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Final Antigenic Melan-A Peptides Produced Directly by the Proteasomes Are Preferentially Selected for Presentation by HLA-A*0201 in Melanoma Cells

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The melanoma-associated protein Melan-A contains the immunodominant CTL epitope Melan-A26/27–35/HLA-A*0201 against which a high frequency of T lymphocytes has been detected in many melanoma patients. In this study we show that the in vitro degradation of a polypeptide encompassing Melan-A26/27–35 by proteasomes produces both the final antigenic peptide and N-terminally extended intermediates. When human melanoma cells expressing the corresponding fragments were exposed to specific CTL, those expressing the minimal antigenic sequence were recognized more efficiently than those expressing the N-terminally extended intermediates. Using a tumor-reactive CTL clone, we confirmed that the recognition of melanoma cells expressing an N-terminally extended intermediate of Melan-A is inefficient. We demonstrated that the inefficient cytosolic trimming of N-terminally extended intermediates could offer a selective advantage for the preferred presentation of Melan-A peptides directly produced by the proteasomes. These results imply that both the proteasomes and postproteasomal peptidases limit the availability of antigenic peptides and that the efficiency of presentation may be affected by conditions that alter the ratio between fully and partially processed proteasomal products. The Journal of Immunology, 2004, 173: 6033–6040.

In addition to their essential functions in cellular homeostasis, proteasomes play a critical role in the generation of most antigenic peptides recognized by CTL. Proteasomes are nucleocytoplasmic multicatalytic protease complexes that degrade proteins into peptides ranging from 3–24 aa in length. Most of these proteasomal products are rapidly degraded into amino acids by aminopeptidases of the cytoplasm. However, some of the peptides escape from complete proteolysis and are loaded onto HLA class I molecules in the endoplasmic reticulum and transported to the cell surface for recognition by CD8+ T lymphocytes. In vitro, proteasomes generate either fully processed antigenic peptides, displaying both exact N and C termini, or intermediates with exact C termini and N-terminal extensions of varying length. Frequently, the production of fully processed antigenic peptides by the proteasomes is accompanied by the concomitant generation of N-terminally extended intermediates. Whereas antigenic peptides that are produced as intermediates require additional postproteasomal trimming by amino- and oligopeptidases of the cytoplasm, those that are produced in their final size of 9–10 aa are immediately available for loading onto HLA class I molecules. However, results from several studies have inferred that the presence of N-terminally elongated intermediates would be advantageous for Ag processing because the presence of additional N-terminal amino acids will simultaneously protect the antigenic core sequences from premature destruction by highly active aminopeptidases and will improve or at least not interfere with TAP transport (1–5).

In recent years much attention has been paid to the melanoma-associated Ag Melan-A/MART-1 (hereafter termed Melan-A), an intracellular transmembrane protein expressed in melanocytes and melanomas, because of the identification of an immunodominant epitope recognized frequently by CD8+ tumor-infiltrating T lymphocytes (TIL) from HLA-A*0201+ melanoma patients (6). The antigenic peptide is located within the transmembrane domain of Melan-A and requires the activities of the standard proteasomes for its presentation (7, 8). Although the nonamer peptide Melan-A26–35 AAGIGILTV has been isolated from HLA-A*0201+ melanoma cells (9), many tumor-reactive CTL recognize the decamer Melan-A26–35 (EAAGIGILTV) with equal or increased efficiency (10, 11). Therefore, the cognate antigenic peptides will be referred to as peptides Melan-A26–35 throughout the text. Earlier work had also shown that the substitution of Ala27 for Leu within the decamer sequence (position 2 of the decamer) increased the binding affinity of the peptide to HLA-A*0201 and led to a more efficient recognition by specific CTL and a stronger induction of CTL responses (10, 12). Most importantly, these CTL were tumor-reactive.

In this study we analyzed the degradation of wild-type and mutated Melan-A26–35 precursors by standard proteasomes in vitro. We observed that the proteasomes produced the antigenic peptides (nona- and decamers) in their final sizes along with N-terminally extended intermediates.
Materials and Methods

**Digestion of synthetic peptides, analyses by on-line HPLC-mass spectrometry (HPLC-MS/MS), and quantification**

Wild-type and A27L substituted precursor peptides (3.5 μg), Melan-A_{5-26}, and Melan-A_{5-26}^{A27L}, respectively, were digested by 4 μg of proteasomes isolated from human erythrocytes in 13 μl of 25 mM Tris, pH 7.6. Proteasome affinity purification and quantification were performed as previously described (8, 13, 14). The purity of the proteasome preparations was assessed by SDS-PAGE and staining. The reaction was stopped after 1 h with 3.5 mM phenylmethylsulfonyl fluoride (TPF). After lyophilization, the digestes were resuspended in 12 μl of H₂O/CH₃CN (50/50, v/v) and diluted to 48 μl in CH₃CN/H₂O/HCOOH (5/95/0.05, v/v/v). Twelve microliters of a 20 mM solution of an internal standard peptide in CH₃CN/H₂O/HCOOH (5/95/0.05, v/v/v) was then added to the digest. Fifteen microliters were analyzed by online HPLC-UV-electro-spray ionization-MS/MS. Peptides were separated on a Pepmap LC Packings (Dionex, Amsterdam, The Netherlands) C18 column (1 mm × 15 cm) at 1 l/min. The flow rate of 40 μl/min was achieved with a gradient elution of 10–50% for 40 min (A is 5/95/0.05 CH₃CN/H₂O/HCOOH, B is 70/30/0.05 CH₃CN/H₂O/HCOOH, v/v/v). UV absorption was measured online at 215 nm with electro-spray ionization-MS/MS detection performed on an LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Data acquisition was performed in a data-dependent mode consisting of a full-scan MS over the m/z range 200–2,000 and followed by a zoom-scan MS and a full-scan MS/MS in a dynamic exclusion mode of the most intense ion detected in the full-scan MS. MS/MS data were acquired using a 3 m/z isolation window, a relative collision energy of 35%, and a dynamic exclusion duration of 0.5 min. After identification by mass spectrometry, quantification of the peptides was performed by measuring the surface area (millivolts/s) of the relevant peaks on the UV chromatograms and by measuring the ion current of arbitrary units; corresponding to a specific m/z value in the full-scan MS. For nonoverlapping UV peaks, the variations in relative abundances of different peptide species determined by UV and MS analyses were similar. In case of peptide coelution during the HPLC separation, quantification was based on MS data only.

**Construction of plasmids and recombinant vaccinia**

The ubiquitin/protein/reference (UPR)-based pEGFP vector used in this study is identical with the vector described previously (12), except that the GFP coding sequence was replaced by the enhanced GFP (EGFP). All constructs were obtained by annealing complementary synthetic oligonucleotides containing at their 5' and 3' ends SacII and BamHI restriction sites, respectively. The oligonucleotides contained two codons for Gly at the 5' end (except for construct shown in Fig. 2B; see below) and a stop codon at the 3' end. Insertion of these fragments into the SacII-BamHI sites of pEGFP (strain U2817, Stratagene) yielded the full-length plasmids directing the expression of the reference protein EGFP-Ub and the Melan-A fragments spanning aa 26–35 (M26–35/27L), aa 26–40 (M26–40/27L), aa 20–35 (M20–35/27L), and aa 22–33 (M22–33/27L; Fig. 2A). In the construct Ub-M26–35/27L (Fig. 2B), the two C-terminal Gly of ubiquitin were removed, inhibiting the cleavage of EGFP-Ub by ubiquitin peptidase (15). In all constructs, the amino acid Ala² of Melan-A was substituted by a leucine (L). The sequence of all inserts was confirmed by DNA sequence analysis.

The generation of recombinant vaccinia virus was described previously (4, 8). A27L-substituted precursor peptides (3.5 μg) were digested with 4 μg of proteasomes isolated from human erythrocytes in 13 μl of 25 mM Tris, pH 7.6. Proteasome affinity purification and quantification were performed as previously described (8, 13, 14). The purity of the proteasome preparations was assessed by SDS-PAGE and staining. The reaction was stopped after 1 h with 3.5 mM phenylmethylsulfonyl fluoride (TPF). After lyophilization, the digestes were resuspended in 12 μl of H₂O/CH₃CN (50/50, v/v) and diluted to 48 μl in CH₃CN/H₂O/HCOOH (5/95/0.05, v/v/v). Twelve microliters of a 20 mM solution of an internal standard peptide in CH₃CN/H₂O/HCOOH (5/95/0.05, v/v/v) was then added to the digest. Fifteen microliters were analyzed by online HPLC-UV-electro-spray ionization-MS/MS. Peptides were separated on a Pepmap LC Packings (Dionex, Amsterdam, The Netherlands) C18 column (1 mm × 15 cm) at 1 l/min. The flow rate of 40 μl/min was achieved with a gradient elution of 10–50% for 40 min (A is 5/95/0.05 CH₃CN/H₂O/HCOOH, B is 70/30/0.05 CH₃CN/H₂O/HCOOH, v/v/v). UV absorption was measured online at 215 nm with electro-spray ionization-MS/MS detection performed on an LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). Data acquisition was performed in a data-dependent mode consisting of a full-scan MS over the m/z range 200–2,000 and followed by a zoom-scan MS and a full-scan MS/MS in a dynamic exclusion mode of the most intense ion detected in the full-scan MS. MS/MS data were acquired using a 3 m/z isolation window, a relative collision energy of 35%, and a dynamic exclusion duration of 0.5 min. After identification by mass spectrometry, quantification of the peptides was performed by measuring the surface area (millivolts/s) of the relevant peaks on the UV chromatograms and by measuring the ion current of arbitrary units; corresponding to a specific m/z value in the full-scan MS. For nonoverlapping UV peaks, the variations in relative abundances of different peptide species determined by UV and MS analyses were similar. In case of peptide coelution during the HPLC separation, quantification was based on MS data only.

Flow cytometry

Flow cytometry was performed on FACSscan devices using CellQuest software (BD Biosciences, San Jose, CA). Transfected cells were washed twice, resuspended in PBS/3% FCS, and directly analyzed. Identification of live cells was based on the forward and side scatter of mock-transfected cells. Sorting of transfected cells was performed using a FACSAnia cell sorter (BD Biosciences). Cells (10⁵) were washed twice, resuspended in PBS/3% FCS, and directly sorted according to their EGFP expression level into EGFP⁺¹⁰ and EGFP⁺⁰⁰ subpopulations. Sorted cells were allowed to recover for 60 min in complete medium before the addition of specific CTL.

**Pepptide digestion by incomplete cytosol, HPLC separation, and MS**

Purified synthetic peptides were quantified by amino acid analysis. Five nanomoles of peptide Melan-A_{5-26}, Melan-A_{5-26}^{A27L}, Melan-A_{5-26}^{A27L}, and RUL_{30-42} were digested in 40 μl of incomplete cytosol (cytosol without proteasome) corresponding to 3 × 10⁶ cell equivalents. Preparation of proteasome-depleted cytosol was described previously (15) and contained <5% of the remaining proteasomal activities, based on the proteolysis of fluorogenic substrates (data not shown). For each peptide, an aliquot was removed at time zero and served as the reference for quantitative analysis. Digestions were allowed to proceed for 2.5, 5, 10, and 20 min at 37°C. At each time point, the reaction was stopped with 1% TFA. The digests were diluted with acetic acid/water (50/50, v/v) to a final volume of 120 μl, and 100 μl was injected on the HPLC. HPLC separations were performed on an Agilent system (1100 series) using a C₁₈ small pore Vydac (250 × 4 mm, 5 μm; 2015S84; Hewlett-Packard) column. The column was eluted at a flow rate of 1 ml/min by a linear gradient of acetic acid/TFACaceto-nitrite/H₂O (0.1/0.005/80/20, v/v/v/v) on acetic acid/TFACaceto-nitrite/H₂O (0.1/0.005/100, v/v/v/v), rising within 60 min from 0 to 100%. The OD of the eluant was monitored at 215 nm with a photodiode array detector (model DAD-10; Hewlett-Packard). The peaks were determined by integration of the absorbance profile.
eluate was monitored at 214 nm. Fractions were collected using an Agilent (G1364A) fraction collector on 96-well plates and subjected to MALDI-TOF mass spectrometric analysis as previously described (15).

Results
Degradation of Melan-A26–35 and Melan-A26–35A27L precursors by proteasomes produces minimal and N-terminally extended antigenic peptides

We first analyzed and compared degradation of the wild-type and mutated Melan-A26–35 precursors by purified proteasomes in vitro. The 26-aa-long precursors, encompassing aa 15–40, were digested with standard proteasomes and subjected to on-line HPLC-UV-MS/MS analyses. As shown in Fig. 1, the major single proteasomal cleavage site was detected at the exact C terminus of the wild-type and mutated antigenic peptides, producing the fragments Melan-A15–35 and Melan-A27–35 (and its complementary fragment ILGVL). Five fragments were generated with that C terminus: the final antigenic peptides Melan-A26–35 and Melan-A27–35, two N-terminally extended fragments corresponding to the peptide Melan-A22–35 and Melan-A20–35, and the 8-mer Melan-A28–35, a fragment too short to bind to HLA-A*0201 (Fig. 1, A and B). The peptides Melan-A28–35, Melan-A27–35, Melan-A26–35, and Melan-A22–35 were produced in similar amounts, whereas less of the fragment Melan-A20–35 was generated. The same cleavages were also observed with the mutated precursor Melan-A15–40A27L, although the relative ratio of Melan-A26–35A27L vs Melan-A27–35A27L was strongly biased in favor of the 10-mer (Fig. 1, C and D). The N-terminally extended precursors were also produced, as was the internal 8-mer Melan-A28–35. We concluded from this experiment that both wild-type and mutated minimal antigenic Melan-A peptides were produced by the proteasomes along with two N-terminally extended intermediates.

Previous studies reported that peptide fragments produced by proteasomes in vitro could also be isolated from whole-cell extracts, suggesting that the production of peptides in vitro is representative of the intracellular processing (18, 19). Because the final Melan-A peptides and intermediates were produced by the proteasomes in vitro, we determined which one, if any, would be preferentially selected for loading onto HLA-A*0201 molecules in melanoma cells.

Recognition of cells expressing Melan-A26–35 and extended precursors by specific CTL

Based on the peptide fragments described in Fig. 1D, we constructed a series of plasmids to express the corresponding fragments in mammalian cells (Fig. 2). We selected four sequences, two corresponding to the fragments carrying additional four and six N-terminal amino acids, respectively, and one corresponding to the final antigenic decamer. Because the proteasomes have been shown to limit the supply of antigenic peptides, we also compared the presentation efficiency of a peptide that only required proteasomal processing with those that only required N-terminal trimming. Therefore, we included a plasmid coding for a fragment that displayed the final N terminus, but carried an additional five amino acids at the C terminus of the antigenic peptides.
availability of the antigenic peptide. Surprisingly, cells expressing proteasomal cleavage for the production of the antigenic peptide, IFN-\(\gamma\)-responsive protein. This construct produces equimolar amounts of two distinct polypeptides, EGFPha-Ub and the Melan-A peptides. The arrow indicates the cleavage site of the Ub-specific protease.

These results suggest that conversion of the N-terminally extended peptide. All constructs were expressed from a UPR-based plasmid, which produces equimolar amounts of the reference protein EGFP-Ub and the fragment of interest (14, 20). This strategy circumvents the need for N-terminal Met and allows measurement of the transfection efficiency and expression levels of the EGFP-Ub moieties by flow cytometry. We focused on the processing of Melan-A peptides carrying the A27L mutation because we had previously shown that the recognition of transiently transfected cells expressing the wild-type antigenic peptide Melan-A\(_{26-35}\) did not efficiently stimulate the production of cytokines by specific CTL (7, 12).

Human NA8-MEL melanoma cells (HLA-A*0201 positive, Melan-A negative) were transiently transfected with those constructs. Transfection efficiency and expression level were assessed by flow cytometry (Fig. 3A). Approximately 20% of the cells expressed EGFP-Ub, and the level of expression was similar for each construct, as judged by the mean fluorescence of the fluorescent cell pool. Nevertheless, the expression level in individual cells varied significantly within a transfected population, as evidenced by the width of the fluorescence histogram.

Cells from the same transfection as that used for flow cytometry were assayed for their recognition by specific anti-Melan-A CTL (Fig. 3B). CTL were incubated for 24 h with the transfected cells at a ratio of 3:1, and the secretion of IFN-\(\gamma\) by the CTL was measured by ELISA. Cells expressing the antigenic decamer (M\(_{26-35}\)) were efficiently recognized, as indicated by the level of IFN-\(\gamma\) release. This level was similar to that obtained by incubating CTL with cells loaded with saturating amounts of exogenous Melan-A\(_{26-35}\) peptide. The recognition of cells expressing the C-terminally extended precursor M\(_{26-40}\), which requires a single proteosomal cleavage for the production of the antigenic peptide, was lower, confirming that the proteosomal cleavage is limiting the availability of the antigenic peptide. Surprisingly, cells expressing the two N-terminally extended precursors, M\(_{20-35}\) and M\(_{22-35}\), were recognized less efficiently than cells expressing M\(_{26-35}\). These results suggest that conversion of the N-terminally extended intermediates into the final antigenic Melan-A peptide is inefficient in these cells. Alternatively, rapid proteolytic cleavage within the antigenic peptide could limit presentation of the N-terminally extended products.

Sorting of the subpopulations of transfected cells expressing M\(_{26-35}\) or the full-length protein Melan-A\(_{27L}\) and exhibiting bright or dim fluorescence intensities by FACS (Fig. 3C) showed that both populations could be recognized by anti-Melan-A CTL, but that the amount of IFN-\(\gamma\) secreted by CTL was 3 times higher when incubated with bright cells than with dim cells (Fig. 3D). In both subpopulations, cells expressing M\(_{26-35}\) were recognized more efficiently than those expressing the full-length protein.

To control that the efficient presentation of cells expressing M\(_{26-35}\) was caused by release of the EGFP-Ub moiety from the final antigenic peptide and not by rapid degradation of the uncleaved fusion protein, we introduced a mutation in the plasmid M\(_{26-35}\), which resulted in the production of an uncleaved fusion protein, EGFP-Ub-M\(_{26-35}\) (Fig. 2B). This mutation, which removes the two C-terminal Gly residues of ubiquitin, prevents cleavage of the EGFP-Ub moiety by ubiquitin proteases. The effects of this mutation have been described previously (15). NA8-MEL cells were transfected with this plasmid and exposed to anti-Melan-A CTL (Fig. 3B). Recognition of the transfected cells by CTL was poor, confirming that the efficient recognition of cells expressing M\(_{26-35}\) was due to the efficient release of the antigenic peptide in its final form. This also confirmed that the differences observed between the fully processed peptide and the N-terminally extended ones were due to the peptide-flanking sequences liberated in the cytosol.

**Trimming of Melan-A\(_{26-35}\) and Melan-A\(_{20-35}\) precursors by cytosolic proteases**

A possible cause of the apparent inefficient presentation of the antigenic Melan-A peptide in cells expressing the N-terminally extended precursors could be their different sensitivities to cytosolic peptidases. To address this question, we incubated synthetic peptides corresponding to the 14-mers Melan-A\(_{22-35}\) and Melan-A\(_{26-35}\) as well as the 10-mers Melan-A\(_{26-35}\) and Melan-A\(_{26-35}\) with NA8-MEL cytosol depleted of proteasomes. As control, we incubated an N-terminally extended 14-mer precursor of the HLA-B51-restricted peptide RU1\(_{34-42}\). This precursor was shown previously to be trimmed to the antigenic nonamer by the sequential activity of two different cytosolic peptidases, tripeptidyl peptidase II and puroycin-sensitive aminopeptidase (13, 15). The two 14-mer Melan-A wild-type and mutated peptides were degraded at a significantly lower rate than the RU1 peptide and the 10-mer Melan-A peptides (Fig. 4, A and B, respectively). The half-life of the 14-mer Melan-A peptides was ~10 min, whereas the half-life of the 14-mer RU1 peptide was ~5 min, similar to the half-life of the antigenic 10-mers. At each time point, the peptide digest was separated by HPLC, and the content of each detected peak was identified by mass spectrometry. As shown in Fig. 4B, the species corresponding to the final antigenic peptide sequence 26–35 was produced from the precursor Melan-A\(_{22-35}\) and was detectable after 20 min degradation. For Melan-A\(_{22-35}\), the final antigenic peptide could be identified after 5 min. In sharp contrast, the fragment corresponding to the final antigenic peptide RU1\(_{34-42}\) was already clearly quantifiable after 2.5 min. The exact composition of the fractions containing the final antigenic peptides for each of the precursors is detailed in Fig. 4C. For Melan-A\(_{22-35}\) (upper row), deletion fragments were already detected at time zero. However, several other fragments appeared over time, including the final antigenic peptide at 20 min. For Melan-A\(_{22-35}\) (middle row), the final antigenic peptide was already detectable at 5 min, among other fragments. Finally, the final antigenic peptide produced from the RU1 precursor (lower row) was detected at 0, 2.5, and 5 min.

**FIGURE 2.** DNA constructs used in the study. A, Plasmids coding for the two N-terminally extended intermediates M\(_{20-35}\) and M\(_{22-35}\), the antigenic peptide M\(_{26-35}\), and the C-terminally extended fragment M\(_{26-40}\), were generated. All constructs were expressed from an UPR-based plasmid, which contained the ha-tagged EGFPha-Ub as reference protein. This construct produces equimolar amounts of two distinct polypeptides, EGFPha-Ub and the Melan-A peptides. The arrow indicates the cleavage site of the Ub-specific protease. B, The antigenic peptide M\(_{26-35}\) expressed from a noncleavable UPR-based plasmid.
Despite its detection by mass spectrometry at time zero, the final antigenic peptide is nevertheless produced in the course of the digestion, and its actual amount can be derived from the HPLC profile shown in Fig. 4B, because no other fragment coeluted. The RU12 9–42 peptide was completely digested at 20 min. The antigenic 9-mer Melan-A27–35 (m/z 815) and Melan-A27L (m/z 857) could not be detected by MALDI-TOF MS, because this region contains several intense peaks corresponding to matrix ions. Because MALDI-TOF mass spectrometry does not allow the identification of fragments below a mass of 850 Da, we cannot completely rule out that a rapid internal cleavage could take place in the cytosol. Nevertheless, the degradation kinetics of the Melan-A precursors correlated well with the efficiency by which the final antigenic peptides were generated.

Melan-A26–35 is inefficiently converted into Melan-A27–35 by intracellular proteases

To confirm that the expression of an N-terminally extended Melan-A precursor leads to poor recognition of target cells by specific CTL, we took advantage of the fine specificity of a previously described CTL clone, clone A42 (17). This clone was shown to recognize Melan-A27–35 more efficiently than Melan-A26–35, which can be considered, under these circumstances, as an N-terminally extended intermediate. We first established that this tumor-reactive clone indeed recognizes the nonamer Melan-A27–35 more efficiently than the decamer Melan-A26–35 (Fig. 5A). We then tested whether melanoma cells expressing the nonamer Melan-A27–35 or the decamer Melan-A26–35 would be recognized with similar efficiency. To this end, we infected N8-MEL cells with recombinant vaccinia virus coding for the wild-type versions of the decamer Melan-A26–35, the nonamer Melan-A27–35, and the full-length Melan-A protein and incubated them with CTL clone A42 (Fig. 5B). As expected, the cells expressing the antigenic nonamer were efficiently recognized by the CTL clone A42, but not cells expressing the decamer Melan-A26–35. N8-MEL cells infected with recombinant vaccinia virus coding for the 10-mer Melan-A27L were not recognized either (Fig. 5C). However, cells expressing the full-length proteins Melan-A and Melan-A27L were recognized by the CTL clone A42. To ensure that the cells used for the experiment depicted in Fig. 5C were expressing similar amounts of Melan-A proteins or peptides, we took advantage of our UPR-based constructs, in which Melan-A is expressed as C-terminal fusions of the ha-tagged reporter GFP-Ub (12, 20). We therefore performed a Western blot analysis on an aliquot of cells used in Fig. 5C. As shown in Fig. 5D, infected cells were expressing similar amounts of the reporter proteins. Because the reporter and the Melan-A products are expressed from the same mRNA, the amount of detected reporter is equimolar to the amount of Melan-A in the cells. This observation led us to conclude that although the decamers Melan-A26–35 and Melan-A26–35 are expressed, they are not efficiently converted into the antigenic nonamers in infected cells, supporting our previous findings that cells expressing the N-terminally elongated precursors of Melan-A were inefficiently recognized by CTL. However, we cannot completely rule out that the decamer Melan-A26–35 escapes N-terminal trimming by rapid binding to HLA-A*0201 molecules. Cells expressing the full-length protein were also recognized by the CTL.

**FIGURE 3.** Expression and presentation of Melan-A peptides to specific CTL. A, N8-MEL cells were transiently transfected with the constructs described in Fig. 2. The transfection efficiency (percentage) and the average expression level (MF) were calculated based on the fluorescence emitted by the reference protein EGFPha-Ub. The constructs are indicated above each plot. B, Transfected cells were incubated in duplicate with Melan-A-specific CTL at an E:T cell ratio of 3:1, and the production of IFN-γ by the CTL was measured by ELISA. As controls, cells transfected with EGFPha-Ub (−) and mock-transfected cells loaded with saturating amounts of synthetic peptide Melan-A27L were used. A representative result of three independent experiments is shown. C, Cells transiently transfected with M26–35A27L and Melan-A27L were sorted by FACS into an EGFP high and EGFP low subpopulation, based on fluorescence intensity emitted by the EGFPha-Ub moiety. D, The bright (EGFP high) and dim (EGFP low) subpopulations expressing either M26–35A27L or Melan-A27L were separately incubated with specific CTL, at an E:T cell ratio of 3:1, and the production of IFN-γ was measured by ELISA. Mock-transfected cells (−) were used as a negative control. A representative result of two independent experiments is shown.
FIGURE 4. Degradation of Melan-A peptides by cytosolic peptidases. A, Synthetic peptides corresponding to Melan-A_{22-35} and Melan-A_{26-35} (left panel) as well as Melan-A_{22-35}^{A27L} and Melan-A_{26-35}^{A27L} (right panel) were incubated with proteasome-depleted cytosol for 0, 2.5, 5, 10, and 20 min at 37°C. The 14-mer peptide RU1_{29-42} was used as a reference. At each time point, the reaction was stopped, and the peptide was separated by HPLC. The percentage of precursor peptide remaining at each time point is calculated based on the amount of peptide present at time zero. Average values (n = 2) with mean error are shown. The dotted line indicates the 50% level.

B, Samples shown in A were separated by HPLC, and the HPLC profiles at 0, 2.5, 5, and 20 min are shown for Melan-A_{22-35} (left column), Melan-A_{26-35}^{A27L} (middle column), and RU1_{29-42} (right column). The main peak of each digestion contained the precursor peptide and served to determine the degradation curve shown in A. The species detected in the relevant fractions are indicated as P for the precursor and as P-1, P-2, and P-3 for fragments lacking one, two, and three N-terminal amino acids, respectively. The gray boxes correspond to the final antigenic 10-mer peptide of Melan-A and 9-mer of RU1.

For digestion of the RU1 peptide, a detailed view of the relevant region (Figure legend continues...
clone A42, suggesting that along with other fragments, these cells produce the antigenic nonamer from the full-length protein. In parallel, cells infected with the same constructs were incubated with the CTL line EAA that indiscriminately recognizes the nonamer and decamer peptides. Contrary to what was observed with clone A42, cells expressing the different constructs were recognized with similar efficiency, indicating that infected NA-8 MEL cells infected with recombinant vaccinia expressing Melan-A<sub>27–35</sub>, Melan-A<sub>26–35</sub>, and full-length Melan-A. The left panel represents the target cell lysis by CTL clone A42. The right panel corresponds to the target cell lysis by CTL line EAA, which recognizes Melan-A<sub>27–35</sub>/HLA-A*0201 and Melan-A<sub>26–35</sub>/HLA-A*0201 with similar efficiency. C. Same as in B, except that NA8-MEL cells infected with recombinant vaccinia virus coding for the mutated Melan-A<sub>27L</sub> and Melan-A<sub>26–35</sub> were included. D. Expression level of the reference protein GFP<sub>ha</sub>-Ub in NA8-MEL cells infected with recombinant vaccinia virus. An aliquot of the cells used in C was lysed and subjected to Western blot analysis using the anti-ha mAb that detects the UPR-based reference protein GFP<sub>ha</sub>-Ub (see text for details). The relative intensity of the band corresponding to GFP<sub>ha</sub>-Ub is indicated below each lane. The intensity of the band detected in cells expressing Melan-A<sub>27–35</sub> was arbitrarily set at 1. For each panel, a representative result of two independent experiments is shown.

Discussion
The proteasomal degradation of a Melan-A peptide encompassing the region involved in the HLA-A<sup>*0201</sup>-restricted CTL recognition produces similar amounts of antigenic peptides (Melan-A<sub>26–35</sub> and Melan-A<sub>27–35</sub>) and N-terminally elongated intermediates. Functional CTL assays indicated that the final antigenic peptides produced directly by the proteasomes were more efficiently presented than the elongated intermediates. We show that the reason for this preference probably resides in the fact that the trimming of the N-terminally elongated Melan-A fragments is inefficient.

Over the last few years an increasing number of peptidases have been implicated in the postproteasomal processing of antigenic peptides, including tripeptidyl peptidase II, puromycin-sensitive aminopeptidase, bleomycin hydrolase in the cytosol (1, 15, 21–23), and ERAPI/ERAAP in the endoplasmic reticulum (24, 25). However, none of these studies determined the roles of these peptidases when antigenic peptides were produced by the proteasomes both in their final form and as N-terminally extended intermediates. It has been shown that the degradation of OVA precursors by 20S proteasomes in vitro produces both fully processed and N-terminally extended antigenic peptides (26). Based on the analysis of the OVA fragments produced by 26S proteasomes in vitro and in cells, it was later suggested that most antigenic peptides are produced as N-terminally extended intermediates that require postproteasomal trimming (4, 5). Although this conclusion was consistent with the idea that such extensions would protect the antigenic peptides from trimming, recent studies have shown that this trimming is not essential for the presentation of melanoma antigens.
core sequences from premature destruction by cytoplasmic proteases, it was not determined experimentally which pool of the N-terminally elongated or minimal peptides of OVA served as the source of CTL epitopes. Similar to OVA, Melan-A is degraded by the proteasomes into minimal antigenic peptides and extended intermediates. In the case of Melan-A, our results suggest that peptides directly produced by the proteasomes are preferentially selected for presentation by HLA-A*0201 molecules in melanoma cells. This preference could be imposed by the limiting amount of specific peptides present within melanoma cells or by the presence of unfavorable amino acids within the N-terminal extension of Melan-A.

Evidence of possible negative effects of N-terminal extensions was reported previously (27). In this study the authors analyzed the presentation of a subdominant CTL epitope derived from OVA and showed that the subdominance was directly linked to the flanking N-terminal amino acid sequence. These results led the authors to suggest that N-terminal trimming played a significant role in this process. Other studies also showed that amino acids preceding the antigenic sequences could affect the presentation of epitopes (28, 29). More recently, it was shown that cytosolic peptides can limit the availability of antigenic peptides by overdigesting the precursors (30). As shown in this study, it appears that the proteasomes can limit the availability of antigenic peptides by overdigesting the precursors (28, 29). More recently, it was shown that cytosolic peptides can limit the availability of antigenic peptides by overdigesting the precursors (30). As shown in this study, it appears that the proteasomes can limit the availability of antigenic peptides by overdigesting the precursors (28, 29).

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References


Altogether, our results indicate that the postproteasomal processing of Melan-A peptides does not contribute significantly to the pool of antigenic peptides loaded and presented by HLA-A*0201 molecules at the surface of melanoma cells. Moreover, our findings suggest that the presentation of the Melan-A epitope in melanoma cells is affected not only by the efficiency by which proteasomes generate the appropriate C terminus, but also by the ratio between N-terminally elongated fragments and final antigenic peptides produced by the proteasomes. It is therefore reasonable to postulate that aside from proteasomes, postproteasomal processing peptides impose a second limiting step in the availability of antigenic peptides for presentation.

Processing of Melan-A/MART-1 influences the processing of its HLA-A2-restricted epitope. J. Biol. Chem. 276:43189.