anti- $\beta 1i$ was performed after the $\beta 5$ depletion. The commercial anti- $\beta 1$ antibodies used were of mouse origin and therefore in some cases the secondary antibody cross-reacted with the light chain of the mouse mAb used for the immunoprecipitation (Upper bands). The control showing complete depletion of $\beta 5$ -containing proteasomes is shown on Fig. S8.

mediate proteasomes containing $\beta 1i$ and $\beta 5i$, and probably also proteasomes containing $\beta 1$ and $\beta 5i$. To confirm the presence of the latter, we repeated depletion of the melanoma lysate with anti- $\beta 5$ antibodies and performed a second depletion using our $\beta 1i$ -specific antibodies. The remaining proteasomes contained both $\beta 5i$ and $\beta 1$, confirming the presence of proteasomes containing $\beta 5i$ as the only inducible subunit in those melanoma cells (Fig. 1B). We also analyzed the proteasomes immunoprecipitated with anti- $\beta 5$ antibodies: they did not contain $\beta 1i$, further excluding the presence of proteasomes $\beta 1i$ - $\beta 2$ - $\beta 5$ (Fig. S5A). In conclusion, this analysis demonstrated the presence of two intermediate proteasome types in these melanoma cells, one with catalytic subunits $\beta 1$, $\beta 2$, and $\beta 5i$ (proteasome $\beta 5i$) and one with catalytic subunits $\beta 1i$, $\beta 2$, and $\beta 5i$ (proteasome $\beta 1i$ - $\beta 5i$). Similar results were obtained with melanoma lines LB39-MEL and LB1751-MEL, and myeloma line

L363. Lung carcinoma line NCI-H460 was also analyzed and was found to contain only the intermediate proteasome β 1i- β 5i.

To determine the relative abundance of the different proteasome types in those cell lines, we repeated the depletion approach and quantified the proteasomes after each step using a sandwich ELISA (30). Additional quantifications were performed by testing total lysates directly in sandwich ELISA using the anti- β 5, anti- β 1i, anti- β 2i or anti- β 5i antibodies for detection. Intermediate proteasomes represented 9 to 17% of the proteasome content of melanoma cells and comprised a majority of proteasome β 5i (Table 1). Lung carcinoma line NCI-H460 and myeloma line L363 contained about 20% of proteasome β 1i- β 5i.

In normal human tissues, we observed high proportions of intermediate proteasomes: these represented between one-third and one-half of the proteasome content of liver, kidney, small bowel, and colon (Table 1). Liver and kidney essentially contained standard proteasomes and intermediate proteasome β 5i. Small bowel mostly contained immunoproteasomes, together with both types of intermediate proteasomes, whereas colon contained equally high amounts of immunoproteasomes and intermediate proteasome β 5i. Heart mostly contained standard proteasomes (Table 1). It should be stressed that, because of the difficulty in obtaining normal human tissues, these estimates are based on small numbers of samples and should be taken as indications rather than precise and final quantifications.

Intermediate proteasomes were also abundant in monocyte-derived dendritic cells (Table 1). Proteasome β 5i was the most abundant form, accounting for about 40% of the proteasome content of dendritic cells. These proportions were relatively similar in immature and mature dendritic cells, but large variations were observed between different donors, possibly reflecting differences in the inflammatory conditions prevailing in vivo in the donors of blood monocytes.

Activity of Intermediate Proteasomes. To study the function of intermediate proteasomes, we produced cells whose proteasome content was composed exclusively of intermediate proteasomes β 5i or intermediate proteasomes β 1i- β 5i. This was achieved by

transfecting 293-EBNA cells, which contain only standard proteasomes, with strong expression vectors encoding β 1i or β 5i. The overexpression of the transfected subunit ensured a complete replacement of standard proteasomes by intermediate proteasomes β 5i in the β 5i-transfected cells 293- β 5i, or by proteasomes β 1i- β 5i in doubly transfected cells 293- β 1i- β 5i (Fig. S24). When tested on fluorogenic substrates, the activity of intermediate proteasomes purified from those cells was similar, although not identical, to that of the immunoproteasome with regard to the chymotrypsin- and trypsin-like activities (Fig. S6). The caspase-like activity, which is attributed to the β 1 subunit, was predictably different for the two intermediate proteasomes: high for proteasome β 5i, like the standard proteasome, and very low for the proteasome β 1i- β 5i, like the immunoproteasome.

Exclusive Processing of a MAGE-A10 Tumor Peptide by the Intermediate Proteasome β 1i- β 5i. Peptide GLYDGMEL₂₅₄₋₂₆₂ is an HLA-A2-restricted tumor antigenic peptide encoded by gene *MAGE-A10* and currently used in cancer vaccine trials (31). The presentation of this antigen is proteasome dependent (Fig. S7). We compared the production of this antigenic peptide by the different proteasome types using two complementary approaches. First, we transfected our panel of 293 cell lines expressing either proteasome type with increasing amounts of *MAGE-A10* cDNA. Only cells containing intermediate proteasomes β 1i- β 5i were efficiently recognized by the MAGE-A10/HLA-A2-specific CTL clone (Fig. 2A). Second, we used proteasomes purified from the different 293 cells to digest a precursor peptide of 24 amino acids encompassing the antigenic peptide. Using HPLC coupled to mass spectrometry (HPLC-MS), we observed production of the antigenic peptide in the digest performed with intermediate proteasomes β 1i- β 5i, but not in the other digests (Fig. 2B). The main fragments observed in the other digests resulted from a cleavage within the sequence of the antigenic peptide, between D₂₅₇ and G₂₅₈ (Fig. 2C). These fragments were much less abundant in the digest obtained with proteasomes β 1i- β 5i. This result indicates that proteasome β 1i- β 5i is less active at destroying the antigenic peptide. As a result, fragments with the proper N terminus or C terminus of the antigenic

Table 1. Quantification of intermediate proteasomes in human cells and tissues

Cell type	% standard proteasome ($\pm$ SD)	% immunoproteasome ($\pm$ SD)	Intermediate proteasomes	
			% β 5i ($\pm$ SD)	% β 1i- β 5i ($\pm$ SD)
Tumor lines				
Melanoma EB81-MEL*	87	0	10	3
Melanoma LB1751-MEL*	91	0	7	2
Melanoma LB1751-MEL [†]	83	<0.7%	9	8
Melanoma SK23-MEL [†]	89	<0.7%	7	4
Nonsmall cell lung carcinoma NCI-H460*	80	0	0	20
Myeloma L363 [†]	72	<2.5%	5	23
Normal tissues				
Liver [†] (n = 3)	31 ($\pm$ 3)	15 ($\pm$ 2)	50 ($\pm$ 8)	4 ($\pm$ 6)
Kidney (cortex) [†] (n = 1)	61	9	30	0
Small bowel (mucosa) [†] (n = 1)	0	64	15	21
Colon (mucosa) [†] (n = 2)	2 ($\pm$ 4)	49 ($\pm$ 7)	49 ($\pm$ 10)	0 ($\pm$ 0)
Heart [†] (n = 3)	83 ($\pm$ 3)	12 ($\pm$ 8)	2 ($\pm$ 4)	3 ($\pm$ 5)
Antigen-presenting cells				
Monocytes [†] (n = 1)	15	31	0	54
Immature dendritic cells [†] (n = 3)	4 ($\pm$ 4)	29 ($\pm$ 19)	42 ($\pm$ 5)	24 ($\pm$ 12)
Mature dendritic cells (cytokines) [†] (n = 2)	8 ($\pm$ 0.4)	44 ($\pm$ 7)	42 ($\pm$ 2)	6 ($\pm$ 9)
Mature dendritic cells (LPS) [†] (n = 3)	4 ($\pm$ 1)	47 ($\pm$ 4)	39 ($\pm$ 23)	10 ($\pm$ 17)

The proportions of four proteasomes types were determined by two different sandwich ELISAs, indicated by* and[†], both using MCP21 as capture antibody (39). *This ELISA quantified all proteasomes remaining after depletions as described in Fig. 1B and used as detection antibody a polyclonal recognizing all proteasome types as described (30). Samples from n individuals were tested.

[†]This ELISA used the β 5-, β 1i-, β 2i-, and β 5i-specific antibodies described here as detection antibodies and was applied directly to cell/tissue lysates. The calculation method for the different proteasome types is detailed in *Materials and Methods*.

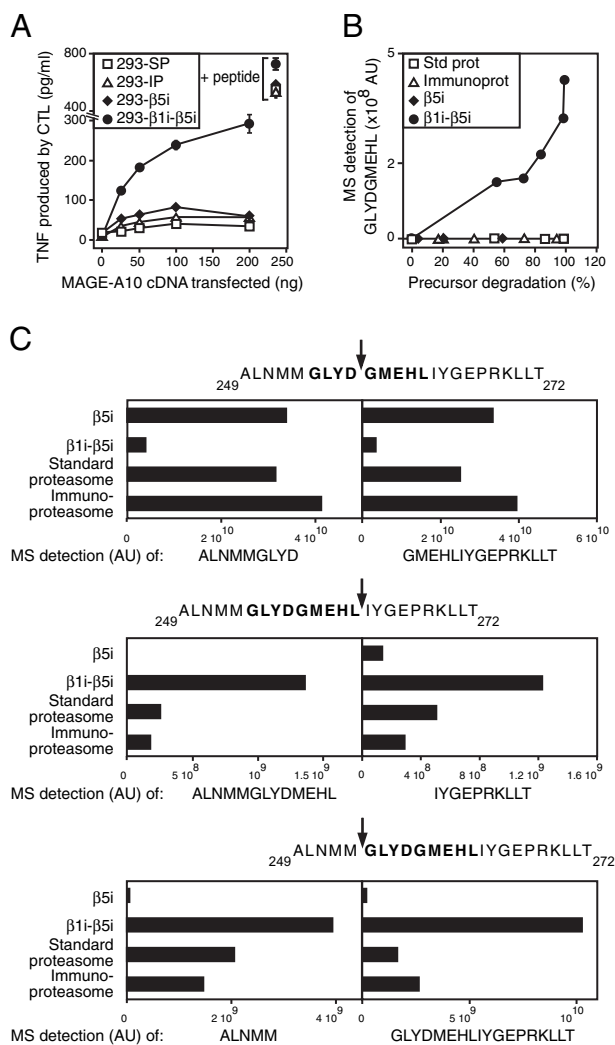


Fig. 2. Exclusive processing of peptide MAGE-A10_{254–262} by the intermediate proteasome $\beta 1i$ - $\beta 5i$. (A) Processing of peptide GLYDGM_{254–262} by 293-EBNA cells containing different proteasome subtypes (characterized in Fig. S2). 293-SP, untransfected 293-EBNA cells containing only standard proteasomes (SP); 293- $\beta 1i$, 293-EBNA cells transfected with a construct encoding $\beta 1i$; 293- $\beta 1i$ - $\beta 5i$, transfected with $\beta 1i$ and $\beta 5i$; 293-IP, transfected with $\beta 1i$, $\beta 2i$, and $\beta 5i$, and containing only immunoproteasomes (IP). Cells were transfected with an HLA-A2 construct and the indicated amount of plasmid encoding MAGE-A10. As positive control, cells were loaded with the synthetic peptide at 0.5 μ g/mL MAGE-A10-specific CTL was added and TNF production was measured. (B) Detection of antigenic peptide GLYDGM_{254–262} in digests obtained by incubating precursor peptide ALNMMGLYDGM_{254–262} with purified 20S proteasomes. Digests were analyzed by mass spectrometry coupled to HPLC, and the detection of the relevant ion was plotted as a function of the degradation of the precursor peptide. AU, arbitrary units. (C) MS detection of the indicated peptide fragments in the digests shown in B, analyzed at a time point corresponding to a precursor degradation of 59% ($\beta 5i$), 55% ($\beta 1i$ - $\beta 5i$), 53% (SP), and 40% (IP).

peptide become prominent in the digests obtained with proteasome $\beta 1i$ - $\beta 5i$ (Fig. 2C). We concluded that most proteasome types, except proteasome $\beta 1i$ - $\beta 5i$, destroy the antigenic peptide by internal cleavage. The lack of destructive cleavage by proteasome $\beta 1i$ - $\beta 5i$ may explain its exclusive capacity to produce the antigenic peptide. Those results imply that the recognition of tumor cells by the MAGE-A10-specific CTL is made possible by their expression of proteasome $\beta 1i$ - $\beta 5i$. Remarkably, melanoma line LB1751-MEL, which is recognized by the CTL, contains only 2–8% of proteasome $\beta 1i$ - $\beta 5i$ (Table 1), indicating that a low proportion of the appro-

prate proteasome type is sufficient to produce enough antigenic peptide for CTL recognition.

Exclusive Processing of a MAGE-A3 Tumor Peptide by the Intermediate Proteasome $\beta 5i$. We analyzed the processing of another tumor peptide widely used in cancer vaccine trials, the MAGE-A3-derived peptide FLWGPRALV_{271–279} presented by HLA-A2 (32). The processing and presentation of this antigen seems unconventional: although some HLA-A2⁺ tumor cells expressing MAGE-A3 were clearly recognized by CTL specific for this peptide, others were not, leading to the proposal that this peptide was poorly processed (33, 34). Moreover, the treatment of tumor cells with proteasome inhibitors was surprisingly able to induce their recognition by the CTL (ref. 34 and Fig. 3A). We transfected our various 293 cells with increasing amounts of a MAGE-A3 cDNA and observed that only cells containing intermediate proteasomes $\beta 5i$ were recognized by the specific CTL clone (Fig. 3B). When we digested a 23-amino-acid-long precursor peptide with purified proteasomes of either type and analyzed the digests by HPLC-MS, we detected the production of the antigenic peptide FLWGPRALV after digestion with proteasome $\beta 5i$, but not with proteasomes $\beta 1i$ - $\beta 5i$ or immunoproteasomes, and only weakly with standard proteasomes (Fig. 3C, Left). In the presence of proteasome inhibitor epoxomicin, the digest obtained with standard proteasomes became strongly positive, whereas the other digests remained unchanged (Fig. 3C, Left). Similar results were obtained when the digests were loaded onto HLA-A2⁺ target cells and tested for recognition by specific CTL (Fig. 3C, Right). These results suggested that standard proteasomes destroyed the antigenic peptide by internal cleavage through an epoxomicin-sensitive activity, whereas an epoxomicin-resistant activity was responsible for production of the antigenic peptide. They also suggested that the destructive activity was absent in proteasome $\beta 5i$.

Among the activities monitored with fluorogenic substrates, the least sensitive to epoxomicin is the caspase-like activity, which is exerted by the $\beta 1$ subunit and cleaves after acidic and branched residues including valine, which is present at the C terminus of the MAGE-A3/HLA-A2 peptide (Fig. S6B) (3). This activity is high in standard proteasomes and proteasomes $\beta 5i$, which have the $\beta 1$ subunit, whereas it is low in immunoproteasomes and proteasomes $\beta 1i$ - $\beta 5i$, which have the $\beta 1i$ subunit (Fig. S6). The $\beta 1$ subunit could therefore be responsible for the epoxomicin-resistant activity needed to produce the C-terminal cleavage of the antigenic peptide. Indeed, when we analyzed the digests by HPLC-MS, we found that only standard proteasomes and proteasomes $\beta 5i$ were able to perform the C-terminal cleavage after valine (Fig. 3D). The data also confirmed that this cleavage was not clearly prevented by epoxomicin. Rather, the abundance of the fragments resulting from this C-terminal cleavage was increased by epoxomicin in the digests obtained with standard proteasomes, likely because epoxomicin prevented a prominent “competitive” destructive cleavage within the antigenic peptide. Indeed, a prominent cleavage was observed between F₂₇₁ and L₂₇₂ in the digests obtained with standard proteasomes, but not in the other digests (Fig. 3D), suggesting an involvement of subunit $\beta 5$ in this cleavage. This was further supported by the fact that this cleavage was strongly reduced by epoxomicin. Altogether, these results indicate that efficient processing of the MAGE-A3/HLA-A2 peptide requires the presence of subunit $\beta 1$ to make the proper C terminus and the absence of subunit $\beta 5$ to avoid a prominent destructive cleavage within the peptide. These conditions are met naturally with intermediate proteasomes $\beta 5i$ and artificially with standard proteasomes exposed to epoxomicin, which blocks $\beta 5$ but not $\beta 1$ activity.

Discussion

The existence of intermediate proteasomes $\beta 5i$ and $\beta 1i$ - $\beta 5i$ reported here is consistent with the rules of cooperative assembly of inducible catalytic subunits (15–18). These intermediate pro-

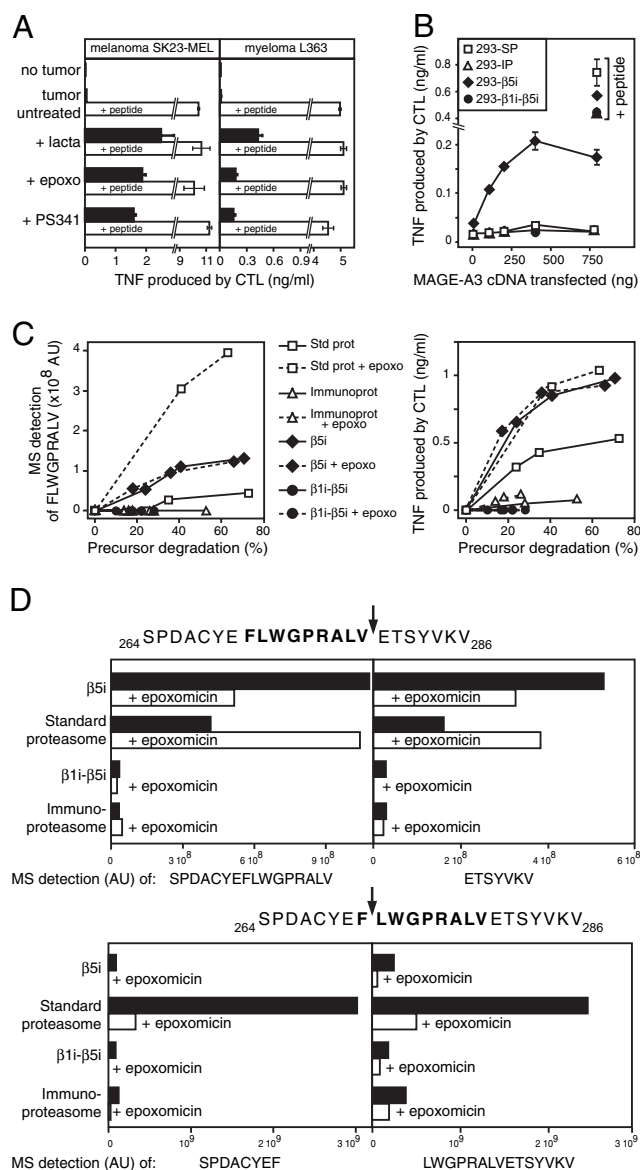


Fig. 3. Exclusive processing of peptide MAGE-A3₂₇₁₋₂₇₉ by the intermediate proteasome β5i. (A) Restoration of the presentation of MAGE-A3 peptide FLWGPRALV₂₇₁₋₂₇₉ by tumor cells after treatment with proteasome inhibitors. Melanoma SK23-MEL and myeloma L363 were treated with lactacystin (50 μM), epoxomicin (1 μM), or PS341 (50 nM) for 1 h before addition of the MAGE-A3₂₇₁₋₂₇₉-specific CTL. The synthetic peptide (0.2 μg/mL) was loaded on tumor cells as positive control. Production of TNF was measured after 24 h. (B) Processing of peptide FLWGPRALV₂₇₁₋₂₇₉ by 293-EBNA cells containing different proteasome subtypes. Cells were transfected with HLA-A2 and the indicated amount of plasmid encoding MAGE-A3. As positive control, cells were loaded with the peptide at 1 μg/mL. MAGE-A3-specific CTL was added and TNF production was measured. (C) Detection of antigenic peptide SPDACYEFLWGPRALV₂₇₁₋₂₇₉ obtained by incubating precursor peptide SPDACYEFLWGPRALV₂₇₁₋₂₇₉ with purified 20S proteasomes, in the absence (solid lines) or in the presence (dotted lines) of 1 μM epoxomicin. (Left) Digests were analyzed by mass spectrometry coupled to HPLC, and the detection of the relevant ion was plotted as a function of the degradation of the precursor peptide. (Right) The same digests were loaded onto HLA-A2* melanoma cells LB2667-MEL. MAGE-A3-specific CTL was added and TNF production was measured. Similar results were obtained when lactacystin was used instead of epoxomicin. (D) MS detection of the indicated peptide fragments in the digests shown in C, analyzed at a time point corresponding to a precursor degradation of 41% (β5i), 36% (β5i + epoxo), 35% (SP), 41% (SP + epoxo), 22% (β1i-β5i), 28% (β1i-β5i + epoxo), 28% (IP), and 26% (IP + epoxo). Similar results were obtained when lactacystin was used instead of epoxomicin.

teasomes all have β5i, which is needed for the maturation of β1i and β2i, and none of them has β2i, which cannot incorporate without β1i. The abundance of intermediate proteasomes β5i in human liver and colon is in line with the previous detection of high amounts of subunit β5i with low amounts of β1i and β2i in these tissues (25). Human heart mostly contained standard proteasomes, in line with a recent study performed in rat heart (24).

Our study focused initially on the characterization of proteasomes in tumor cell lines lacking β2i (Fig. 1). Our results exclude the presence of proteasomes β1i-β2-β5 and demonstrate the presence of intermediate proteasomes β1-β2-β5i and β1i-β2-β5i. Cells containing β2i might theoretically contain proteasomes β1-β2i-β5, β1i-β2i-β5, and/or β1-β2i-β5i. Although the rules of cooperative assembly preclude the formation of such proteasomes, one study suggested the possible assembly of proteasomes β1-β2i-β5i (18). We searched for such proteasomes in human liver by analyzing the proteasomes immunoprecipitated with anti-β2i antibodies: we found β1i and β5i but no β1 or β5, indicating that all of the β2i subunits are associated with immunoproteasomes, and confirming the absence of intermediate proteasomes bearing β2i (Fig. S5C).

The mature proteasome particle is made of two α rings and two β rings, arranged in a symmetrical order αββα. The existence of proteasomes with an asymmetrical assortment of inducible and standard catalytic subunits is conceivable. Such asymmetric proteasomes were recently observed in HeLa cells transfected with a tagged β1 subunit and exposed to IFNγ (21). The question remains whether asymmetrical proteasomes exist in untransfected cells and normal tissues. The approach we followed to characterize intermediate proteasomes in tumor lines ensured that we detected symmetrical intermediate proteasomes, as any asymmetrical particle containing both β5 and β5i, or both β1 and β1i, would have been depleted with the antibodies to β5i, β5, or β1i (Fig. 1B). We can therefore confirm the existence of symmetrical intermediate proteasomes β5i and β1i-β5i. These results, however, do not exclude the existence of asymmetrical proteasomes. We performed additional experiments where we analyzed proteasomes immunoprecipitated with anti-β5 in immunoblots probed with anti-β5i antibodies. We did not observe proteasomes bearing β5 together with β5i in melanoma cells nor in normal kidney, indicating the absence of asymmetrical proteasomes β5/β5i in these samples (Fig. S5B).

Although the activity profile of intermediate proteasomes on fluorogenic substrates largely fits the catalytic activities previously ascribed to each subunit (3), our results illustrate limitations in the definition of activities with fluorogenic substrates and their ascription to a given subunit. The replacement of subunit β1 by β1i decreased the caspase-like activity, as expected (Fig. S6). The chymotrypsin-like activity is expected to increase upon replacement of β5 by β5i. We did observe an increased chymotrypsin-like activity in proteasomes containing β5i, although this was clearer with one of the two fluorogenic substrates tested (Fig. S6). The trypsin-like activity, which is considered to be exerted by subunits β2/β2i, is increased in intermediate proteasomes β5i and β1i-β5i as compared with standard proteasomes, even though they all have subunit β2, suggesting that several distinct catalytic subunits contribute to a given activity. In addition, the classification into chymotrypsin-like, trypsin-like, and caspase-like proteasome activities does not consider the sequence flanking the cleavage site in the peptidic substrate. Flanking residues located before or after the cleavage site were shown to influence cleavage efficiency (35). Altogether, these considerations may explain the unexpected destructive cleavage of the MAGE-A10/HLA-A2 peptide by the immunoproteasome after an internal aspartic acid (Fig. 2), despite a low caspase-like activity measured on fluorogenic substrates (Fig. S6). In a similar fashion, the destructive cleavage of the MAGE-A3/HLA-A2 peptide between F271 and L272 is exerted by subunit β5 and not by β5i, despite the higher chymotrypsin-like activity of β5i. The study of preferred cleavage sites of protein enolase indicated that immunoproteasomes, as opposed

to standard proteasomes, favor cleavage after hydrophobic residues but disfavor cleavage before hydrophobic residues, suggesting that subunit $\beta 5i$ —as opposed to $\beta 5$ —does not cleave efficiently in the middle of hydrophobic stretches (35). This conclusion, which was supported by our analysis of the processing of other antigenic peptides (30), explains the destructive cleavage of the MAGE-A3 peptide reported here, which occurs between two hydrophobic residues and is exerted by subunit $\beta 5$ and not $\beta 5i$.

The existence of intermediate proteasomes, their presence in tumor and dendritic cells, their abundance in normal tissues, and their different activity profiles may have important bearings on the development of CD8⁺ immune responses. We describe two tumor antigens whose processing is dependent on the presence of intermediate proteasomes. It is likely that other antigens involved in host defense or autoimmunity are also processed differently by the intermediate proteasomes. The repertoire of antigens presented by a given cell is therefore, at least partly, dependent on its proteasome content. In a clinical context, it may become important to determine the proteasome content of cells that are targeted by immunotherapeutic approaches. A change in this proteasome content may modulate the antigenic repertoire, preventing or ensuring immune recognition of the cells. A clear illustration is the example of the MAGE-A3/HLA-A2 peptide, for which conflicting data were reported in the literature regarding its presentation by tumor cells (32–34). Our results provide an explanation for this controversy, by showing that the processing of this antigenic peptide is dependent on the presence of intermediate proteasomes $\beta 5i$. They also explain the reported paradoxically increased presentation of the MAGE-A3 peptide upon treatment with protea-

some inhibitors (34), which efficiently block the activity of subunit $\beta 5$, responsible for destruction of the antigenic peptide, but not that of $\beta 1$, responsible for production of the C terminus of the antigenic peptide. The presentation of other antigenic peptides is increased by lactacystin (36–38) and, in two cases, by transfection of $\beta 5i$ (36, 38), suggesting that the processing of these peptides also depends on the intermediate proteasome $\beta 5i$.

An interesting question for further studies will be whether, in addition to changing the antigenic repertoire, modulation of the proteasome content also affects other proteasome functions such as the activation of transcription factors, the production of inflammatory cytokines, the regulation of the cell cycle, or the degradation of oxidized proteins.

Materials and Methods

Quantification of Proteasome Subtypes by Sandwich ELISA. The proportions of proteasome subtypes were determined on the basis of the ELISA results, as follows: total amount of proteasomes = $\beta 5 + \beta 5i$; amount of immunoproteasomes = $\beta 2i$; amount of intermediate proteasomes $\beta 5i = \beta 5i - \beta 1i$; amount of intermediate proteasomes $\beta 1i - \beta 5i = \beta 1i - \beta 2i$. More details are provided in *SI Materials and Methods*.

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