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Analysis of the Processing of Seven Human Tumor Antigens by Intermediate Proteasomes

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We recently described two proteasome subtypes that are intermediate between the standard proteasome and the immunoproteasome. They contain only one (β5i) or two (β1i and β5i) of the three inducible catalytic subunits of the immunoproteasome. They are present in tumor cells and abundant in normal human tissues. We described two tumor antigenic peptides that are uniquely produced by these intermediate proteasomes. In this work, we studied the production by intermediate proteasomes of tumor antigenic peptides known to be produced exclusively by the immunoproteasome (MAGE-A314–122, MAGE-C242–50, MAGE-C236–344) or the standard proteasome (Melan-A26–35, tyrosinase369–377, gp100209–217). We observed that intermediate proteasomes efficiently produced the former peptides, but not the latter. Two peptides from the first group were equally produced by both intermediate proteasomes, whereas MAGE-C236–344 was only produced by intermediate proteasome β1i-β5i. Those results explain the recognition of tumor cells devoid of immunoproteasome by CTL recognizing peptides not produced by the standard proteasome. We also describe a third antigenic peptide that is produced exclusively by an intermediate proteasome: peptide MAGE-C2191–200 is produced only by intermediate proteasome β1i-β5i. Analyzing in vitro digests, we observed that the lack of production by a given proteasome usually results from destruction of the antigenic peptide by internal cleavage. Interestingly, we observed that the immunoproteasome and the intermediate proteasomes fail to cleave between hydrophobic residues, despite a higher chymotrypsin-like activity measured on fluorogenic substrates. Altogether, our results indicate that the repertoire of peptides produced by intermediate proteasomes largely matches the repertoire produced by the immunoproteasome, but also contains additional peptides. The Journal of Immunology, 2012, 189: 3538–3547.

The proteasome is responsible for most of the nonlysosomal degradation of intracellular proteins in eukaryotic cells (1). Some of the peptides resulting from this degradation end up as ligands bound to cell surface MHC class I molecules, and as such represent most of the antigenic peptides presented to CD8 T lymphocytes. The 20S core of the proteasome is made of four stacked rings forming a barrel (2). The two outer rings are identical and contain seven different α subunits each, whereas the two identical inner rings contain seven different β subunits each, three of which, namely β1, β2, and β5, exert catalytic activities. In some lymphoid tissues and in cells exposed to IFN-γ, those standard catalytic subunits are replaced by their inducible counterparts, β1i (LMP2), β2i (MECL1), and β5i (LMP7), which are preferentially incorporated into proteasome particles, leading to the formation of immunoproteasomes (3). When tested on small fluorogenic substrates, immunoproteasomes show a higher ability to cleave after hydrophobic residues (chymotrypsin-like activity ascribed to β5/β5i) and basic residues (trypsin-like activity ascribed to β2/β2i) and a lower ability to cleave after acidic residues (caspase-like activity ascribed to β1/β1i) as compared with standard proteasomes (4).

A number of antigenic peptides are differentially processed by the immunoproteasome and the standard proteasome (5–8). Such differential processing may have important consequences for the development of CD8 T cell responses and for immune escape through Ag loss (6, 9).

A thymus-specific β-catalytic subunit named β5t was also recently described in thymic cortical epithelial cells. It associates with β1i and β2i to form thymoproteasomes, which produce peptides involved in positive selection of CD8+ T lymphocytes (10).

Using new Abs able to recognize β-catalytic subunits in their native and denatured conformation, we recently isolated two intermediate proteasome types, which have incorporated only one or two of the three catalytic subunits of the immunoproteasome (11). Intermediate proteasome β5i and intermediate proteasome β1i-β5i together represent about half of the proteasome content of immature and mature dendritic cells and 30–50% of total proteasomes in normal human liver, kidney, and gut. When tested on fluorogenic substrates, they showed high chymotrypsin- and trypsin-like activities, like the immunoproteasome (11). This was surprising because both intermediate proteasomes contain subunit β2, which is associated with the low trypsin-like activity of the standard pro-
teasome. As expected, the caspase-like activity, which is attributed to subunit β1, was high for proteasome β5i and low for proteasome β1i-β5i. Importantly, we have described two antigenic peptides that are produced only by intermediate proteasomes and not by the standard proteasome nor the immunoproteasome (11).

In this work, we analyze the processing of seven human tumor antigenic peptides by the intermediate proteasomes in comparison with the standard proteasome and the immunoproteasome. Three of these peptides were previously found to be more efficiently produced by the immunoproteasome, and three others more efficiently by the standard proteasome.

Materials and Methods

Cell lines

Melanoma cell line EB81-MEL-2 was described previously (12). CTL clones 6, 16, and 1 recognize peptides MAGE-C2-391–399, MAGE-C2-336–344, and MAGE-C2-50, respectively (12, 13). CTL clone CHAX 526 F7.52 recognizes peptide MAGE-A3-114–122 (7). CTL clones 246/15, 606 C2/1, and IVSB recognize peptides Melan-A-26-35 (15), gp100209–217 (14), and tyrosinase369–377, respectively (15).

Proteasome purification

The proteasomes of EB81-MEL were purified from ~2 × 10^7 cells. Frozen pellets were lysed with four freeze/thaw cycles and resuspended in a 3- to 5-fold volume (v/v) of buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA [pH 7.5], 10 µg DNase) supplemented with 0.1% Nonidet P-40 and 1 mM EDTA. After centrifugation at 100,000 × g for 1 h at 4°C, the supernatants were precipitated with 35% (NH4)2SO4. The precipitates were dissolved in PBS and centrifuged at 3000 × g for 30 min at 4°C. Cleared supernatants were desalted on a HiPrep 26/10 desalting column (GE Healthcare) equilibrated with buffer. Proteasomes were first purified by immunoaffinity chromatography on CNBr-activated 4B Sepharose beads (±3 ml; Amersham Pharmacia Biotech) coupled to mAb M519, which is directed against the α2 subunit of the proteasome (16). Proteasomes were eluted with 20 mM Tris-HCl and 3 M NaCl.

Two-dimensional gel electrophoresis and liquid chromatography-tandem mass spectrometry analysis

For a resolution of the proteasomal subunits, an isoelectric focusing by carrier ampholytes was combined with SDS-PAGE, as previously described (17). The purified proteasomes from tumor cell line EB81-MEL (80 µg) were dialyzed against milliQ water, TCA/acetone precipitated, and then isoelectrofocalsed in a nonlinear ampholate gradient on an immobilized pH gradient strip of 17 cm in length (pH 3–10 NL; Bio-Rad), so that isof orm separation could be achieved. In the second dimension, proteins were separated in a 0.5-mm–thick SDS-PAGE gel (20 × 20 cm) and stained with colloidal blue. For identification, protein spots were excised from the gel, digested with trypsin, and analyzed by nano-liquid chromatography coupled to tandem mass spectrometry (MS/MS) on an ESI-LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific) operating in positive mode, as recently described (18).

Proteasome inhibition

EB81-MEL cells were first incubated with 1 µM epoxomicin for 1.5 h, then with a low dose of epoxomicin (0.0625 µM) overnight at 40,000 cells/well. Half of these cells were loaded with the antigenic peptide (0.1 µg/ml) 1 h before the CTL assay. For this assay, the tumor cells were incubated for 6 h with 10^3 cells of the MAGE-C2391–399-specific CTL clone in the presence of 25 U/ml IL-2, and the production of TNF-α was measured.

Assay of Ag presentation by 293-SP, 293-β5i, 293-β1i-β5i, and 293-IP cells

The 293-EBNA cells expressing different proteasome types were described earlier (11). For the Ag presentation assay, 30,000–100,000 cells were transiently transfected (Lipofectamine) with 50 ng pcDNA3-HLA-A2 and titrated amounts of the indicated cDNAs cloned into the following plasmids: pCP4 for MAGE-C2, pcDNA3 for MAGE-A3, gp100, tyrosinase, and pcDNA-HA/Amp for Melan-A. These DNAs were diluted in empty vector DNA, as needed. Cells of the relevant CTL clone (8,000–30,000) were added after 24–48 h, and IFN-γ or TNF production was measured after 18 h (19).

Digestion of synthetic peptides with purified proteasomes and mass spectrometry analysis

Standard proteasomes and immunoproteasomes were purified, as described earlier (8). Intermediate proteasomes β5i and β1i-β5i were purified from the corresponding transfected 293 cells using the same technique. Peptides were synthesized on solid phase using Fmoc chemistry and purified by reverse-phase HPLC. Precursor peptides (>95% pure; 1 or 2.5 µg per time point) were incubated with proteasomes (1–1 µg/time point) at 37°C for 10 min Tris-HCl (pH 7.4) (25 µl/time point). At each time point, an aliquot was taken from the digestion mixture, added to 2 µl trifluoroacetic acid (TFA) 10%, and lyophilized. For the mass spectrometry (MS) analysis, the digests were injected on a PepMAP C18 0.3/15 reverse-phase chromatography column (LC Packings) and eluted with a 40-min linear gradient of acetonitrile in water (5–50%) containing 0.0% TFA with a flow rate of 4 µl/min. The MS analysis was performed on line with a LCQ Deca XP ion-trap spectrometer equipped with an electrospray ionization source (ThermoFinnigan).

Digestion times

Fig. 2 shows digestion of the MAGE-A3114–122 precursor with standard proteasome for 0, 10, 20, and 30 min; with immunoproteasome for 0, 20, 40, and 60 min; with intermediate proteasome β5i for 0, 240, 480, and 600 min; and with intermediate proteasome β1i-β5i for 0, 20, 40, and 60 min. Fig. 2 illustrates digestion of the MAGE-C232–40 precursor with standard proteasome for 0, 10, 20, and 30 min, and with immunoproteasome for 0, 90, 180, and 360 min; with intermediate proteasome β5i for 0, 120, 240, and 360 min, and with intermediate proteasome β1i-β5i for 0, 30, 60, and 90 min. Fig. 2 also shows digestion of the MAGE-C253–64 precursor with standard proteasome for 0, 10, 20, 30, and 45 min; with immunoproteasome for 0, 120, 240, and 360 min; with intermediate proteasome β5i for 0, 10, 20, 30, and 45 min; and with intermediate proteasome β1i-β5i for 0, 120, 240 and 360 min.

Fig. 3 shows digestion of the Melan-A26–35 precursor with standard proteasome for 0, 30, 60, and 120 min; with immunoproteasome for 0, 30, 60, and 120 min; with intermediate proteasome β5i for 0, 30, 60, and 120 min; and with intermediate proteasome β1i-β5i for 0, 30, 60, and 120 min.
Fig. 3 illustrates digestion of the gp100209–217 precursor with standard proteasome for 0, 30, 60, and 120 min; with immunoproteasome for 0, 60, 120, and 240 min; and with intermediate proteasome β1i-β5i for 0, 120, 240, and 360 min. Fig. 3 also shows digestion of the tyrosinase367–377 precursor with standard proteasome for 0, 90, 120, and 180 min; with immunoproteasome for 0, 180, 360, and 480 min; with intermediate proteasome β5i for 0, 180, 360, and 480 min; and with intermediate proteasome β1i-β5i for 0, 180, 360, and 480 min.

Fig. 7 shows digestion of the MAGE-C2191–200 precursor with standard proteasome for 0, 5, 10, and 20 min; with immunoproteasome for 0, 15, 30, and 45 min; with intermediate proteasome β5i for 0, 20, 40, and 60 min; and with intermediate proteasome β1i-β5i for 0, 30, 60, and 90 min.

Results

Tumor antigenic peptides MAGE-C2136–344 and MAGE-C242–50, derived from cancer germline gene MAGE-C2, are produced efficiently by the immunoproteasome, but not by the standard proteasome (8, 12, 13). Accordingly, CTLs clones directed against these peptides recognized EB81-MEL tumor cells treated with IFN-γ (Fig. 1). Surprisingly, however, untreated EB81-MEL were also recognized by those CTL clones (Fig. 1). We purified 20S proteasomes from untreated EB81-MEL tumor cells and analyzed their composition by two-dimensional gel electrophoresis combined with MS. We observed the presence of β1i and β5i, but not β2i, indicating the presence of intermediate proteasomes (Supplemental Fig. 1). This result confirmed previous analyses showing the absence of immunoproteasome and the presence of standard proteasome and both intermediate proteasomes β5i and β1i-β5i in untreated EB81-MEL tumor cells (11). It suggested that the recognition of untreated EB81-MEL cells might be due to the processing of these MAGE-C2 Ags by intermediate proteasomes. Similarly, we previously reported that peptide MAGE-A3114–122, which is presented by HLA-B40 and depends on the immunoproteasome, was efficiently presented by 293-EBNA cells transfected with immunosubunit β5i, alone or combined with immunosubunit β1i (7). These observations led us to hypothesize that the intermediate proteasomes can process many Ags in a similar way as the immunoproteasome.

Production of immunoproteasome-dependent antigenic peptides by intermediate proteasomes

We first compared the four proteasome types for their ability to produce the immunoproteasome-dependent peptides mentioned above. We previously generated 293-EBNA cells overexpressing each proteasome type (11). We transfected each of these 293 cell lines with increasing amounts of the cDNA encoding MAGE-A3 or MAGE-C2 together with a fixed amount of a plasmid encoding the corresponding HLA molecule (Fig. 2A). We observed that cells bearing either intermediate proteasome presented peptides MAGE-
A3114–122 and MAGE-C242–50 as efficiently as cells equipped with the immunoproteasome (Fig. 2A). In contrast, peptide MAGE-C2336–344 was only presented by cells containing the immunoproteasome or intermediate proteasome β1i-β5i (Fig. 2A).

In a second approach, we purified each proteasome type from 293 cells and used them to digest precursor peptides encompassing the different antigenic peptides. We analyzed the digests by HPLC coupled to MS (HPLC-MS) or MS/MS (HPLC-MS/MS) for the presence of antigenic peptides and plotted the results according to the percentage of degradation of the precursor peptide (Fig. 2B).

In agreement with the CTL activation assays, we observed significant production of peptide MAGE-A3114–122 AELVHFLLL in the digests obtained with the intermediate proteasomes and the immunoproteasome, but not with the standard proteasome (Fig. 2B). The MAGE-C242–50 antigenic peptide was abundant in the digests obtained with proteasome β1i-β5i and the immunoproteasome, but was weak or absent in digests performed with standard proteasome and proteasome β5i. The discordance for the processing of the MAGE-C242–50 peptide between the cellular and the in vitro assay could be due to a weak in vitro cleavage at the N terminus of the peptide by proteasome β5i (Supplemental Fig. 2B). In the cells, this N-terminal cleavage might be produced by a trimming peptidase not related to the proteasome (20). In line with the CTL assays, peptide MAGE-C2 ALKDVEERV336–344 was only observed in the digests obtained with the β1i-β5i proteasome or the immunoproteasome.

**Lack of production of standard-dependent antigenic peptides by the intermediate proteasomes**

We used the same approach to compare the processing of three peptides previously shown to be produced by the standard proteasome, but not by the immunoproteasome. These Ags are derived from melanocytic proteins and presented by HLA-A2 (5, 8). All these peptides, namely Melan-A26–35, gp100209–217, and tyrosinase369–377, were efficiently processed by the standard proteasome, but not by the intermediate proteasomes nor the immunoproteasome (Fig. 3). In most cases, consistent results were obtained in the CTL assays and by in vitro digestion. However, a weak production of the Melan-A and gp100 peptides was observed in vitro after extended digestion with intermediate proteasomes β5i or β1i-β5i, respectively (Fig. 3B), although in the cellular assay these peptides were not processed by these proteasomes. This discrepancy might arise from the difference in nature of the assays. The cellular assay recapitulates all the aspects of the Ag processing pathway in physiological conditions, whereas the in vitro digestion only assesses the role of the proteasome in conditions that can be biased at high precursor degradation rates, due to re-entry of digestion products for successive digestion rounds. It is also possible that the

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**FIGURE 3.** Lack of production of standard-dependent antigenic peptides by the intermediate proteasomes. (A) The 293-EBNA cells containing standard proteasomes (293-SP), immunoproteasomes (293-IP), intermediate proteasomes β5i (293-β5i), or intermediate proteasomes β1i-β5i (293-β1i-β5i) were transfected with a HLA-A2 plasmid and the indicated amounts of plasmid encoding Melan-A, gp100, or tyrosinase. As a positive control, cells were loaded with the antigenic peptide at 3 μg/ml (gp100209–217), 5 μg/ml (Melan-A26–35), or 10 μg/ml (tyrosinase369–377). CTLs against peptides Melan-A26–35, gp100209–217, and tyrosinase369–377 were added 24 h later, and the production of IFN-γ was measured. (B) Detection of antigenic peptides EAAGIGILTV (Melan-A26–35) and YMDGTMSQV (tyrosinase369–377) in digests obtained by incubating purified 20S proteasomes with precursor peptides KGHGHSYTTAEAGIGILTVILGL and NALHIYMVDGTSQVQGSAN. Digests were analyzed by HPLC-MS, and the detection of the relevant ions (in arbitrary units [AU]) was plotted as a function of the degradation of the precursor peptide. The gp100209–217 peptide ITDQVPFSV obtained by incubating precursor peptide SSHAFTTDQVPFSVSQL with purified 20S proteasomes was analyzed by MS/MS coupled to HPLC (HPLC-MS/MS), and the detection of the relevant ion was plotted as a function of the degradation of the precursor peptide. Digestion times are indicated in Materials and Methods.
physiological production of these antigenic peptides requires N-terminally elongated peptides for efficient transfer into the endoplasmic reticulum, which are further trimmed in the endoplasmic reticulum by ERAP. In this case, the production of the N terminus directly by the proteasome might be deleterious for the processing of the peptide. The fact that intermediate proteasomes β5i and β1i-β5i are the most efficient to produce the N terminus of the Melan-A (Supplemental Fig. 3A) and gp100 (Supplemental Fig. 3B) epitopes, respectively, supports this possibility, which might explain the discrepancy between the cellular assay (Fig. 3A) and the MS analysis of in vitro digests (Fig. 3B) for these two peptides.

Unique ability of the standard proteasome to cleave between hydrophobic residues

To understand why the processing of the different antigenic peptides requires particular proteasome(s), we identified by HPLC-MS the fragments contained in digests obtained with each proteasome type. For the MAGE-A3114-122 and MAGE-C242-50 peptides, which are not produced by the standard proteasome, we observed, in the digests obtained with this proteasome, fragments resulting from prominent cleavages destroying the epitope, after F119, L120, and L121 for MAGE-A3114-122 and after L58 for MAGE-C242-50 (Fig. 4A, 4B). These cleavages were very weak in the other digests (Fig. 4A, 4B). Strikingly, these cleavages occur after hydrophobic residues, and are therefore not in line with the lower chymotrypsin-like activity of the standard proteasome measured on fluorogenic substrates (21). However, the analysis of proteasomal digests performed with enolase or with MAGE-C2 peptides indicated that, even though the immunoproteasome cleaves efficiently after hydrophobic residues, it does not between two hydrophobic residues, as opposed to the standard proteasome (13, 22). Our results indicate that the intermediate proteasomes also fail to cleave efficiently between hydrophobic residues. Fragments resulting from cleavage at the C terminus and N terminus of the MAGE-A3114-122 and MAGE-C242-50 peptides are shown on Supplemental Fig. 2. Because the C terminus of both peptides was produced with a similar efficiency by each proteasome type, we concluded that these peptides are not processed by the standard proteasome because of its unique ability to destroy the epitope by cleaving within the hydrophobic stretches that are present in these antigenic peptides (Fig. 4A, 4B).

A similar mechanism might explain the unique processing of the Melan-A26-35 peptide by the standard proteasome. In this study, production of the C terminus of the epitope requires cleavage within a hydrophobic stretch, after V35. Hence, the standard proteasome is the only proteasome able to produce the C terminus of this epitope (Fig. 4C). In addition to the poor ability of the other proteasomes to produce the C terminus of the epitope, we also observed destructive cleavages, as follows: after I30 for proteasome β5i and the immunoproteasome and after L33 for proteasome β1i-β5i (Supplemental Fig. 3A).

In conclusion, our results suggest that the proteasomes that contain subunit β5i cannot cleave within a hydrophobic stretch, as opposed to the standard proteasome, which can easily cleave between two hydrophobic residues. These three examples demonstrate that the substitution of β5i for β5 dramatically modifies the chymotrypsin-like activity, not only in intensity, but also, most importantly, in sequence specificity.

Destructive cleavage of antigenic peptide MAGE-C2336-344 by the standard proteasome and intermediate proteasome β5i

The MAGE-C2336-344 antigenic peptide is produced by the immunoproteasome and intermediate proteasome β1i-β5i, but not by the standard proteasome nor intermediate proteasome β5i (Fig. 2). We observed a prominent destructive cleavage after the aspartic residue in position 339 in the digests obtained with the latter proteasomes (Fig. 5A). Such cleavage was expected to be more efficient with the standard proteasome and intermediate proteasome β5i, as it occurs after an acidic residue and is mediated by the caspase-like activity exerted by subunit β1. In addition, intermediate proteasome β5i appears inefficient to produce the C terminus of this peptide (Fig. 5B). Additional fragments resulting from internal cleavage and from cleavage at the N terminus of the epitope were detected and are shown on Fig. 5C and 5D. Another destructive cleavage was observed after A336 in the digests obtained
with proteasome β1i-β5i (Fig. 5C). However, surprisingly, this cleavage did not preclude presentation of the antigenic peptide by cells expressing this proteasome type (Fig. 2A). We concluded that this destructive cleavage is minor as compared with the destructive cleavage after D339, which appears dominant as it fits with the processing ability of the different proteasome types in the cellular assay. It should be stressed that MS enables a quantitative comparison of a given fragment in various digests, but cannot reliably compare the amount of different fragments in the same digest, due to variations of ionization efficiency of the fragments. A limitation of the MS approach therefore is its inability to define a hierarchy of dominant cleavages.

**Destructive cleavage of peptides gp100209–217 and tyrosinase369–377 by intermediate proteasomes**

The poor processing of these peptides by the intermediate proteasomes appeared to result from destructive cleavages similar to those performed by the immunoproteasome, at least for intermediate proteasome β1i-β5i (Fig. 6). The gp100 peptide is destroyed after I209, whereas the tyrosinase peptide is destroyed after the two internal methionines (Fig. 6). These cleavages are in line with the higher activity of these proteasomes after an isolated hydrophobic residue. With intermediate proteasome β5i, however, prominent destructive cleavages were observed after D211 in the gp100 and after D371 in the tyrosinase peptide (Fig. 6). This is in agreement with proteasome β1i-β5i (Fig. 5C). However, surprisingly, this cleavage did not preclude presentation of the antigenic peptide by cells expressing this proteasome type (Fig. 2A). We concluded that this destructive cleavage is minor as compared with the destructive cleavage after D339, which appears dominant as it fits with the processing ability of the different proteasome types in the cellular assay. It should be stressed that MS enables a quantitative comparison of a given fragment in various digests, but cannot reliably compare the amount of different fragments in the same digest, due to variations of ionization efficiency of the fragments. A limitation of the MS approach therefore is its inability to define a hierarchy of dominant cleavages.

**FIGURE 5.** Analysis of proteasomal digests of a precursor of peptide MAGE-C2336–344. (A–D) MS detection of the indicated peptide fragments in the digests shown in Fig. 2B, analyzed at a time point corresponding to a precursor degradation of 42% (10 min) (β5i), 38% (360 min) (β1i-β5i), 34% (45 min) (standard proteasome), and 36% (120 min) (immunoproteasome).

**FIGURE 6.** Destructive cleavage of peptides gp100209–217 and tyrosinase369–377 by intermediate proteasomes. MS detection of the indicated peptide fragments in the digests shown in Fig. 3B, analyzed at a time point corresponding to a precursor degradation of 47% (360 min) (β5i), 60% (240 min) (β1i-β5i), 73% (60 min) (standard proteasome), and 47% (240 min) (immunoproteasome) for gp100; and 61% (180 min) (β5i), 44% (480 min) (β1i-β5i), 67% (90 min) (standard proteasome), and 43% (480 min) (immunoproteasome) for tyrosinase. Additional fragments are shown in Supplemental Fig. 3B and 3C.
with the higher caspase-like activity associated with the presence of subunit β1. Surprisingly, the fragments resulting from these cleavages were less abundant in the digests obtained with the standard proteasome. Besides, the proper cleavage at the C terminus of both the gp100 and the tyrosinase epitopes was most efficient in the digests obtained with the standard proteasome (Fig. 6). Fragments resulting from cleavage at the N terminus are shown on Supplemental Fig. 3B and 3C. Thus, the better processing of these antigenic peptides by the standard proteasome appears related to the combination of a poor destructive activity and a better production of the C terminus.

Exclusive production of a MAGE-C2<sub>191–200</sub> peptide by intermediate proteasome β1i-β5i

Peptide LLFGLALIEV<sub>191–200</sub> is a second HLA-A2–restricted tumor antigenic peptide encoded by gene MAGE-C2 (12). The

FIGURE 7. Exclusive processing of peptide MAGE-C2<sub>191–200</sub> LLFGLALIEV by intermediate proteasome β1i-β5i. (A) Proteasome inhibition blocks the presentation of peptide MAGE-C2<sub>191–200</sub> by melanoma cells. Melanoma cells EB81-MEL were treated with epoxomicin, as described in Materials and Methods, and incubated with the MAGE-C2<sub>191–200</sub>-specific CTL clone. Left panel shows the actual values of TNF release; right panel shows a relative CTL activation level calculated as the ratio of TNF produced by the CTL incubated with tumor cells to that produced by CTL incubated with the same tumor cells loaded with the antigenic peptide. The relative CTL activation level of untreated cells was set at 100%. (B) The 293-EBNA cells containing standard proteasome (293-SP), immunoproteasome (293-IP), intermediate proteasome β5i (293-β5i), or intermediate proteasome β1i-β5i (293-β1i-β5i) were transfected with a HLA-A2 construct and the indicated amounts of plasmid encoding MAGE-C2. As a positive control, cells were loaded with the relevant antigenic peptide at 0.1 μg/ml. MAGE C2<sub>191–200</sub>-specific CTL was added, and TNF production was measured. (C) Detection of antigenic peptide LLFGLALIEV in digests obtained by incubating precursor peptide LKRAREFME<sub>LLFGLALIEV</sub>GPDHF with purified 20S proteasomes. Digests were analyzed by HPLC/MS-MS, and the detection of the relevant ion was plotted as a function of the degradation of the precursor peptide. Digestion times are indicated in Materials and Methods. The sharp increase observed between 62 and 64% precursor degradation might result from re-entry of digestion products into the proteasome for further rounds of degradation. Such artifactual re-entry is likely to occur at high rates of precursor degradation in vitro conditions. (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 57% (20 min) (β5i), 52% (30 min) (β1i-β5i), 45% (5 min) (SP), and 38% (45 min) (IP). Fragment GPDHF monitoring the production of the C terminus of the epitope, was detected by MS/MS. Its complementary fragment LKRAREFME<sub>LLFGLALIEV</sub>GPDHF was not detected, presumably because of further destructive cleavages during in vitro digestion. Additional fragments are shown in Supplemental Fig. 4.
processing of this peptide has not been characterized. Treatment of EB81-MEL cells with epoxomicin decreased their recognition by the MAGE-C2/HLA-A2–specific CTL clone, suggesting a role for the proteasome in the production of this antigenic peptide (Fig. 7A). When we transfected the panel of 293 cells expressing each proteasome type with increasing amounts of MAGE-C2 cDNA, we observed that only cells containing proteasome β1i-β5i were efficiently recognized by the specific CTL clone (Fig. 7B). We used proteasomes purified from the different 293 cells to digest a precursor peptide of 24 aa encompassing the antigenic peptide. Using HPLC coupled to MS/MS (HPLC-MS/MS), we detected the antigenic peptide in the digests obtained with proteasome β1i-β5i, but not in the other digests (Fig. 7C). Those results imply that these MAGE-C2–specific CTL can only recognize tumor cells containing proteasome β1i-β5i. We further analyzed the digests by HPLC/MS and HPLC/MS-MS to quantify the main fragments associated with the production of the antigenic peptide (Fig. 7D). We observed a destructive cleavage after L195 and L197 in the digests obtained with the standard proteasome and intermediate proteasome β5i, respectively. The fragments resulting from these cleavages were not or weakly detected in the digests obtained with the other proteasomes. The cleavage producing the C terminus of the antigenic peptide was monitored by detection of complementary fragment GPDHF; it was more abundant in digests obtained with the standard proteasome and intermediate proteasomes than with the immunoproteasome (Fig. 7D). Addi-
tional fragments detected are shown in Supplemental Fig. 4. They indicate another destructive cleavage, which occurs after E199 and is performed exclusively by intermediate proteasome β5i. Altogether, these data provide an explanation for the generation of this MAGE-C2 antigenic peptide only in cells expressing proteasome β1i-β5i; destructive cleavages by the standard proteasome and intermediate proteasome β5i and poor production of the C terminus by the immunoproteasome (Fig. 8).

**Discussion**

Because of their ability to produce a unique repertoire of antigenic peptides and their abundance in many tissues and cell types, intermediate proteasomes are likely to shape significantly the CD8+ immune response. First, they increase the size of the repertoire of peptides presented by MHC class I molecules. Second, because the nature of the peptides displayed at the surface of a given cell depends on the proteasome content of that cell, intermediate proteasomes introduce a new level of diversity beyond the well-characterized standard proteasome and immunoproteasome. This has implication for cancer immunotherapy. For instance, a number of tumor Ags that are relevant for tumor rejection are better—sometimes exclusively—produced by the immunoproteasome, particularly those Ags that are targeted by T lymphocytes elicited by Ag spreading, which relies on presentation of tumor Ags by dendritic cells, which contain mostly immunoproteasomes (13, 23). But tumor cells that are not located at inflammatory sites usually do not contain immunoproteasomes, and would therefore not be sensitive to the attack by those T cells. However, our work indicates that tumor cells commonly contain intermediate proteasomes, which usually produce the same peptides as the immunoproteasome. This ensures sensitivity of tumor cells to such T cells, even when tumors are not exposed to IFN-γ. Indeed, we show in this study that melanoma line EB81-MEL, which does not contain immunoproteasomes in the absence of IFN-γ exposure, is recognized by CTL against MAGE-C2136–344 and by CTL against MAGE-C242–50 because it contains intermediate proteasomes that can produce these peptides (11). To date, all the Ags we tested whose processing depends on the immunoproteasome proved to be processed by at least one intermediate proteasome. Moreover, a low proportion of the appropriate proteasome appears sufficient to produce enough peptide for efficient CTL recognition, because EB81-MEL cells, which contain only 3% of intermediate proteasome β1i-β5i, are recognized by

**FIGURE 8.** Summary of the relevant cleavages performed by the different proteasome types in precursor peptides of several tumor Ags. The column entitled “Proteasome production” is based on the cellular assay (Figs. 2A, 3A). The column entitled “Sequence and cleavages” is based on the MS analysis of in vitro digests, and summarizes the major cleavages detected that are relevant to the production of the Ag. Arrowheads indicate cleavages performed by the standard proteasome (blue), intermediate proteasome β5i (green), intermediate proteasome β1i-β5i (black), and the immunoproteasome (red). Weak cleavages are not indicated. *Data from Ref. 11. β5i, Intermediate proteasome β5i; β1i-β5i, intermediate proteasome β1i-β5i; IP, immunoproteasome; SP, standard proteasome.

<table>
<thead>
<tr>
<th>Source</th>
<th>MHC restriction</th>
<th>Proteasome production</th>
<th>Sequence and cleavages</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAGE-A3 hap1-122</td>
<td>HLA-B40</td>
<td>- + +</td>
<td>LSRKVAELVIF L L LKYRAR</td>
</tr>
<tr>
<td>MAGE-A3 *17,17a</td>
<td>HLA-A2</td>
<td>- - -</td>
<td>PACYEF LWPRALY ETSYY*</td>
</tr>
<tr>
<td>MAGE-A10 *204,202</td>
<td>HLA-A2</td>
<td>- - +</td>
<td>ALKMM GLYD GMEHL IYGEp*</td>
</tr>
<tr>
<td>MAGE-C2 *40,50</td>
<td>HLA-B57</td>
<td>- + +</td>
<td>EEASS ASSTLYL YF SPSSF</td>
</tr>
<tr>
<td>MAGE-C2 *16,16b</td>
<td>HLA-A2</td>
<td>- - +</td>
<td>REPMLFLGAL LEV GPDH</td>
</tr>
<tr>
<td>MAGE-C2 *36,344</td>
<td>HLA-A2</td>
<td>- - +</td>
<td>SWYKD LA KVEERV QATID</td>
</tr>
<tr>
<td>Melan-A *35,35</td>
<td>HLA-A2</td>
<td>- + +</td>
<td>YYTAIE EAAGI GIL TV ILGVL</td>
</tr>
<tr>
<td>gp100 *38,38</td>
<td>HLA-A2</td>
<td>- + +</td>
<td>SSAFT I TV QVFPVS SVQSL</td>
</tr>
<tr>
<td>Tyrosinate *69,67</td>
<td>HLA-A2</td>
<td>- + +</td>
<td>NALHIM D GM OV QGSA</td>
</tr>
</tbody>
</table>

- standard proteasome
- proteasome β5i
- proteasome β1i-β5i
- immunoproteasome
CTL against peptide MAGE-C2\textsubscript{191–200}, which is produced exclusively by this proteasome type (11). Altogether, these findings indicate that immunotherapy strategies that elicit T cells directed against immunoproteasome-dependent peptides should be able to target tumors even when these tumors are not exposed to inflammatory conditions.

We previously described two tumor Ags that were produced only by intermediate proteasome $\beta$1i-$\beta$5i (peptide MAGE-A10\textsubscript{254–262}) or by intermediate proteasome $\beta$5i (peptide MAGE-A3\textsubscript{271–279}) (11) (Fig. 8). In this study, we report a third tumor Ag (peptide MAGE-C2\textsubscript{191–200}) whose processing depends exclusively on an intermediate proteasome, in this case proteasome $\beta$1i-$\beta$5i. The presence of intermediate proteasomes in dendritic cells (11) should ensure efficient priming of T cells recognizing such Ags. Intermediate proteasomes, however, did not produce the three Ags we tested that are known to be produced by the standard proteasome better than by the immunoproteasome. These Ags derive from melanocyte-differentiation proteins Melan-A, gp100, and tyrosinase (Fig. 8). As previously discussed, the limited amounts of standard proteasomes in mature dendritic cells might prevent efficient priming of T cells against such Ags, and suggest the use of vaccination strategies that bypass the need for processing by the proteasome of dendritic cells (5, 6, 9).

Altogether, it appears that the repertoire of antigenic peptides produced by intermediate proteasomes comprises that produced by the immunoproteasome, but is larger.

The analysis of peptide digests obtained with the different proteasomes provides new insights into the cleavage specificities of each proteasome type. The caspase-like activity commonly attributed to subunit $\beta$1 of the standard proteasome decreases when $\beta$1 is replaced by $\beta$1i (2, 4, 24). Our results confirm this assertion. Intermediate proteasomes $\beta$5i cleaved efficiently after the aspartate residues of MAGE-C2\textsubscript{336–344}, MAGE-A10\textsubscript{254–262}, gp100\textsubscript{209–217}, and tyrosinase\textsubscript{569–577}, whereas these cleavages were weaker in the digests obtained with proteasomes $\beta$1i-$\beta$5i (summarized in Fig. 8). However, despite the presence of subunit $\beta$1i, immunoproteasomes cleaved after the aspartate residue contained in MAGE-A10\textsubscript{254–262} as efficiently as standard proteasomes (11) (Fig. 8). These data suggest that not only the subunit composition, but also the structural features of each proteasome can impact its cleavage activity, in agreement with previous results showing the importance of the immunoproteasome structure for the processing of a viral Ag derived from hepatitis B virus (25).

The chymotrypsin-like activity is mainly exerted by subunits $\beta$5 and $\beta$5i (4). Assays with fluorogenic substrates indicated that replacement of $\beta$5 by $\beta$5i increased the chymotrypsin-like activity (26, 11). However, it appears that the sequence specificity of the chymotrypsin-like activity is different between the standard proteasome and the immunoproteasome. The analysis of proteasomal digests of enolase-1 indicated that the immunoproteasome favors cleavage after a hydrophobic residue, but disfavors this cleavage when another hydrophobic residue is located at the P1 position, that is, after the cleavage site. The chymotrypsin-like activity of the immunoproteasome is therefore characterized by an inability to cleave between two bulky hydrophobic residues (22). This inability was also observed for the MAGE-A3\textsubscript{114–122} and MAGE-C2\textsubscript{42–50} antigenic peptides (7, 13) (Fig. 8). These Ags contain at their C terminus a hydrophobic stretch of four to five residues, FLLL and LYLVF, respectively. The MAGE-A3\textsubscript{271–279} has a hydrophobic stretch of three residues, FLW, located at the N terminus (Fig. 8). Our results indicate that all the proteasomes that contain subunit $\beta$5i are unable to cleave between these hydrophobic residues, as opposed to the standard proteasome, which often destroys these epitopes by cleaving within their hydrophobic stretches. For some Ags, such as the Melan-A Ag, the higher capacity of the standard proteasome to cleave between two hydrophobic residues is critical to the efficient production of the C terminus of the epitope. We conclude that the sequence specificity of the chymotrypsin-like activity of the intermediate proteasomes and the immunoproteasome, exerted by subunit $\beta$5i, is different from that of the standard proteasome, exerted by subunit $\beta$5.

Our results to date indicate that the Ags produced by the immunoproteasome are also produced by at least one intermediate proteasome (Fig. 8). It follows that subunit $\beta$2i is not required for the processing of such Ags.

Immunoproteasomes were recently reported to be particularly suited to the degradation of oxidized proteins (27). It will be interesting to determine whether intermediate proteasomes exert a similar activity. As opposed to immunoproteasomes, which are only abundant in inflammatory conditions, intermediate proteasomes are abundant in normal tissues in steady state conditions (11). Thus, one of their functions could be to degrade oxidized proteins in situations of oxidative stress that are not accompanied by inflammation, and thereby contribute to maintain protein homeostasis.

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**Disclosures**

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