The Production of a New MAGE-3 Peptide Presented to Cytolytic T Lymphocytes by HLA-B40 Requires the Immunoproteasome

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Abstract

By stimulating human $CD8^+$ T lymphocytes with autologous dendritic cells infected with an adenovirus encoding MAGE-3, we obtained a cytotoxic T lymphocyte (CTL) clone that recognized a new MAGE-3 antigenic peptide, AELVHFLLL, which is presented by HLA-B40. This peptide is also encoded by *MAGE-12*. The CTL clone recognized MAGE-3–expressing tumor cells only when they were first treated with IFN-γ. Since this treatment is known to induce the exchange of the three catalytic subunits of the proteasome to form the immunoproteasome, this result suggested that the processing of this MAGE-3 peptide required the immunoproteasome. Transfection experiments showed that the substitution of β5i (LMP7) for β5 is necessary and sufficient for producing the peptide, whereas a mutated form of β5i (LMP7) lacking the catalytically active site was ineffective. Mass spectrometric analyses of in vitro digestions of a long precursor peptide with either proteasome type showed that the immunoproteasome produced the antigenic peptide more efficiently, whereas the standard proteasome more efficiently introduced cleavages destroying the antigenic peptide. This is the first example of a tumor-specific antigen exclusively presented by tumor cells expressing the immunoproteasome.

Key words: β5i • proteasome • mass spectrometry • tumor • HLA-B40

Introduction

Therapeutic vaccinations of cancer patients aim at stimulating the immune system, in particular the CTL, so as to eliminate cancer cells efficiently and safely (1). Various immunization modalities are being applied in vaccination trials: injection of antigenic peptides, either alone or loaded onto dendritic cells, injection of recombinant proteins or recombinant viral vectors encoding either the full-length protein or only a single peptide. The dendritic cells most probably play a crucial role in the activation of the CTL precursors, and they have to present efficiently the antigenic peptides at their surface. Once activated, the CTLs have to reach the tumor site and destroy the tumor cells expressing the same antigenic peptides. Such peptides are presented by MHC class I molecules, and derive from intracellular proteins that are degraded by the proteasome, a proteolytic complex playing a critical role in this antigen processing pathway.

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The catalytic core of the standard proteasome has the shape of a barrel made of four rings, with the three catalytically active subunits, β1, β2, and β5 located in each of the two inner rings (2). It was recently shown that mature dendritic cells express a different sort of proteasome in which the three catalytic subunits are replaced by their IFN-γ–inducible homologs, β1i (LMP2), β2i (MECL1), and β5i (LMP7) (3, 4). The resulting proteasome, named immunoproteasome, has slightly different catalytic activities, which can result in different cleavage specificities (5–7). Therefore, a number of antigenic peptides are not processed with the same efficiency by the immunoproteasome and by the standard proteasome (8). Most nonlymphoid cells, be they normal or tumoral, constitutively express standard proteasomes, and switch to immunoproteasomes when exposed to IFN-γ (2).

Since mature dendritic cells and cancer cells constitutively express different proteasome types, it is crucial for the success of cancer immunotherapy to determine precisely the relative efficiency of the two proteasome types to process the antigenic peptides considered. For viral antigens, there are several examples of peptides that are processed exclusively by the immunoproteasome (9–11). On the opposite, some antigenic peptides derived from self-proteins expressed either ubiquitously or only in the melanocytic lineage appear to be processed more efficiently by the standard proteasome (4, 8).

We report here the characterization of a new tumor-specific peptide which is recognized by CTLs exclusively on melanoma cells expressing the immunoproteasome. This antigenic peptide is encoded by genes *MAGE-3* and *MAGE-12*. These genes are expressed in various tumors but not in most normal tissues and they encode several tumor-specific antigens recognized by T lymphocytes (12). The new MAGE-3 antigen described here was identified in the course of an effort to identify additional tumor-specific peptides of clinical interest.

Materials and Methods

Cell Lines, Media, Reagents, and Antibodies. The Epstein-Barr virus–immortalized B (EBV-B)* cell lines and the melanoma cell lines were cultured in IMDM supplemented with 10% FCS. COS-7 cells were maintained in DMEM medium supplemented with 10% FCS. All culture media were purchased from Life Technologies and supplemented with 0.24 mM L-asparagine, 0.55 mM L-arginine, 1.5 mM L-glutamine (AAG), 100 U/ml penicillin, and 100 μg/ml streptomycin. Human recombinant IL-2 was purchased from Eurocetus. 1 U/ml of IL-2 is the concentration needed to obtain half-maximal proliferation of mouse CTLL-2 cells. IL-7 was purchased from Genzyme, GM-CSF (LEUCOMAX) from Novartis Pharma, and IFN-γ from R&D Systems. Human recombinant IL-4, IL-6, and IL-12 were produced in our laboratory. 1 U/ml of IL-6 is the concentration needed to obtain half-maximal proliferation of mouse 7TD1 cells (13). Flow-cytometric analyses were performed with the following antibodies: CD3-FITC, CD4-FITC, and CD8-FITC (Becton Dickinson).

Recombinant Viruses. The vaccinia virus encoding MAGE-3 (vaccinia MAGE-3) was provided by Vincenzo Cerundolo (Molecular Immunology Group, University of Oxford, UK). Before infection, the vaccinia virus samples were sonicated for 30 s. For the construction of the recombinant adenovirus adeno-MAGE-3, the plasmid pAd-CMVIcpA-MAGE-3, containing the *MAGE-3* cDNA under the control of the cytomegalovirus promoter, was obtained by inserting the complete *MAGE-3* cDNA into the NotI site of vector pAd-CMVIcpA (provided by Celia Garcia and Thierry Ragot, Institut Gustave Roussy, Paris, France). The recombinant adeno-MAGE-3 was constructed in cell line 293 by in vivo homologous recombination between pAd-CMVIcpA-MAGE-3 and adeno-βgal genomic DNA (14). The recombinant adenovirus was plaque purified, propagated in 293 cells, and purified by double cesium chloride density centrifugation. The construction of the retroviral construct coding for MAGE-3 and the transfection of cell lines was done as described previously (15).

Dendritic Cells and $CD8^+$ Responder T Cells. Peripheral blood was obtained from hemochromatosis patient LB1841 as standard buffy coat preparations, which were laid down on a 15-ml Lymphoprep layer (Nycomed Pharma) in 50-ml tubes. To minimize contamination of the PBMCs by platelets, the tubes were first centrifuged at 1,000 rpm for 20 min at room temperature. After removal of the top 20–25 ml, containing most of the platelets, the tubes were centrifuged at 1,500 rpm for 20 min at room temperature. The interphase containing the PBMCs was harvested and washed three times (or more) in cold phosphate buffer solution with 2 mM EDTA in order to eliminate the remaining platelets. To generate autologous dendritic cells, PBMCs were depleted from T lymphocytes by rosetting with sheep erythrocytes (Bio Mérieux) treated with 2-aminoethylisothiouronium (Sigma-Aldrich). Rosetted T cells were treated with NH_4CL (160 mM) to lyse the sheep erythrocytes and washed. $CD8^+$ T lymphocytes were isolated from rosetted T cells by positive selection using an anti-CD8 mAb coupled to magnetic microbeads (Miltenyi Biotec). They were then sorted through a magnet and subsequently frozen. The day before the first stimulation, $CD8^+$ T cells were thawed and grown overnight in IMDM supplemented with 10% human serum, AAG, and antibiotics (hereafter referred to as complete IMDM) in the presence of 10 U/ml of IL-2. The lymphocyte-depleted PBMCs were left to adhere for 2 h at 37°C in culture flasks (FALCON; Becton Dickinson) at a density of 10^6 cells per cm^2 in RPMI 1640 supplemented with Hepes (2.38 g/liter), AAG, antibiotics, and 10% FCS (hereafter referred to as complete RPMI medium). Nonadherent cells were discarded and adherent cells were cultured in the presence of IL-4 (100 U/ml) and GM-CSF (100 ng/ml) in complete RPMI medium. Cultures were fed on days 2 and 4 by removing 1/3 of the volume and adding fresh medium with IL-4 (100 U/ml) and GM-CSF (100 ng/ml). On day 7, >95% of the cells were $CD14^-CD83^-CD86^{lo}HLA\text{-}DR^{lo}$ as assessed by flow cytometry (FACScan™; Becton Dickinson) after labeling with CD14-FITC, CD83-PE, CD86-PE, or HLA-DRPE (Becton Dickinson). They were frozen on day 7.

Mixed Lymphocyte–Dendritic Cells Culture. Autologous dendritic cells from donor LB1841 (2×10^6) were thawed and infected with the adeno-MAGE-3 virus, at a multiplicity of infection of 500, in 200 μl of complete RPMI medium at 37°C under 5% CO_2. The infected dendritic cells were washed after 2 h. Autologous responder $CD8^+$ T lymphocytes (150,000) and infected dendritic cells (30,000) were cocultured in U-bottomed micro-

Abbreviation used in this paper: EBV-B cell, Epstein-Barr virus–immortalized B cell.

wells in 200 μl of complete IMDM in the presence of IL-6 (1,000 U/ml) and IL-12 (10 ng/ml). The $CD8^+$ lymphocytes were stimulated once per week with autologous dendritic cells freshly infected with the adeno-MAGE-3 virus, and grown in complete IMDM supplemented with IL-2 (10 U/ml) and IL-7 (5 ng/ml).

Cytotoxicity Assay. To test the MAGE-3 reactivity of the microcultures, autologous EBV-B cells were infected for 2 h with either the parental vaccinia virus or the vaccinia MAGE-3 construct using a multiplicity of infection of 20. The infected target cells were then labeled with 100 μCi of $Na(^{51}Cr)O_4$ for 1 h, washed, and added to the responder cells. Unlabeled K562 cells were also added (5×10^4 per V-bottomed microwell) to block natural killer activity. The individual microcultures were tested in duplicate on each target at an effector/target ratio of ~40:1. Chromium release was measured after incubation at 37°C for 4 h. To test the lytic activity of CTL 52, no unlabeled K562 cells were added during the assay. The melanoma cell lines were treated or not with 100 U/ml of IFN-γ for 48 h and labeled with $Na(^{51}Cr)O_4$ as described above.

Isolation of CTL Clones Specific for MAGE-3. 1 microculture containing cells with anti–MAGE-3 reactivity was cloned by limiting dilution in 96-well plates using irradiated autologous EBV-B cells transduced with a retrovirus coding for MAGE-3 as stimulating cells (5,000–15,000 cells per well), and irradiated allogeneic LG2-EBV as feeder cells (5,000–15,000 cells per well), in the presence of IL-2 (50 U/ml). CTL clones were maintained in culture in 24-well plates by weekly restimulation with either autologous EBV-B cells transduced with a retrovirus coding for MAGE-3 (10^5 cells) or purified PHA (0.5 μg/ml) (HA 16; Murex Biotech) in complete IMDM supplemented with 50 U/ml IL-2, in the presence of allogeneic LG2-EBV as feeder cells (10^6 cells).

Transfection of COS-7 Cells and TNF Assay. COS-7 cells (1.5×10^4) were distributed in microwells and cotransfected using 1 μl of LIPOFECTAMINE (Life Technologies) with the indicated cDNAs cloned into expression plasmids that multiply episomally in COS-7 cells (16). The *MAGE-1*, *3*, *4*, *8*, *9*, *10*, *11*, and *12* cDNAs were inserted into pcDNAI/Amp (Invitrogen). The *MAGE-2* and *6* cDNAs were inserted into pcDSR-α (17). The *HLA-B*4001* cDNA was inserted into pcDNA3 (Invitrogen). The HLA coding sequences were isolated from various individuals and were also inserted into pcDNA3. The COS-7 cells were incubated for 24 h at 37°C and 8% CO_2. Transfectants were tested for their ability to stimulate the production of TNF by CTL 52. Briefly, 2,500 CTLs were added to the transfectants, in a total volume of 100 μl of complete IMDM containing 25 U/ml of IL-2. After 24 h, the supernatant was collected and its TNF content was determined by testing its cytotoxic effect on WEHI-164 clone 13 cells in a MTT colorimetric assay (18–20).

Site-directed Mutagenesis of β5i. In vitro–directed mutagenesis was performed on the β5i cDNA cloned into vector pEF-BOSpuro (PL3) with the Gene editor in vitro site–directed mutagenesis kit (Promega) using the mutagenesis primer 5′-P-GCCCATGGCGCCACCACGCTC-3′ and the top strand selection primer. The mutation (G instead of A) introduced in the primer changes a Threonine into an Alanine at position 73 of the full-length β5i.E2 protein (i.e., position 1 of the mature protein) (21, 22).

Transfection of 293-EBNA Cells and IFN-γ Assay. Human 293-EBNA cells (15,000) were transiently transfected in microwells with 150 ng of cDNA and 1.3 μl of LIPOFECTAMINE. β1i was cloned in expression vector pcDNA3 (Invitrogen), β2i in pEF/myc/cyto (Invitrogen), β5i, and mutated β5i in pEF-BOSpuro (PL3) (4, 23). cDNAs coding for MAGE-3, HLA-B*4001, β1i, β2i, β5i, or mutated β5i were added in different combinations in the DNA mix at 30 ng each, and β galactosidase (β Gal) was added to reach a total of 150 ng. 2 d after transfection, 7,000 cells of CTL 52 were added to the transfected cells. IFN-γ production was measured by ELISA after 20 h of coculture. SK28-MEL were treated with IFN-γ (100 UI/ml) for 48 h where indicated.

Determination of the Antigenic Peptide. Peptides were synthesized on solid phase using F-moc for transient NH_2-terminal protection and were characterized using mass spectrometry. All peptides were >90% pure, as indicated by analytical HPLC. Lyophilized peptides were dissolved at 2 mg/ml in 10 mM acetic acid and 10% DMSO, and stored at –20°C. The first screening was performed with autologous EBV-B cells incubated with peptides at a concentration of 1 μg/ml, and tested for recognition by CTL 52 at an effector/target ratio of 5:1.

Purification and Quantification of 20S Proteasomes. Frozen pellets of LB1751 cells and of LB1751 cells treated with IFN-γ for 10 d (100 U/ml) were lysed on ice in Tris 25 mM NaCl 50 mM, pH = 7.5, and homogenized in a Dounce homogenizer. Unsolubilized material was removed by centrifugation at 40,000 *g*. Proteasomes were purified from cleared supernatants by immunoaffinity chromatography on MCP21 (a mAb directed against subunit α2, ECACC96030418) covalently linked to CNBr-activated 4B sepharose beads (Amersham Pharmacia Biotech) as described previously (24). The purity of the proteasome was checked by SDS-PAGE and was >95%. Purified proteasomes were quantified with a BCA Protein Assay (Pierce Chemical Co.). A second quantification was performed by a sandwich ELISA: 96-well microtiter plates (Nunc-Immuno Plate and MaxiSorp; Life Technologies) were coated with the MCP-21 (5 μg/ml) by overnight incubation at 4°C in 100 μl of PBS. Plates were then washed three times with PBS containing 0.1% Tween 20 (PBS-T), blocked with PBS containing 2% BSA for 1 h at room temperature, and washed again three times with PBS-T. 100 μl of serial dilutions of purified proteasomes were added to the plates and incubated for 2 h at room temperature. After this incubation step, the plates were washed three times with PBS-T and incubated with a rabbit antiserum (1 μg/ml) raised against human proteasome for 1 h at room temperature. Subsequently, plates were washed three times and incubated with 50 μl of AP-labeled goat anti–rabbit (1:1,000) (Southern Biotechnology Associates) for another 1 h at room temperature. Enzymatic reactions were performed at room temperature by adding p-nitrophenyl phosphate (Sigma-Aldrich). Color development was measured at 405 nm in a microplate reader. Quantification of proteasomes was performed in triplicates. Concentration was determined by regression analysis based on a reference preparation of standard proteasome from human erythrocytes.

Digestion of Synthetic Precursor Peptide and CTL Assay. 5 μg of 19-amino-acid-long precursor peptide LSRKVAELVHFLLLKYRAR were incubated with 4 μg of either standard or immunoproteasomes at 37°C in 86 μl of Tris buffer (25 mM, pH 7.6). At different incubation times (0, 30, 60, 120 min), an aliquot (20 μl) corresponding to the digestion of 1.25 μg precursor peptide with 1 μg of proteasomes was removed from the digestion mixture and the reaction stopped by addition of 2 μl of 10% trifluoroacetic acid. Aliquots were then lyophilized and stored at −20°C. Lyophilized digests were resuspended in 12 μl of cold PBS. 5 μl were diluted to 155 μl in X-vivo 10 medium and 50 μl of this dilution were pulsed in triplicates on 30,000 LB1841 EBV-B cells distributed in microculture plates. CTL 52 was

added (6,000 cells per well) with IL-2 (25 U/ ml) in a final volume of 150 μl of X-vivo 10 medium. The supernatants were collected after 20 h and their IFN-γ content was measured by ELISA using reagents from Biosource.

Mass Spectrometry. Digestions were performed with 20 μg of the long precursor peptide and 4 μg of either standard or immunoproteasomes at 37°C in 86 μl of Tris buffer (25 mM, pH 7.6). At different incubation times (0, 30, 60, 120 min), an aliquot (20 μl) corresponding to the digestion of 5 μg precursor peptide with 1 μg of proteasomes was removed from the digestion mixture and the reaction stopped by addition of 2 μl of 10% trifluoroacetic acid. Aliquots were then lyophilized and stored at −20°C. Lyophilized digests were resuspended in 12 μl of cold PBS. 5 μl were used for mass spectrometric analysis. Peptides were separated on a Pepmap™ LC Packings C18 column (1 mm × 10 cm) at a flow rate of 40 μl/min. The separation was achieved with a gradient elution of 5–55% B for 45 min (A is 5/95/0.05 CH3CN/H2O/HCOOH, B is 50/50/0.05 CH3CN/H2O/HCOOH vol/vol/vol). Mass spectrometric analyses were performed on a TSQ 700 triple quadrupole (Finnigan Mat) equipped with an electrospray source. Mass spectra were obtained by scanning the range of masses corresponding to *m/z* between 200 and 1,950 every 3 s. Peptide quantitation was obtained from the peak height of a given mass/charge ratio in Reconstructed Ion Current analysis, using an internal calibrating peptide. Results are reported as relative intensities, corresponding to the measured intensity of each fragment corrected for the measured intensity of the precursor peptide at $t = 0$.

Results

Dendritic cells obtained from donor LB1841 were infected with a recombinant adenovirus containing the entire MAGE-3 coding sequence (adeno-MAGE-3). 96 microcultures were set up with 3×10^4 of these stimulator dendritic cells and 1.5×10^5 autologous $CD8^+$ T cells, in the presence of IL-6 and IL-12. The T cells were restimulated weekly with autologous dendritic cells infected with adeno-MAGE-3, in the presence of IL-2 and IL-7. They were tested on day 28 for their lytic activity on autologous EBV-B cells infected with a vaccinia virus encoding MAGE-3 (vaccinia-MAGE-3). A vaccinia recombinant virus was used to avoid detecting CTLs directed against adenoviral antigens. As a negative control, we used EBV-B cells infected with the parental vaccinia virus.

5 microcultures displaying anti–MAGE-3 reactivity were obtained. Microculture F7 was cloned by limiting dilution in the presence of IL-2, irradiated allogeneic EBV-B cells as feeder cells and irradiated autologous EBV-B cells transduced with a retrovirus coding for MAGE-3 as stimulator cells. Several CTL clones displaying anti-MAGE-3 reactivity were isolated, such as CTL clone LB 1841 526/F7.52 hereafter referred to as CTL 52. This $CD3^+$ $CD8^+$ $CD4^-$ clone lysed autologous EBV-B cells infected with vaccinia-MAGE-3 but neither EBV-B cells infected with the parental vaccinia virus nor K562 cells (Fig. 1).

*A New MAGE-3 Antigenic Peptide Presented by HLA-B*4001.* Donor LB1841 was serologically typed HLA-A3, B35, B60, Cw3, and Cw4. Genetic typing for the HLA-B loci revealed that B60 corresponds to B*4001. To identify the HLA molecule that presents the MAGE-3 peptide recognized by CTL 52, COS-7 cells were transiently transfected with the *MAGE-3* cDNA together with each of the cDNAs encoding these HLA molecules. Transfected cells were subsequently tested for recognition by CTL 52. Only these cells that were transfected with both *MAGE-3* and *HLA-B*4001* stimulated CTL 52 to produce TNF above the level obtained by transfecting only *MAGE-3* (Fig. 2).

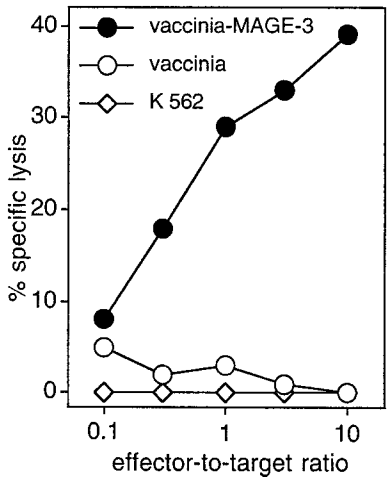

Figure 1. Lysis by CTL 52 of autologous EBV-B cells infected with vaccinia-MAGE-3. LB1841-EBV-B cells were infected for 2 h with either vaccinia-MAGE-3 or parental vaccinia at a multiplicity of infection of 20. The infected EBV-B cells and K 562 cells were ^{51}Cr-labeled for 1 h, and incubated with the CTL clone at the indicated effector-to-target ratios. Chromium release was measured after 4 h.

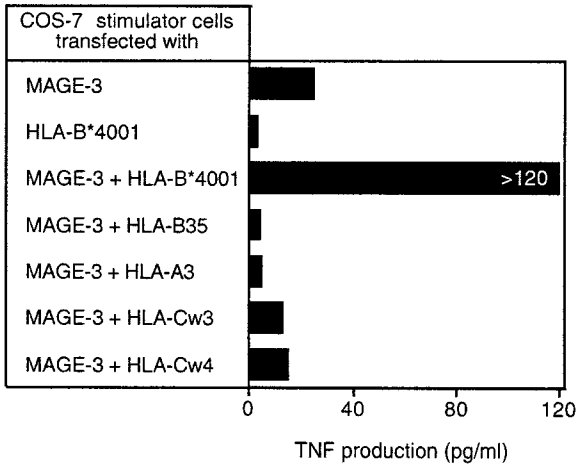

Figure 2. The MAGE-3 antigenic peptide is presented by HLA-B40 molecules. Monkey COS-7 cells were transiently transfected with a *MAGE-3* cDNA and each of the cDNAs coding for putative HLA-presenting molecules. Transfections were performed in microwells with 15,000 COS-7 cells, 50 ng of each cDNA and 1 μl of LIPOFECTAMINE. 1 d after transfection, 2,500 cells of CTL 52 were added to the transfected cells. TNF production was measured after overnight coculture by testing the toxicity of the supernatants for TNF-sensitive WEHI-164 clone 13 cells.

To determine whether the peptide recognized by CTL 52 is also encoded by other *MAGE* genes, COS-7 cells were transiently transfected with an HLA-B*4001 cDNA together with cDNAs corresponding to other genes of the *MAGE-A* family that are expressed in tumors (25). We observed that cells transfected with *MAGE-12* were also recognized by CTL 52 (Fig. 3).

Identification of the Antigenic Peptide. To identify the antigenic peptide recognized by CTL 52 on HLA-B40 molecules, a set of MAGE-3 peptides was screened. These 16 amino-acid long peptides overlapped by 12 residues and covered the entire MAGE-3 protein sequence. Autologous EBV-B cells were incubated with each of these peptides at a concentration of 1 μg/ml and tested for lysis by CTL 52. 36% of lysis was observed with cells pulsed with peptide AALSRKVAELVHFLLL compared with <7% using the other peptides. Nonapeptide AELVHFLLL (MAGE-$3_{114–122}$ and MAGE-$12_{114–122}$), which corresponds to the COOH-terminal part of this peptide, contains the consensus anchor motif for HLA-B40 (B60), namely E in position 2 and L in position 9 (26). It produced half-maximal lysis of the pulsed target cells at a concentration of <1 nM (Fig. 4). Octapeptide AELVHFLL was not recognized by the CTL (data not shown).

Lysis of HLA-B40-positive Tumor Cell Lines Expressing MAGE-3. 3 melanoma cell lines that were obtained from HLA-B40 patients and that expressed MAGE-3 were tested for their recognition by CTL 52 in a chromium release assay. None of them was lysed. However, all these cells were lysed after treatment with IFN-γ during 48 h before the test (Fig. 5). The three melanoma lines were well recognized, even in the absence of IFN-γ treatment, when they were pulsed with peptide AELVHFLLL, indicating that HLA-B40 molecules were present and that the effect of IFN-γ did not simply result from their induction. We also observed that the IFN-γ treatment did not increase the level of expression of *MAGE-3*, as measured by semiquantitative RT-PCR (data not shown).

The Production of the MAGE-3 Antigenic Peptide Requires the Immunoproteasomes. The effect of IFN-γ on the recognition of the B40 cell lines suggested that the immunoproteasome was required for the production of the MAGE-3.B40 antigen. To prove this, we transfected 293-EBNA cells with low amounts of *MAGE-3* and *HLA-B40* cDNAs, together with the cDNAs coding for immunoproteasome subunits β1i, β2i, and β5i. In the absence of those subunits, there was no recognition of the transfected cells by CTL 52. The cotransfection of β5i was sufficient to allow recognition of the transfected cells, whereas the transfection of β1i and β2i had no effect (Fig. 6). Transfection of the three immunoproteasome subunits together did not result in a better recognition of the cells than transfection of β5i alone. Cells transfected with a mutated form of β5i lacking the threonine of the catalytic site were unable to activate CTL 52, indicating that the catalytic activity of β5i is required for the production of the antigenic peptide.

To study further the mechanisms leading to the difference in the production of the antigenic peptide by the two types of proteasome, we compared the results of the digestion of a long precursor peptide by either immunoproteasomes or standard proteasomes, which were purified from melanoma cells treated or not with IFN-γ, respectively. The presence of subunits β1i, β2i, and β5i in the immunoproteasome preparation and their almost complete absence in the standard proteasome batch were confirmed by im-

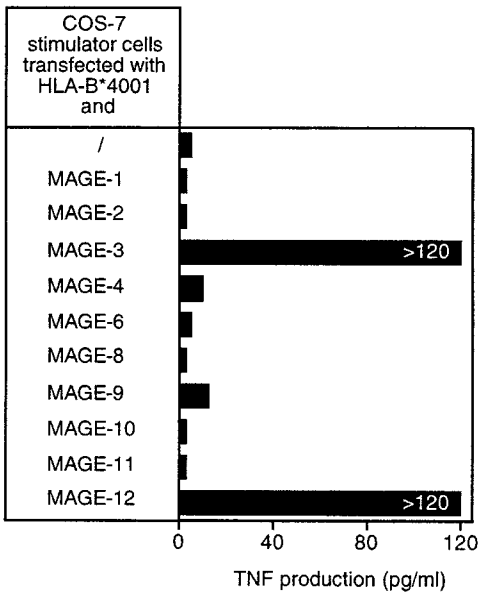

Figure 3. The peptide recognized by CTL 52 is encoded by *MAGE-3* and *MAGE-12*. Monkey COS-7 cells were transiently transfected with an *HLA-B*4001* cDNA and each of the *MAGE-A* cDNAs. Transfections were performed in microwells with 15,000 COS-7 cells, 50 ng of each cDNA and 1 μl of LIPOFECTAMINE. 1 d after transfection, 2,000 cells of CTL 52 were added to the transfected cells. TNF production was measured after overnight coculture by testing the toxicity of the supernatants for TNF-sensitive WEHI-164 clone 13 cells.

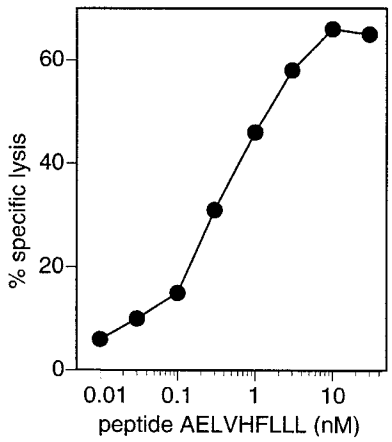

Figure 4. Titration of peptide AELVHFLLL. LB1841-EBV-B cells were ^{51}Cr-labeled and incubated for 30 min with threefold dilutions of the synthetic peptide. Autologous CTL 52 was subsequently added at an effector/target ratio of 5:1. Chromium release was measured 4 h later. The concentrations indicated in the figure correspond to the concentrations during the 4-h incubation.

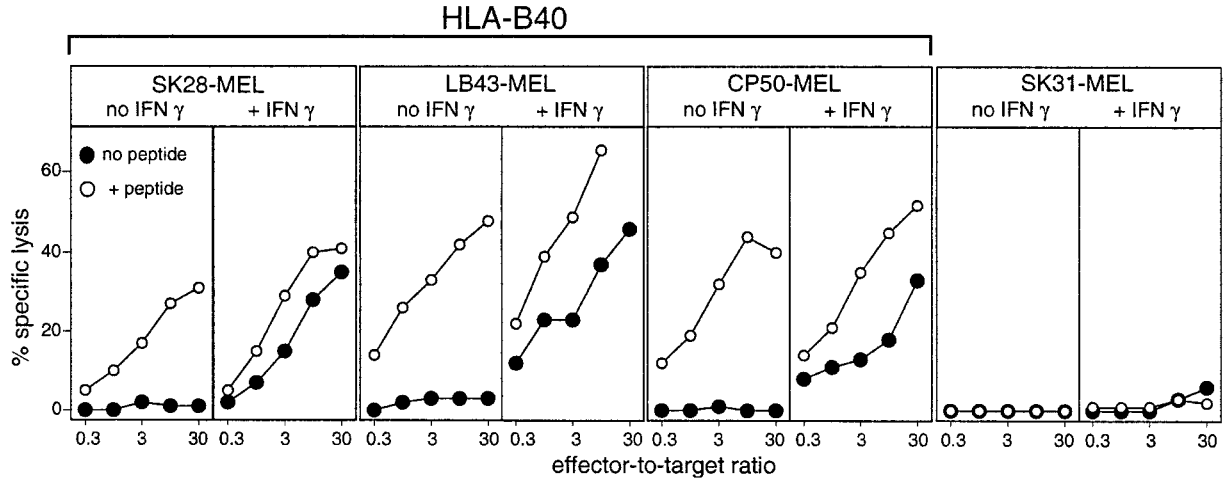

Figure 5. Lysis of tumor cell lines by CTL 52. MAGE-3–expressing melanoma cell lines SK28-MEL, LB43-MEL, CP50-MEL, and SK31-MEL were treated or not with IFN-γ (100 U/ml) for 48 h and used as targets. Target cells were ^{51}Cr-labeled for 1 h, and incubated with CTLs at various effector/target ratios. Chromium release was measured after 4 h. Lines SK28-MEL, LB43-MEL, and CP50-MEL were HLA-B*4001-positive, SK31-MEL was not.

munoblotting (data not shown). The precursor peptide was 19-residue long, corresponding to position 109–127 of the MAGE-3 protein, encompassing antigenic peptide $_{114}$AELVHFLLL$_{122}$ and its natural flanking residues. Digests obtained after different incubation times were pulsed onto HLA-B40-positive cells and tested for recognition by CTL 52. In accordance with our previous data, the digests obtained with immunoproteasomes stimulated CTL 52 much more efficiently than those obtained with standard proteasomes (Fig. 7).

To understand why the processing of this peptide requires the immunoproteasome, the proteasome-mediated digests were analyzed by mass spectrometry. The cleavage products obtained after digestion of 5 μg of peptide were separated by reverse-phase HPLC, identified, and quanti-

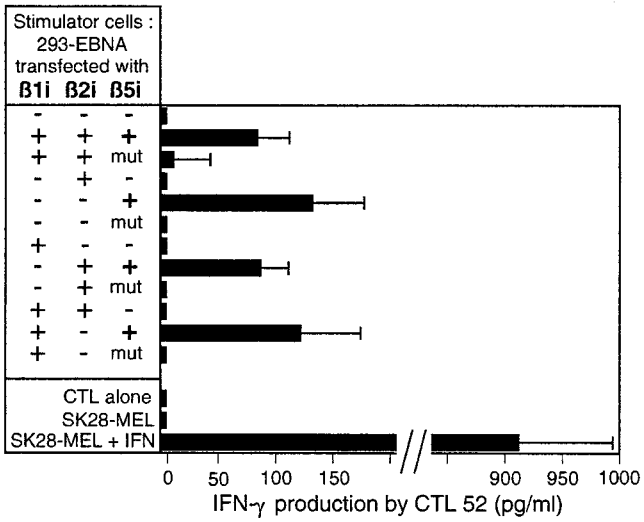

Figure 6. Recognition by CTL 52 of cells transfected with cDNAs coding for MAGE-3, HLA-B40, and the subunits of the immunoproteasome. Human 293-EBNA cells (15,000) were transiently transfected in microwells with 150 ng of cDNA in the presence of 1.3 μl of LIPOFECTAMINE. cDNAs coding for MAGE-3, HLA-B40, β1i, β2i, β5i, or mutated β5i were added in different combinations in the DNA mix at 30 ng each, and cDNA coding for β galactosidase (β Gal) was added to reach a total of 150 ng. 2 d after transfection, 7,000 cells of CTL 52 were added to the transfected cells. IFN-γ production was measured by ELISA after 20 h of coculture. The results shown represent the average and SD of triplicate cocultures. SK28-MEL were treated with IFN-γ (100 UI/ml) for 48 h where indicated.

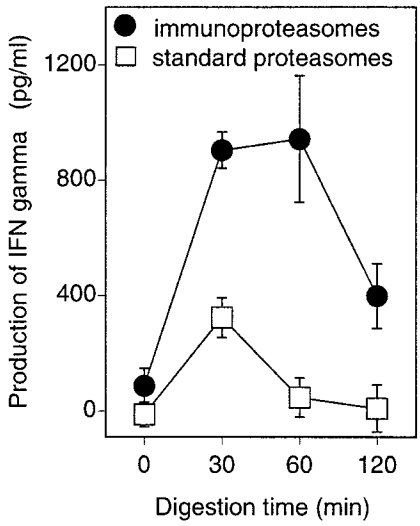

Figure 7. Recognition by CTL 52 of peptide digests performed with purified proteasomes. A 19 amino acid-long precursor peptide, $_{109}$LSRKVAELVHFLLLKYRAR$_{127}$, encompassing antigenic peptide $_{114}$AELVHFLLL$_{122}$, was digested in vitro with different proteasome preparations. Immunoproteasomes were purified from LB1751-MEL melanoma cells treated for 10 d with IFN-γ (100 U/ml), and standard proteasomes were obtained from untreated LB1751-MEL cells. Digestions were performed with 1 μg of proteasomes and 1.25 μg of the precursor peptide. One-seventh of the digests was pulsed for 1 h on 30,000 LB1841-EBV-B cells, which were then cocultured with autologous CTLs 52 (6,000 cells per well). IFN-γ production was measured by ELISA after overnight coculture. The results shown represent the averages and standard deviations of triplicate cultures.

fied by online electrospray ionization/mass spectrometry. The kinetics of degradation of the precursor peptide indicated that the overall activity of both proteasome preparations was similar (Fig. 8 A). The fragments resulting from a single cleavage at the exact NH_2 terminus or at the exact COOH terminus of antigenic peptide $_{114}$AELVHFLLL$_{122}$ were more abundant after digestion with immunoproteasomes (Fig. 8 B and C). The final antigenic peptide was also present in the digests and was much more abundant after digestion with immunoproteasomes (Fig. 8 D). In contrast, standard proteasomes were more active than immunoproteasomes to cleave within the antigenic peptide, after Leu_{120} and Phe_{119} (Fig. 8 E and F). This internal cleavage could explain why the standard proteasome produces less of the antigenic peptide.

Discussion

Although a number of antigenic peptides of viral origin were shown to require the immunoproteasome for their processing, the nonviral class I antigenic peptides studied so far are either poorly processed by the immunoproteasome or processed with the same efficiency by the two proteasome types (8). We previously showed that an antigenic peptide derived from ubiquitous protein RU1 and an antigenic peptide from melanocytic differentiation protein Melan-A/^{MART1} are not processed efficiently by the immunoproteasome (4). A tumor-specific antigenic peptide derived from the NY-ESO1 protein was shown by Chen et al. to be processed equally by the two proteasome types (27). The MAGE-3.B40 antigen we describe here is the first example of a tumor-specific antigen that is exclusively presented by cells carrying immunoproteasomes.

The fact that this is the first tumor antigenic peptide shown to be more efficiently processed by the immunoproteasome could result from the approach that was used to isolate the CTLs. The classical way to derive antitumor CTLs is to stimulate lymphocytes from cancer patients with autologous tumor cells, which usually do not express immunoproteasomes. This procedure clearly favors the isolation of CTLs directed against peptides that are processed efficiently by the standard proteasome. In this study we have used a different procedure, which is based on the stimulation of lymphocytes with autologous dendritic cells infected with a MAGE-3 recombinant virus. Since dendritic cells express immunoproteasomes (3), this protocol should favor the induction of CTLs directed against peptides processed efficiently by the immunoproteasome.

From the mass spectrometric analysis of our peptide digests, it appears that the major reason why the immunoproteasome processes the MAGE-3 antigenic peptide much more efficiently than the standard proteasome is that, as opposed to the latter, it does not destroy the antigenic peptide by cleaving after Phe_{119} and Leu_{120}. These two residues are followed by two hydrophobic residues. Interestingly, the presence of hydrophobic residues immediately downstream from a cleavage site was recently suggested to impair cleavage by the immunoproteasome but not by the standard proteasome (5). This could explain the difference of efficiency of the two proteasome types to destroy the MAGE-3 antigenic peptide by internal cleavage. The cleavage after the COOH terminus of the antigenic peptide, on the other hand, is performed efficiently by the immunoproteasome, even though it occurs after a leucine like the internal cleavage. This further confirms the importance of the context in determining the efficiency of a given cleavage.

Remarkably, the replacement of only the β5 subunit of the standard proteasome by β5i is sufficient for efficient processing of the MAGE-3 antigenic peptide. A simple explanation for the reduced internal cleavage of this peptide by the immunoproteasome would be that this cleavage is performed by subunit β5 and not by β5i. The incorporation of β5i, catalytically active or not, would then be sufficient to prevent the destructive cleavage, as suggested for an influenza peptide (21). This scenario is not supported by our finding that the incorporation of a mutated catalytically inactive β5i subunit does not allow the processing of the antigenic peptide. Although it does not exclude that β5i prevents the internal cleavage, this result indicates that the

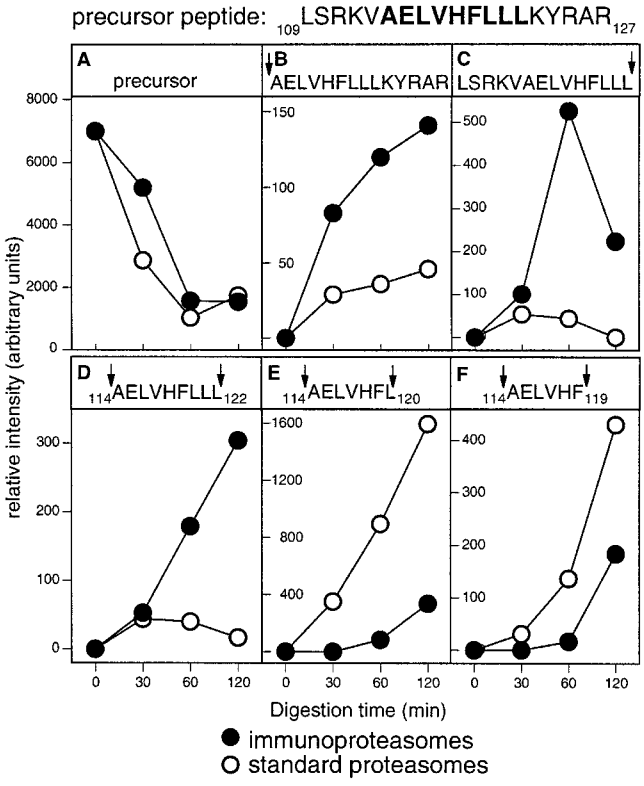

Figure 8. Mass spectrometric analyses of the fragments observed after proteasome digestion of a MAGE-3 precursor peptide. The cleavage products obtained after digestion of the precursor peptide with either proteasome type were separated by reverse-phase HPLC, identified, and quantified by online electrospray ionization/mass spectrometry. The kinetics shown represent the disappearance of the precursor peptide (A) and the appearance of the main fragments, including fragments with the proper NH_2 terminus (B), with the proper COOH terminus (C), the final antigenic peptide (D), and shorter fragments resulting from internal cleavages (E and F).

catalytic activity of β5i is required for the production of the MAGE-3 antigenic peptide. Our data also differ from those described for an Hepatitis B virus peptide, whose processing was recently found to require the presence of the three immunoproteasome subunits, although the catalytic activity of β5i was not necessary (22).

The fact that the processing of the MAGE-3.B40 antigen requires only the incorporation of β5i and not the other subunits of the immunoproteasome is unusual, as a similar situation has only been observed for two viral antigens (21, 28). β5i is the only immunoproteasome subunit that can be incorporated into the proteasome in the absence of the two others, leading to the formation of proteasomes containing β1, β2, and β5i (29). Such proteasomes were isolated recently from rat skeletal muscles (30), and they might be induced by TNF-β in human endothelial cells (31, 32). We also found some β5i in a renal cell carcinoma line in the absence of IFN-γ (data not shown). The function of such proteasomes is unclear, but our results suggest that their presence could increase or at least modify the repertoire of antigenic peptides presented at the cell surface.

Given the β5i requirement for the processing of the MAGE-3 antigen in human melanoma cells, it is surprising that the antigen can be processed and presented by transfected COS cells, as we observed in Figs. 2 and 3. Although we have not checked the proteasome type present in COS cells, these monkey cells most likely contain standard proteasomes as they are not of lymphoid origin and were not exposed to IFN-γ. Their capacity to process the MAGE-3.B40 antigen might result from the high expression levels achieved after transient transfection of expression vectors that multiply episomally in COS cells (16). High levels of expression of the MAGE-3 protein could offset the low processing efficiency of the standard proteasome and allow for sufficient amounts of peptide to be presented to the CTL clone. This would be consistent with the report of Gileadi et al., showing that the lack of processing of an influenza epitope by the standard proteasome can be overcome by increasing the turnover of the parental protein (21).

Whereas it is widely accepted that the proteasome is required for the production of the COOH terminus of most peptides, there is increasing evidence that the NH_2-terminal cleavage can be performed by other peptidases, the nature of which is under investigation (33, 34). Such NH_2-terminal trimming by distinct peptidases is probably not required for the processing of the MAGE-3 antigenic peptide, as we clearly detected fragments with the proper NH_2 terminus within the digests obtained with immunoproteasomes.

The new MAGE-3 antigenic peptide is presented by HLA-B*4001, a class I molecule expressed by 8% of Caucasians and 10% of Asians (35). Interestingly, this new antigenic peptide is not only encoded by *MAGE-3* but also by *MAGE-12*. These two genes are expressed in 76 and 62% of metastatic melanomas, respectively. The fact that the same peptide is encoded by two distinct genes should reduce the probability of emergence of tumor variants having lost expression of this antigen.

Vaccination strategies against cancer should take into account the differential processing of tumor antigens by the two proteasome types. Given the high expression of immunoproteasomes in mature dendritic cells, the MAGE-3 peptide described here should be efficiently produced by dendritic cells and, consequently, strong CTL responses should be obtained. Tumor cells, however, need to be exposed to IFN-γ in order to express the immunoproteasome and present this antigen. CTLs against this antigen should therefore be useful only once an immune response has been initiated at the tumor site. Optimal vaccination strategies could combine the use of peptides more efficiently produced by the standard proteasome with peptides more efficiently produced by the immunoproteasome.

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