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# Destructive Cleavage of Antigenic Peptides Either by the Immunoproteasome or by the Standard Proteasome Results in Differential Antigen Presentation<sup>1</sup>

Jacques Chapiro,\* Stéphane Claverol,<sup>2†</sup> Fanny Piette,\* Wenbin Ma,\* Vincent Stroobant,\* Benoît Guillaume,\* Jean-Edouard Gairin,<sup>3†</sup> Sandra Morel,<sup>4\*</sup> Odile Burlet-Schiltz,<sup>†</sup> Bernard Monsarrat,<sup>†</sup> Thierry Boon,\* and Benoît J. Van den Eynde<sup>5\*</sup>

The immunoproteasome (IP) is usually viewed as favoring the production of antigenic peptides presented by MHC class I molecules, mainly because of its higher cleavage activity after hydrophobic residues, referred to as the chymotrypsin-like activity. However, some peptides have been found to be better produced by the standard proteasome. The mechanism of this differential processing has not been described. By studying the processing of three tumor antigenic peptides of clinical interest, we demonstrate that their differential processing mainly results from differences in the efficiency of internal cleavages by the two proteasome types. Peptide gp100<sub>209–217</sub> (ITDQVPSFV) and peptide tyrosinase<sub>369–377</sub> (YMDGTSQV) are destroyed by the IP, which cleaves after an internal hydrophobic residue. Conversely, peptide MAGE-C2<sub>336–344</sub> (ALKDVEERV) is destroyed by the standard proteasome by internal cleavage after an acidic residue, in line with its higher postacidic activity. These results indicate that the IP may destroy some antigenic peptides due to its higher chymotrypsin-like activity, rather than favor their production. They also suggest that the sets of peptides produced by the two proteasome types differ more than expected. Considering that mature dendritic cells mainly contain IPs, our results have implications for the design of immunotherapy strategies. *The Journal of Immunology*, 2006, 176: 1053–1061.

Cytolytic T lymphocytes recognize peptides derived from intracellular proteins and presented by MHC class I molecules. The peptides are usually produced in the cytosol as a result of protein degradation by the proteasome, a large multicatalytic protease complex whose 20S core has the shape of a cylinder composed of four rings (1). The two outer rings are identical and made of seven different  $\alpha$  subunits. The two inner rings are also identical and are composed of seven different  $\beta$  subunits, three of which ( $\beta_1$ ,  $\beta_2$ , and  $\beta_5$ ) being responsible for the catalytic activity. Catalytic subunits  $\beta_1$ ,  $\beta_2$ , and  $\beta_5$  are present in most cell types and bear the activity of the ubiquitous standard proteasome

(SP).<sup>6</sup> In the presence of IFN- $\gamma$ , these subunits are replaced by their inducible counterparts  $\beta_{1i}$  (LMP2),  $\beta_{2i}$  (MECL1), and  $\beta_{5i}$  (LMP7), also called immunosubunits, to form slightly different particles named immunoproteasomes (IP) (2). IPs are found in most cell types after exposure to IFN- $\gamma$ , but are also present in a constitutive manner in APCs such as dendritic cells (3–6).

Using fluorogenic peptides, it was found that the IP had a higher activity of cleavage after hydrophobic residues (chymotrypsin-like activity) and after basic residues (trypsin-like activity), but a much lower ability to cleave after acidic residues (postacidic or caspase-like activity) (7–9). Because C-terminal hydrophobic residues—as opposed to acidic residues—are important anchor residues for binding to MHC class I molecules, these differences in enzymatic activity were predicted to favor the production of MHC-binding peptides by the IP, thereby increasing the efficiency of Ag presentation in cells harboring this type of proteasome (10, 11). Indeed, it was shown that a number of antigenic peptides, mainly derived from viral proteins, were presented more efficiently, sometimes exclusively, by cells harboring IPs (12–19).

The IP is the main proteasome type present in mature dendritic cells, which play a crucial role in the initiation of immune responses. Consequently, it was expected that T cells directed against IP-dependent peptides would dominate immune responses. This was confirmed in a set of elegant studies by Chen et al. (20), who compared the immunodominance hierarchies of CD8 T cells responding to a influenza virus infection in wild-type and  $\beta_{1i}^{-/-}$  mice, which have no IPs. The peptides that triggered the strongest response in wild-type mice (dominant peptides) were not dominant in  $\beta_{1i}^{-/-}$  mice, and vice-versa. This appeared to result, at least in part, from differences in the efficiency of presentation of the peptides

\*Ludwig Institute for Cancer Research, Brussels Branch, and Cellular Genetics Unit, Université Catholique de Louvain (UCL), Brussels, Belgium; and <sup>†</sup>Institut de Pharmacologie et de Biologie Structurale, Centre National de la Recherche Scientifique, Toulouse, France

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<sup>2</sup> Present address: Plateforme Génomique Fonctionnelle Bordeaux, F-33076 Bordeaux cedex, France.

<sup>3</sup> Present address: Unité Mixte de Recherche Centre National de la Recherche Scientifique - Pierre Fabre, F-31400 Toulouse, France.

<sup>4</sup> Present address: GlaxoSmithKline Biologicals, B-1330 Rixensart, Belgium.

<sup>5</sup> Address correspondence and reprint requests to Dr. Benoît J. Van den Eynde, Ludwig Institute for Cancer Research, Avenue Hippocrate 74, UCL 7459, B-1200 Brussels, Belgium. E-mail address: benoit.vandeneinde@bru.lir.org

<sup>6</sup> Abbreviations used in this paper: SP, standard proteasome; IP, immunoproteasome; MS, mass spectrometry.

by APCs of both mouse strains, which might result from differential processing of those peptides. A recent comparison of a dominant and a subdominant epitope from lymphocytic choriomeningitis virus clearly confirmed this notion by showing that the subdominant epitope was poorly produced by the IP and poorly presented by dendritic cells (21, 22). Differential processing of antigenic peptides by the two proteasome types is therefore an important factor contributing to the immunodominance hierarchy of CD8 T cell responses.

It has become clear that the immunosubunits are not required for the production of all antigenic peptides, because cells lacking  $\beta_1$ i and  $\beta_2$ i were shown to express MHC class I molecules and present many antigenic peptides (23, 24). Moreover, we have observed that some antigenic peptides are efficiently produced by the SP only. This occurred for an antigenic peptide derived from an ubiquitous human protein named RU1, and also for a peptide derived from human melanocytic protein Melan-A (4). Another example was reported recently with a murine viral peptide (21). Therefore, it appears that the set of peptides presented at the cell surface is dependent on the type of proteasome present inside the cell (25). This leads to potentially important consequences in physiological situations where APCs and target cells do not express the same type of proteasome.

The mechanism responsible for the lack of production of antigenic peptides by the IP is unknown. In this study, through the description of three new examples of clinically relevant tumor antigenic peptides that are differentially processed, we provide a mechanistic explanation based on the analysis of cleavage sites by each type of proteasome.

## Materials and Methods

### Cell lines and culture conditions

CTL IVSB and 210/9 are two human CTL clones recognizing HLA-A\*0201-restricted tyrosinase peptides 369–377 (YMDGTSQV) and 1–9 (MLLAV LYCL), respectively (26, 27). CTL clone 52 recognizes peptide MAGE-A3<sub>114–122</sub> (16). CTL clone 16 recognizes peptide MAGE-C2<sub>336–344</sub> (28). CTL clones 446/A11 (see Fig. 4A) and 606 C2/1 (see Fig. 4, B and D) both recognize peptide gp100<sub>209–217</sub> (29, 30). Cell line 721.174 transfected with TAP1 was kindly provided by V. Cerundolo (University of Oxford, U.K.).

### Cell lines 293-SP and 293-IP

We used human embryonic kidney 293 cells expressing the tetracycline repressor (T-Rex-293; Invitrogen Life Technologies) as recipient cells for stable transfection of inducible expression plasmid pcDNA4/TO, which contained, under the control of a CMV promoter and a tetracycline-resistance operon, a tricistronic construct containing the cDNA of human proteasome subunit  $\beta_1$ i, the internal ribosome entry site of hepatitis C virus (kindly provided by A.-M. Delisse and T. Cabezón, GlaxoSmithKline, Rixensart, Belgium), the cDNA of proteasome subunit  $\beta_2$ i, the internal ribosome entry site of Theiler virus (kindly provided by T. Michiels, Institute of Cellular Pathology, Brussels, Belgium), and the cDNA of proteasome subunit  $\beta_5$ i. Transfected cells were selected with bleocin (60  $\mu$ g/ml) (Calbiochem), and further transfected with plasmid pEF/myc/cyto (Invitrogen Life Technologies) containing  $\beta_5$ i. They were selected alternatively with 3.5  $\mu$ g/ml neomycin (Promega) and 1  $\mu$ g/ml puromycin (Sigma-Aldrich). Transfected clone 6, referred to in this study as 293-IP, contained, upon tetracycline exposure, proteasomes bearing only the immunosubunits. Because 293-IP cells expressed immunosubunits also in the absence of tetracycline, we used parental T-Rex-293 as control cell line expressing only SPs (293-SP).

### Assay of Ag presentation by 293-SP and 293-IP cells

293-SP cells or 293-IP cells treated with tetracycline (1  $\mu$ g/ml, 6 days) were plated in flat-bottom 96-well microplates (70,000–100,000/well) 48 h before transfection. They were transfected using Lipofectamine (Invitrogen Life Technologies) with DNA from the indicated cDNAs cloned into plasmid pcDNA3 (pcDNA1 for MAGE-3) and titrated by dilution in empty vector DNA. Twenty-four hours after transfection, 2,500–10,000 cells of the relevant CTL clone were added in IMDM 10% human serum with IL-2 (25 U/ml).

After 18 h of coculture, the supernatant was collected and its content in TNF- $\alpha$  or IFN- $\gamma$  was measured by ELISA (Immunotech and BioSource International).

### Proteasome purification and characterization

SPs were purified from human erythrocytes (one packed red cells), and IPs were purified from LCL-721 cells ( $\sim 4 \times 10^9$  cells) treated at least 1 wk with IFN- $\gamma$  (100 U/ml) (31). Frozen pellets were lysed with four freeze/thaw cycles and resuspended in a 3- to 5-fold volume (w/v) of buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA; pH 7.5) supplemented with 0.01% Nonidet P-40 and 1 mM DTT. After centrifugation at  $4,000 \times g$  for 15 min at 4°C, the supernatants were fractionated by a double precipitation with  $(\text{NH}_4)_2\text{SO}_4$  (30/65%). The 65% precipitates were dissolved in buffer supplemented with 1 mM DTT, and centrifuged at  $15,000 \times g$  for 30 min at 4°C. Cleared supernatants were desalted on a Sephadex G25 Fine gel column equilibrated with buffer. Proteasomes were first purified by immunoaffinity chromatography on CNBr-activated 4B Sepharose beads ( $\pm 3$  ml; Amersham Pharmacia Biotech) coupled to mAb MCP21, which is directed against the  $\alpha_2$  subunit of the proteasome (32). Proteasomes were eluted with 20 mM Tris-HCl, 3 M NaCl, 1 mM EDTA (pH 7.5) at 0.25 ml/min, and subjected to gel filtration on a Superdex 200 column (Amersham) equilibrated in 20 mM Tris-HCl (pH 7.5) containing 100 mM NaCl at a flow rate of 0.5 ml/min. Proteasomes were further purified on a MonoQ HR 5/5 column (Amersham) and eluted with a 10-min linear gradient (50–600 mM) of NaCl in buffer (Tris-HCl 20 mM,  $\text{Na}_2\text{S}_3$  3 mM, glycerol 0.5%) at 1 ml/min. Eluted samples (300–350 mM NaCl) were kept at  $-80^\circ\text{C}$ . For human erythrocytes, an additional chromatography step was applied before  $(\text{NH}_4)_2\text{SO}_4$  precipitation, to remove hemoglobin: lysates were mixed with DEAE-cellulose (DE 52; Whatmann) equilibrated with 20 mM Tris-HCl, 20 mM NaCl, 1 mM EDTA (pH 7.5), and eluted with 0.5 M NaCl in equilibration buffer after extensive washes. Purified proteasomes were quantified by BCA protein assay (Pierce) and calibrated by ELISA as described previously (16).

### Western blot

Proteasomes from 293-SP cells or 293-IP cells treated with tetracycline (1  $\mu$ g/ml, 12 days) were purified using the same protocol. One microgram of proteasomes was analyzed by 12% SDS-PAGE and transferred to nitrocellulose (see Fig. 1A) (Hybond-C Extra; Amersham Pharmacia Biotech). Human proteasome subunits  $\beta_1$ ,  $\beta_2$ ,  $\beta_1$ i, and  $\beta_2$ i were detected with mouse mAbs PW9255, PW8145, PW8840, and PW8845 (Affinity Research Products), respectively, at a 1/1,000 dilution. Subunits  $\beta_5$  and  $\beta_2$ i were detected using rabbit polyclonal antisera PW8895 (1/4,000) and PW8350 (1/10,000), respectively (Affiniti). Bound Abs were revealed with goat anti-mouse-HRP or goat anti-rabbit-HRP (Santa Cruz Biotechnology) using chemiluminescence (Pierce). Cells were lysed (see Fig. 1B) in Tris 20 mM (pH 7.4), Nonidet P-40 1%, and NaCl 150 mM, and 30  $\mu$ g or 60  $\mu$ g (ERAP1 and ERp57) of proteins from postnuclear lysates were separated by SDS-PAGE, transferred as described above, and incubated with mAb against human TAP1 (mAb 148.3), TAP2 (mAb 429.3), ERAP1 (mAb 2C4) (all kindly provided by P. van Endert, Institut National de la Santé et de la Recherche Médicale U580, Université René Descartes, Paris, France; Ref. 33), calnexin or ERp57 (Abcam), or with polyclonal rabbit Abs against PA28 $\alpha$  (Affiniti; PW8185) or Tapasin (Abcam).

### Digestion of synthetic peptides with purified proteasomes and CTL assay

Peptides were synthesized on solid phase using Fmoc chemistry and purified by reversed phase-HPLC, lyophilized and dissolved in DMSO at 10 mg/ml. Precursor peptides were  $>95\%$  pure. Precursor peptides (1.25  $\mu$ g/time point, unless otherwise stated) were incubated with SPs or IPs (1  $\mu$ g/time point, unless otherwise stated) at  $37^\circ\text{C}$  in 10 mM Tris-HCl (pH 7.5) (20  $\mu$ l/time point). Digestion experiments were repeated using different batches of purified proteasomes. At each time point, an aliquot (20  $\mu$ l) was taken from the digestion mixture and added to 2  $\mu$ l of trifluoroacetic acid 10%. After lyophilization, the digests were resuspended in 10  $\mu$ l of cold water; 5  $\mu$ l were diluted to 155  $\mu$ l in serum-free medium X-vivo-10, and  $3 \times 50$   $\mu$ l of this dilution were loaded onto T2 cells (31) seeded in triplicate flat-bottom microwells (30,000/well in 50  $\mu$ l). CTL were added (5–10,000/well) immediately. Both T2 and CTL cells were washed with X-vivo-10 before use to remove any trace of serum. Cells were incubated 16 h in 150  $\mu$ l of X-vivo-10 containing IL-2 (25 U/ml). The supernatants were collected, and their content in IFN- $\gamma$  was measured by ELISA. Results from one representative experiment of at least four are shown.



### Online HPLC/mass spectrometry (MS) analysis of peptide digests

Digestions were performed as described above except that we used here 2.5  $\mu$ g of precursor peptide instead of 1.25  $\mu$ g for each time point. For peptide tyrosinase<sub>364–382</sub>, lyophilized digests were resuspended in 30  $\mu$ l of solvent A (5/95/0.05 CH<sub>3</sub>CN/H<sub>2</sub>O/HCOOH) before addition of 10  $\mu$ l of calibrating peptide, FQIVNPILL, at a final concentration of 5  $\mu$ M. For peptides gp100<sub>204–222</sub> and MAGE-C2<sub>331–349</sub>, lyophilized digests were resuspended in 12  $\mu$ l of H<sub>2</sub>O/CH<sub>3</sub>CN 50/50 before addition of 48  $\mu$ l of solvent A and 12  $\mu$ l of calibrating peptide at a final concentration of 3.3  $\mu$ M. Twelve microliters were separated on a Pepmap LC Packings C18 column (1 mm  $\times$  15 cm) at a flow rate of 40  $\mu$ l/min, with a gradient elution of 5–30% B for 40 min for the Tyrosinase<sub>364–382</sub> digest, and 10–50% B for 40 min for the gp100<sub>204–222</sub> digest (B is 70/30/0.05 CH<sub>3</sub>CN/H<sub>2</sub>O/HCOOH). Mass spectrometric analyses were performed online on a TSQ 700 triple quadrupole (ThermoFinnigan) equipped with a standard electrospray ionization source. Mass spectra were obtained by scanning the range of masses corresponding to  $m/z$  between 200 and 1950 every 3 s. Quantification was performed by measuring the ion current corresponding to a specific  $m/z$  value in the mass spectrum, and corrected for run-to-run variations using the signal of the constant amount of calibrating peptide. Quantification was confirmed where indicated by measuring the surface area (mV $\cdot$ s) of the relevant peaks on the UV chromatograms (215 nm). For the MS analysis of the MAGE-C2 digest, we separated on a C18 column (0.3 mm  $\times$  15 cm) at a flow rate of 4  $\mu$ l/min with a 35-min linear gradient of 10–55% acetonitrile in water, both with 0.05% trifluoroacetic acid, and analyzed online with a LCQ Deca XP Plus ion-trap mass spectrometer (ThermoFinnigan). The data presented are from one representative experiment of 3 or 5 (see Fig. 6) performed with similar results.

## Results

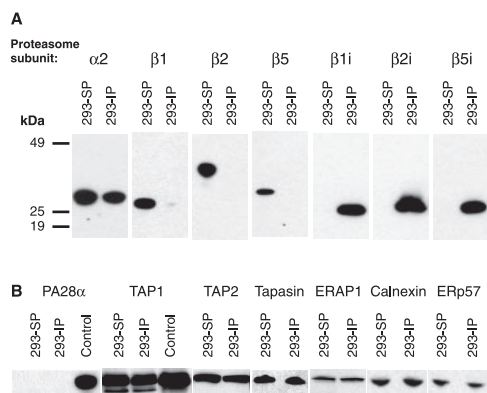
To compare the processing of antigenic peptides by the two proteasome types, we used two complementary approaches. The first is based on testing presentation of the Ag by human cell lines that contain exclusively either SPs or IPs. The second involves *in vitro* digestion of precursor peptides by 20S proteasomes of the two types followed by MS analysis of the products.

For the cellular approach, we selected human embryonic kidney cell line 293, which contains exclusively SPs and will be referred to hereafter as 293-SP (Fig. 1A). We transfected 293-SP cells with plasmid constructs encoding the three catalytic subunits of the IP, and we analyzed by Western blot the proteasome content of several transfected clones. It was reported previously that in murine transfected cells, overexpressed immunosubunits could be cooperatively incorporated into newly formed proteasome particles and

lead to a complete replacement of SPs by IPs (13, 14, 34). Also, in our human system, we identified a transfected 293 clone, hereafter called 293-IP, that contained proteasomes bearing exclusively catalytic immunosubunits  $\beta_1$ i,  $\beta_2$ i, and  $\beta_5$ i (Fig. 1A). We also compared by Western blot the expression of PA28, TAP1, TAP2, Tapasin, ERAP1, calnexin, and ERp57, which are also involved in Ag processing and regulated by IFN- $\gamma$ , and we found no difference between 293-SP and 293-IP cells (Fig. 1B).

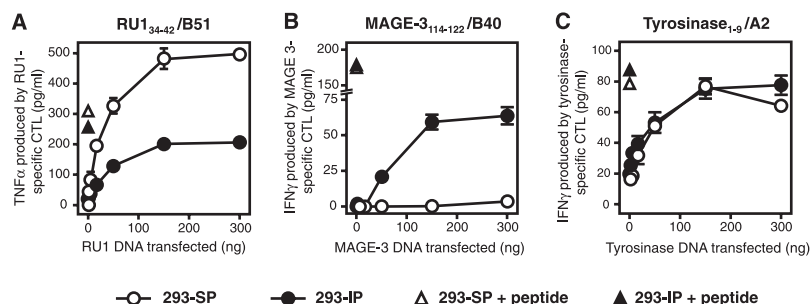
To validate this cellular approach, we used it to assess the processing of antigenic peptides previously studied by other methods. These included peptide RU1<sub>34–42</sub>, known to be better processed by the SP than by the IP, peptide MAGE-A3<sub>114–122</sub>, which is better produced by the IP, and peptide tyrosinase<sub>1–9</sub>, which is derived from the signal sequence and is therefore neither proteasome- nor TAP-dependent (4, 16, 35). 293-SP and 293-IP cells were transiently transfected with a constant amount of plasmid encoding the HLA-presenting molecule and increasing amounts of plasmid encoding the parental gene, either *RUI1*, *MAGE-A3*, or *tyrosinase*. One day after transfection, the cells were tested for recognition by the relevant CTL clone. As expected, the RU1 peptide was presented to CTL more efficiently by 293-SP than 293-IP cells, whereas the MAGE-A3 peptide was only presented by 293-IP cells (Fig. 2, A and B). When 293-SP and 293-IP were transfected with tyrosinase, they presented the signal peptide with the same efficiency (Fig. 2C). We concluded that the two cell lines did not differ significantly with regard to the other components of the Ag presentation machinery, and that the difference in their ability to present the RU1 and MAGE-A3 peptides could be fully attributed to their different proteasome content. Additional controls confirmed the similar transfection efficiency of 293-SP and 293-IP, using FACS analysis of cells transfected with a plasmid encoding GFP (data not shown). We also verified that, after transfection with the HLA coding sequence and loading with synthetic antigenic peptides, the two cell lines stimulated the CTL equally well (Fig. 2). This cellular approach therefore appears appropriate to compare the processing of antigenic peptides by the two types of proteasomes, while avoiding the use of IFN- $\gamma$ , which alters cellular physiology in multiple ways. This approach integrates not only the catalytic activity of the 20S proteasome but also its modulation by the 19S regulatory particle (36) and the potential involvement of additional proteases (37). However, the cellular approach does not address the mechanism of the differential processing.

To investigate the mechanism, we used an *in vitro* approach based on the digestion of synthetic precursor peptides with purified 20S proteasomes, followed by the analysis of the resulting fragments by HPLC coupled to MS (38). For these comparisons, it was essential to have highly purified preparations of SP and IP. We developed an optimized purification protocol using several chromatography steps based on affinity chromatography, size exclusion, and anion exchange as detailed in *Materials and Methods*. SPs were purified from human erythrocytes, and IPs were purified from EBV-transformed B cell line LCL-721 treated with IFN- $\gamma$  (31). The purity of the proteasome preparations was evaluated by two-dimensional gel electrophoresis, followed by MS identification of each spot stained with Coomassie blue (39). Every spot corresponded to a subunit of the 20S proteasome (Fig. 3), suggesting the absence of significant contaminants. Notably, we did not find any subunit belonging to the cap of the 26S proteasome, nor did we detect the presence of other proteases. Thus, our 20S proteasome preparations were purified to apparent homogeneity. The SP contained no catalytic subunits of the IP. The IP preparation essentially contained immunosubunits  $\beta_1$ i,  $\beta_2$ i, and  $\beta_5$ i, with a



**FIGURE 1.** Characterization of parental cells 293-SP and clone 293-IP, obtained by transfection of cDNAs encoding immunosubunits  $\beta_1$ i,  $\beta_2$ i, and  $\beta_5$ i. *A*, Proteasomes purified from 293-SP and 293-IP were analyzed by Western blot using Abs directed against the indicated proteasome subunits. mAb directed against subunit  $\alpha_2$  was used as loading control. *B*, Cleared lysates from 293-SP and 293-IP were analyzed by Western blot using the indicated Abs. The positive control for PA28 was cell line LCL-721 treated with IFN- $\gamma$ , and for TAP1 cell line LCL-721.174 transfected with TAP1.

**FIGURE 2.** Validation of the 293-SP and 293-IP cells for the study of differential processing. Cells were transfected with 50 ng of plasmid encoding the presenting HLA molecule and the indicated amount of plasmid encoding either RU1 (A), MAGE-3 (B), or tyrosinase (C). Cells from the relevant CTL clones were added, and cytokine production was measured. Control cells transfected only with the HLA-encoding construct were loaded with the antigenic peptide (A, 3  $\mu\text{g/ml}$ ; B, 2  $\mu\text{g/ml}$ ; C, 10  $\mu\text{g/ml}$ ) for 1 h before addition of the CTL. Error bars show SEM.



complete absence of  $\beta_5$  and some residual  $\beta_1$  and  $\beta_2$ , in a proportion estimated by densitometry to be  $\sim 4$  and 18% of the corresponding immunosubunit, respectively (Fig. 3).

#### Differential processing of peptide gp100<sub>209–217</sub>

We first studied HLA-A2-binding antigenic peptide ITDQVPFSV, which is derived from melanocytic protein gp100<sup>PMEL17</sup> and is presented to CTL less efficiently by melanoma cells treated with IFN- $\gamma$ , suggesting a poor processing by the IP (4, 29). We transfected 293-SP or 293-IP cells with the HLA-A2 cDNA and with increasing amounts of the gp100 cDNA, and tested them for recognition by a gp100-specific CTL clone (Fig. 4A). Transfected 293-SP cells clearly stimulated the CTL in a dose-dependent manner, whereas little or no CTL stimulation was obtained with 293-IP.

We used the purified 20S proteasomes to digest a synthetic precursor peptide of 19 aas encompassing the sequence of the gp100 antigenic peptide. Digestion times were selected by monitoring the degradation of the precursor peptide by UV detection coupled to HPLC, and were chosen to give  $>90\%$  degradation at the last time point and 40–50% at the penultimate time point. Digests were then loaded onto HLA-A2-expressing target cells T2. The CTL was added, and the production of IFN- $\gamma$  was measured. As shown on Fig. 4B, the CTL clone reacted strongly to the digests obtained with SPs, but not to the digests obtained with IPs.

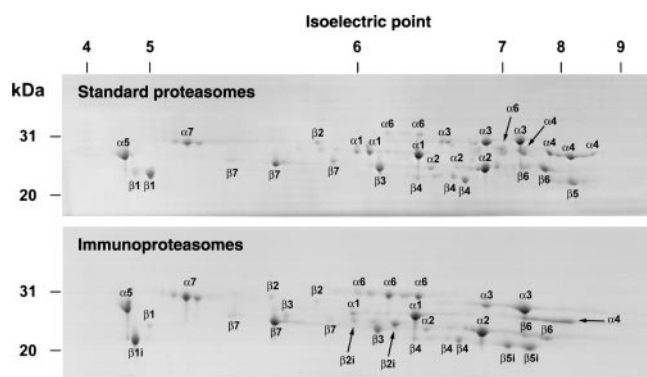
We also analyzed the digests by HPLC coupled to MS. Arrows on top of Fig. 4C indicate the cleavage sites observed in the precursor peptide. All the fragments detected are listed below. Time points corresponding to equal degradation of the precursor peptide by the two proteasome types were selected for a comparison of the

abundance of each fragment in the two digests, as shown in the right part of Fig. 4C. The fully processed antigenic peptide was not detected in any digest. But it is known that, whereas the proteasome is required to produce the final C terminus of antigenic peptides, the N terminus can be further trimmed by aminopeptidases either in the cytosol or in the endoplasmic reticulum (40, 41). Proteasome products may therefore contain N-extended precursors of antigenic peptides. Such a fragment, TITDQVPFSV, which has the final C terminus and an N-terminal extension of 1 aa, was present in the digest obtained with SPs (SP digest), albeit it was only detected with a low intensity. It was not detected in the digest obtained with IPs (IP digest), in agreement with the lack of CTL recognition of this digest. Another fragment, which had the final C terminus and an N-terminal extension of 5 aa, was also more abundant in the SP digest than in the IP digest. These differences could result in part from differences in the ability of the two proteasomes to perform the cleavage producing the C terminus of the antigenic peptide, as suggested by the higher amounts of complementary fragment SVSQL in the SP digest. Another factor might be the difference in the ability to cleave after isoleucine<sub>209</sub>, i.e., within the antigenic peptide. This destructive cleavage produces complementary fragments SSAFTI and TDQVPFSVSVSQL (see Fig. 4C legend), both of which are the major fragments detected in the IP digest, where they are 3–8 times more abundant than in the SP digest. These results suggest that the failure of the IP to produce the gp100 antigenic peptide results from a predominant cleavage after isoleucine<sub>209</sub>, which destroys the antigenic peptide. Occurring after a hydrophobic residue, the destructive cleavage is due to the chymotrypsin-like activity, which is known to be higher in the IP than in the SP (7, 8). The stronger destructive cleavage of the gp100 peptide by the IP is therefore perfectly in line with the known differences in the catalytic activities of the two proteasome types.

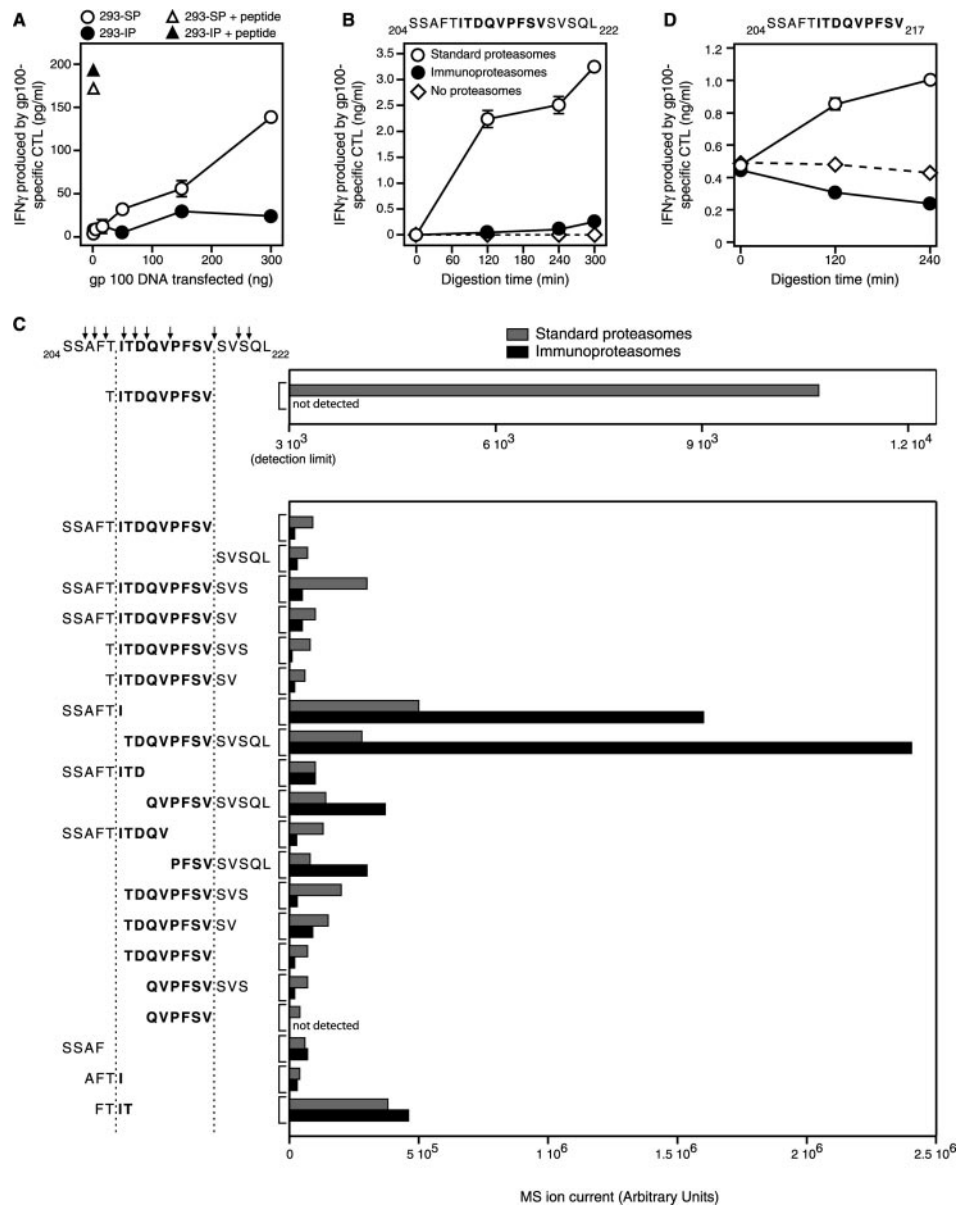
To determine whether this destructive cleavage was the main explanation or whether the observed difference in the intensity of the cleavage producing the C terminus also played an important role, we performed digestions of another gp100 precursor peptide, SSAFTITDQVPFSV, which corresponded to the antigenic peptide with its final C terminus and an N-terminal extension of 5 residues. This precursor, which does not require C-terminal cleavage, was poorly recognized by the CTL. Recognition strongly increased after digestion with SPs (Fig. 4D). In contrast, after digestion with IPs, recognition did not increase and even dropped below the recognition of the undigested precursor. These results are consistent with a destructive cleavage by the IP being the major mechanism accounting for the poor processing of the gp100 peptide by the IP.

#### Differential processing of peptide tyrosinase<sub>369–377</sub>

Peptide YMDGTMSQV is derived from tyrosinase, a melanocytic protein, and is presented to CTL by HLA-A2 (26, 27). We transfected 293-SP and 293-IP cells with the cDNAs encoding HLA-A2



**FIGURE 3.** Evaluation by two-dimensional gel electrophoresis of the purity of 20S proteasome preparations. SPs were purified from human erythrocytes, and IPs were purified from EBV-transformed B cell line LCL721 treated with IFN- $\gamma$ . Gels were loaded with 17  $\mu\text{g}$  of SPs or 15  $\mu\text{g}$  of IPs, and stained with Coomassie blue. By peptide mass fingerprinting using MALDI MS and data base search, each spot was identified as a proteasome subunit, showing the absence of detectable contaminant.



**FIGURE 4.** Differential processing of peptide gp100<sub>209–217</sub> (ITDQVPFSV). **A**, Presentation of peptide gp100<sub>209–217</sub> by 293-SP and 293-IP cells. Cells were transfected with 50 ng of an HLA-A2 plasmid construct and the indicated amount of plasmid encoding gp100. The gp100-specific CTL clone was added, and IFN- $\gamma$  production was measured. Control cells transfected with HLA-A2 only were loaded with the antigenic peptide ITDQVPFSV (3  $\mu$ g/ml) for 1 h before addition of the CTL. Error bars show SEM. **B**, CTL recognition of peptide digests obtained by incubation of gp100 precursor peptide SSAFTITDQVPFSVSVS SQL with SPs or IPs. Digests were loaded onto target cells T2, and gp100-specific CTL clone was added. Error bars show SEM. **C**, Peptide fragments detected by HPLC coupled to MS in digests obtained with SPs and IPs. The sequence of the 19-aa precursor peptide is shown on top, with the sequence of the epitope in bold and the arrows indicating the observed cleavage sites. Below are listed all the fragments detected after a 3-h digestion that allowed 54 and 57% degradation of the precursor peptide with SPs and IPs, respectively. The abundance of each fragment is shown in the right part of the figure. It should be noted that the quantitative values obtained by MS allow an accurate comparison of the amount of a given fragment in different digests, but are less reliable for comparison of different fragments with each other, because of potential variation in their ionization efficiency. We therefore confirmed the hierarchy of the destructive cleavages by measuring the UV absorbance of peaks corresponding to fragments SSAFTI, SSAFTITD, and SSAFTITDQV. In the SP digest we found the following: SSAFTI = 37,114 mV $\cdot$ s; SSAFTITD = 27,082 mV $\cdot$ s; SSAFTITDQV = 9,992 mV $\cdot$ s. In the IP digest we found the following: SSAFTI = 173,018 mV $\cdot$ s; SSAFTITD = 22,579 mV $\cdot$ s; SSAFTITDQV = 0 mV $\cdot$ s. **D**, CTL recognition of digests obtained by incubating proteasomes with gp100 precursor peptide SSAFTITDQVPFSV. Digests obtained with 0.2  $\mu$ g of precursor peptide and 0.15  $\mu$ g of proteasomes per time point were loaded onto T2 target cells and tested as in **B**. Symbols are defined in **B**.

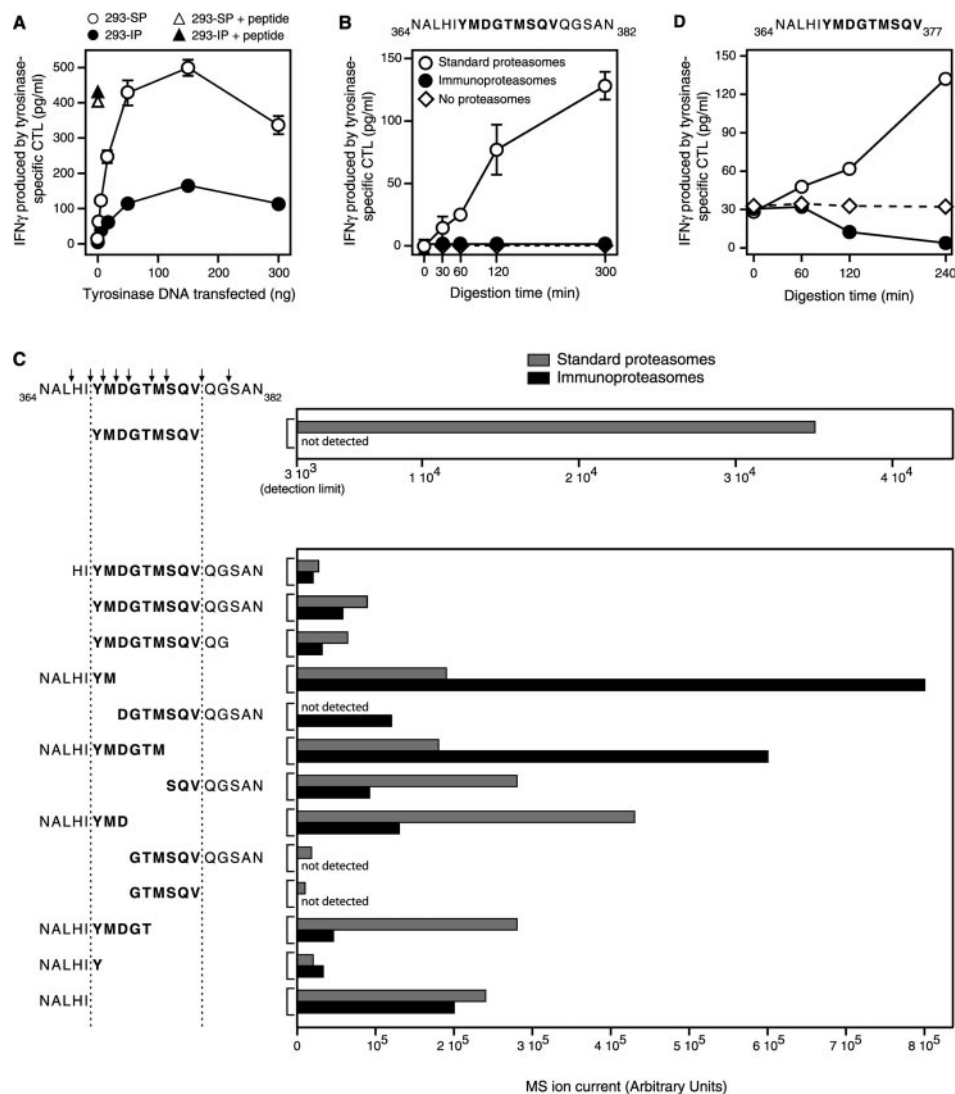
and tyrosinase, and we compared the ability of the two transfected cell types to stimulate the tyrosinase-specific CTL clone. Although both cell types were able to stimulate the CTL, 293-SP cells presented the peptide  $\sim$ 4 times more efficiently than 293-IP cells (Fig. 5A).

We then digested a synthetic peptide corresponding to the antigenic peptide extended by 5 aas at both the N and the C ter-

minus. After loading the digests onto HLA-A2-positive target cells, we observed that those obtained with SPs were efficiently recognized by the CTL, whereas those obtained with IPs were not recognized (Fig. 5B). These results confirmed the poor processing of this peptide by the IP. We then analyzed the digest by HPLC and MS (Fig. 5C). The fully processed antigenic peptide YMDGTMSQV was clearly detected in the SP digest, even though it was



**FIGURE 5.** Differential processing of peptide tyrosinase<sub>369–377</sub> (YM-DGTMSQV). **A**, Presentation of peptide tyrosinase<sub>369–377</sub> by 293-SP and 293-IP cells. Cells were transfected with HLA-A2 and the indicated amount of plasmid encoding tyrosinase before addition of tyrosinase-specific CTL clone IVSB. Control cells transfected with HLA-A2 only were loaded with the antigenic peptide YMDGTMSQV (6  $\mu$ g/ml) for 1 h before addition of the CTL. **B**, CTL recognition of peptide digests obtained by incubation of tyrosinase precursor peptide NALHIYMDGTMSQVQGSAN with SPs or IPs. Digests were loaded onto target cells T2 and tyrosinase-specific CTL clone IVSB was added. **C**, Peptide fragments detected by online HPLC/MS in digests obtained with SPs and IPs. Shown are all the fragments detected after a 5-h digestion that allowed 65 and 66% degradation of the precursor peptide with SPs and IPs, respectively. The abundance of each fragment is shown in the *right part* of the figure. The hierarchy of destructive cleavages could not be confirmed in this case by UV because the relevant fragments coeluted from the HPLC. **D**, CTL recognition of digests obtained by incubating proteasomes with gp100 precursor peptide NALHIYMDGTMSQV. Digests obtained with 1  $\mu$ g of precursor peptide and 1  $\mu$ g proteasomes per time point were loaded onto T2 target cells and tested as in **B**. Symbols are defined in **B**.



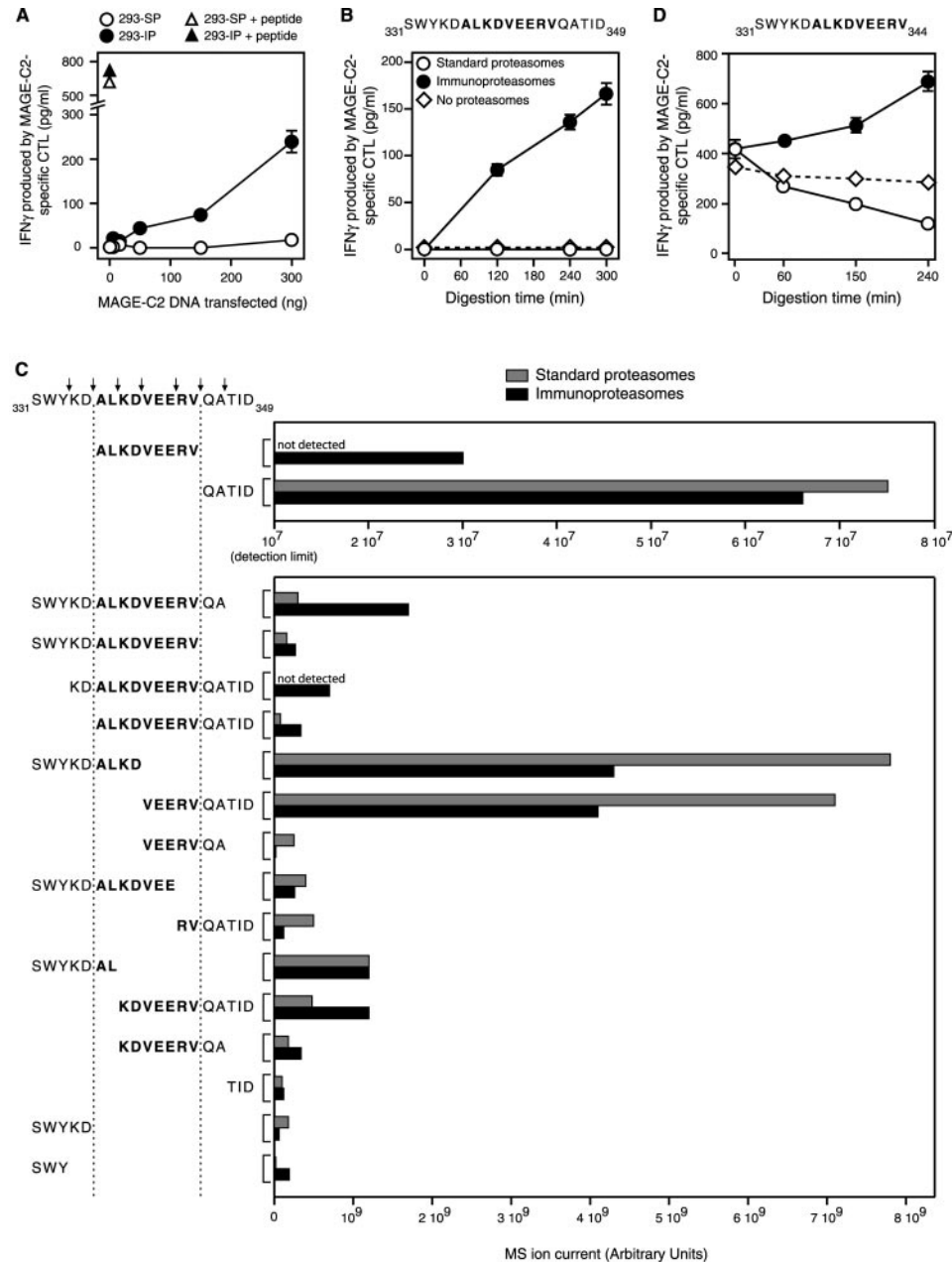
not abundant. It was not detectable in the IP digest, in line with the CTL stimulation data. Again, important differences between the two digests were observed in the abundance of fragments produced by cleavages occurring within the sequence of the antigenic peptide and resulting in its destruction. In particular, fragments produced by cleavage after methionine 370 and, to some extent, after methionine 374 were more abundant in the IP digest (Fig. 5C). Because our HPLC conditions did not allow the detection of the highly hydrophilic fragment QGSAN, we could not evaluate the relative efficiency of the C-terminal cleavage. Therefore, to evaluate whether destructive cleavages by the IP were the main explanation for the poor processing of the Ag by the IP or whether differences in C-terminal cleavage efficiency also played a role, we digested a precursor peptide extended at the N terminus only (NALHIYMDGTMSQV). The digests were loaded onto target cells and tested for recognition by the tyrosinase-specific CTL clone (Fig. 5D). The precursor peptide was weakly recognized by the CTL, and this recognition increased upon digestion with SPs, while it decreased upon digestion with IPs. These results were consistent with the notion that the stronger destructive cleavages observed were the main reasons for the poor processing of this peptide by the IP. Again, these cleavages occurred after hydrophobic residues and can be explained by the higher chymotrypsin-like activity of the IP. Other cleavages were also observed within the antigenic peptide, some of which (after D<sub>371</sub> and T<sub>373</sub>) being apparently

stronger with the SP. These internal cleavages appeared to play a minor role in the differential processing of this Ag, because they were obviously not strong enough to prevent production of the antigenic peptide by the SP.

#### Differential processing of peptide MAGE-C2<sub>336–344</sub> presented by HLA-A2

Peptide ALKDVEERV is encoded by cancer-germline gene *MAGE-C2*, and was recently identified using a CTL clone isolated from blood lymphocytes of a melanoma patient who showed regression of metastases after immunotherapy (28). Efficient recognition of melanoma cells was dependent on prior treatment with IFN- $\gamma$  (data not shown). After transfection with *MAGE-C2* and HLA-A2, 293-IP cells were strongly recognized by the MAGE-C2-specific CTL, whereas 293-SP cells were not, even when high amounts of *MAGE-C2* cDNA were transfected (Fig. 6A). Those results indicated that this peptide was produced more efficiently by the IP. This was confirmed when we tested the CTL recognition of digests obtained with a precursor peptide extended by 5 residues at both extremities: only the digests obtained with IPs were recognized by the CTL (Fig. 6B). Analysis of the digests by HPLC followed by MS confirmed the presence of the antigenic peptide in the digests obtained with IPs and its absence in the other digests (Fig. 6C). Equal amounts of complementary fragment QATID were found in both digests, indicating that both proteasomes made

**FIGURE 6.** Differential processing of peptide MAGE-C2<sub>336–344</sub> (ALKD VEERV). **A**, Presentation of peptide MAGE-C2<sub>336–344</sub> by 293-SP and 293-IP cells. Cells were transfected with HLA-A2 and the indicated amount of plasmid encoding MAGE-C2 before addition of MAGE-C2-specific CTL clone 16. Control cells transfected with HLA-A2 only were loaded with the antigenic peptide ALKDVEERV (1  $\mu$ g/ml) for 1 h before addition of the CTL. **B**, CTL recognition of peptide digests obtained by incubation of MAGE-C2 precursor peptide SWYK DALKDVEERVQATID with SPs or IPs. Digests were loaded onto target cells T2 and MAGE-C2-specific CTL clone 16 was added. **C**, Peptide fragments detected by online HPLC/MS in digests obtained with SPs and IPs. Shown are all the fragments detected in digests obtained after a 150-min (SPs) or a 360-min (IPs) digestion that allowed 64 and 66% degradation of the precursor peptide, respectively. The hierarchy of the destructive cleavages was confirmed by measuring the UV absorbance of peaks corresponding to fragments SWYKDALKD, SWYKDALKDVEE, and SWYKDAL. In the SP digest we found the following: SWYK DALKD = 4,167,417 mV·s; SWYK DALKDVEE = 214,507 mV·s; SWYKDAL = 801,682 mV·s. In the IP digest we found the following: SWYK DALKD = 2,140,740 mV·s; SWYK DALKDVEE = 136,899 mV·s; SWYKDAL = 711,703 mV·s. **D**, CTL recognition of digests obtained by incubating proteasomes with MAGE-C2 precursor peptide SWYKDALKDVEERV. Digests obtained with 0.12  $\mu$ g of precursor peptide and 0.15  $\mu$ g of proteasomes per time point were loaded onto T2 target cells and tested as in **B**. Symbols are defined in **B**.



the C-terminal cleavage with a similar efficiency, and suggesting that the lack of processing of the antigenic peptide by the SP results from destructive internal cleavage(s). In line with this suggestion, the HPLC/MS analysis revealed a prominent cleavage within the sequence of the antigenic peptide, after the aspartic acid in position 339. Fragments resulting from this cleavage were observed in both digests but were more abundant in the digests obtained with SPs (Fig. 6C). These results indicate that the MAGE-C2<sub>336–344</sub> peptide is produced by the IP but not by the SP, because the latter destroys the Ag by making an internal cleavage with a higher efficiency. This was further confirmed when we tested CTL recognition of digests obtained with a precursor peptide that did not require C-terminal trimming (Fig. 6D). Occurring after an acidic residue, the destructive cleavage results from the caspase-like activity, which is known to be higher in the SP. Differential processing of the MAGE-C2 peptide is, therefore, again in line with the known differences in the activity of the two proteasome types.

# Discussion

We previously described the first two examples of antigenic peptides that are better produced by the SP, namely a peptide derived from human ubiquitous protein RU1 and another one derived from human melanocytic protein Melan-A (4). We present in this study two additional peptides, one derived from gp100 and the other from tyrosinase, both of which are also better produced by the SP. This strengthens the conclusion that the IP is not always the most effective proteasome type in producing antigenic peptides, contrary to previous expectations. Interestingly, these findings are not limited to ubiquitous or differentiation Ags, because recent work in a mouse model indicated that this also applies to a viral peptide derived from lymphocytic choriomeningitis virus (21). It is noteworthy that IFN- $\gamma$ , which is known to promote Ag presentation by multiple mechanisms, also prevents the production of some antigenic peptides through the induction of IPs.



We have investigated the reason for the lack of processing of those antigenic peptides by the IP. Interestingly, we found that the gp100 and tyrosinase peptides were preferentially destroyed by the IP due to cleavage after internal hydrophobic residues, in line with the higher chymotrypsin-like activity of this proteasome type. These destructive cleavages appear to account for the differential processing of these Ags, although differences in the efficiency of the C-terminal cleavage may also play a role. It is generally accepted that the IP favors the production of antigenic peptides because of its higher chymotrypsin-like activity, which should increase the production of peptides that bear hydrophobic residues at their C terminus and therefore can efficiently bind to MHC class I molecules. Our results qualify this concept by showing that, for the same reason, IPs can also destroy antigenic peptides.

We also describe a MAGE-C2 peptide that is better produced by the IP. We found that this is largely due to a preferential destruction of the antigenic peptide by the SP. The destructive cleavage occurs after an acidic residue, and is therefore in line with the higher caspase-like activity of the SP defined with fluorogenic peptides. This result is also in accordance with previous observations, suggesting that the SP could destroy some viral epitopes (17–19).

Many peptides contain internal hydrophobic or acidic residues; consequently, the set of peptides produced by either proteasome type could differ more than expected. However, it is important to underline the influence of the surrounding sequence on proteasomal cleavage sites. A statistical analysis of proteasomal cleavage products has indicated that amino acids located up to five residues before or after a given position may determine whether cleavage occurs at this position or not (42). Additional analyses will therefore be required for predicting the differential processing of a given peptide.

Among the peptide fragments that were observed after digestion with the proteasomes, the antigenic peptide, when detected, always represented a quantitatively minor species. This is in line with recent reports showing the low yield of antigenic peptides produced by intracellular protein degradation (43, 44). This low yield implies that in many cases, the number of antigenic peptides presented at the cell surface is probably not much above the threshold needed for recognition by CD8 T cells. This may help to explain how differences in the processing of a given peptide by the two proteasome types can have a great impact on CTL activation. Thus, poor processing by one proteasome may result in the production of the antigenic peptide in amounts that are below the sensitivity limit of the CTL.

The four antigenic peptides derived from ubiquitous or differentiation proteins that we studied are all poorly processed by the IP. This high proportion might reflect a bias in the T cell repertoire, which might be selectively depleted in T cells recognizing self peptides efficiently produced by the IP. Expression of tissue-specific differentiation proteins was observed in the thymus, whereas thymic cells responsible for presentation of self Ags for negative selection, i.e., medullary thymic epithelial cells and thymic dendritic cells, were found to predominantly contain IPs (6, 45). Hence, CTLs recognizing peptides that are efficiently processed by the IP could be preferentially deleted from the peripheral T cell repertoire, which therefore would be enriched in CTLs recognizing peptides poorly produced by the IP.

So far, the tumor antigenic peptides that we found to be better processed by the IP are all encoded by cancer-germline genes of the *MAGE* family. They include peptides MAGE-C2<sub>336–344</sub>, MAGE-A3<sub>114–122</sub>, and another MAGE-C2 peptide presented by HLA-B57 (Ref. 16; our unpublished data). Cytolytic T lymphocytes directed against these peptides obviously escaped negative selection in the thymus, because they were isolated from blood

lymphocytes of cancer patients. The scenario outlined above could explain this fact only if cancer-germline genes were not expressed in the thymus. However, Kyewski and colleagues (46) recently reported the expression of some cancer-germline genes in medullary thymic epithelial cells. The contrast between differentiation and cancer-germline antigenic peptides remains therefore unexplained. Analysis of additional peptides is needed to confirm this difference. The unique cellular system described in this study should prove a useful tool to evaluate the relative processing of selected Ags by the two proteasome types.

The functional consequences of differential processing are potentially numerous, and some of them have already been discussed (47). Because mature dendritic cells mainly contain IPs, vaccination modalities that are based on full-length proteins or full-length recombinant vectors should induce CTL responses against peptides produced by the IP but not against those only produced by the SP. The poor presentation of peptide gp100<sub>209–217</sub> by dendritic cells infected with a full-length gp100 recombinant virus was confirmed in a recent study (48). The induction of CTL responses against such peptides might require an immunization procedure that bypasses intracellular processing by dendritic cells. This is the case of synthetic peptides or recombinant vectors based on peptide-encoding minigenes. Nevertheless, this requirement may not be absolute, because the poor processing by the IP may be offset by the use of vectors that produce a high expression of the encoding gene (49–51). However, immunization strategies that do not produce high levels of precursors of antigenic peptides in dendritic cells, such as those that are based on recombinant proteins or tumor lysates and rely mainly on cross-priming, may be hampered by poor processing by the IP.

At the tumor site, in the absence of an inflammatory environment, peptides produced by the SP would be presented by the tumor cells. Therefore, only CTLs directed against such peptides would be useful at the onset of the response. It might therefore be important for the success of cancer immunotherapy to use vaccination modalities that initiate effective CTL responses against peptides produced by the SP. However, this issue is complicated by the possibility that, for example, primary tumors and metastatic lesions might differ in their proteasome type. This could explain the occurrence of dissociated or mixed tumor responses to immunotherapy. In conclusion, it appears that a subset of the antigenic peptides presented by tumor cells is dependent on the type of proteasome present in those cells. It follows that the set of antigenic peptides presented by tumor cells displays a certain degree of plasticity, the clinical relevance of which remains to be defined.

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## Disclosures

The authors have no financial conflict of interest.

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