An HLA-A2–restricted Tyrosinase Antigen on Melanoma Cells Results from Posttranslational Modification and Suggests a Novel Pathway for Processing of Membrane Proteins

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Summary

T lymphocytes recognize antigens consisting of peptides presented by class I and II major histocompatibility complex (MHC) molecules. The peptides identified so far have been predictable from the amino acid sequences of proteins. We have identified the natural peptide target of a CTL clone that recognizes the tyrosinase gene product on melanoma cells. The peptide results from posttranslational conversion of asparagine to aspartic acid. This change is of central importance for peptide recognition by melanoma-specific T cells, but has no impact on peptide binding to the MHC molecule. This posttranslational modification has not been previously described for any MHC-associated peptide and represents the first demonstration of posttranslational modification of a naturally processed class I–associated peptide. This observation is relevant to the identification and prediction of potential peptide antigens. The most likely mechanism for production of this peptide leads to the suggestion that antigenic peptides can be derived from proteins that are translated into the endoplasmic reticulum.

Class I molecules of MHC bind to peptides derived from intracellular pathogens or from proteins expressed in tumor cells, and present them on the cell surface to the host immune system (1–3). Identification of the specific peptides that constitute T cell epitopes has been difficult without prior knowledge of the source protein. However, peptides recognized by human melanoma–specific T cells have recently been identified from five proteins using two alternative strategies. One approach has been to generate genomic or cDNA libraries from tumor cells followed by transfection of progressively smaller subsets of these molecular clones into cells that express the appropriate MHC molecule, but not the tumor-specific epitope (4–14). Molecular clones that encode T cell epitopes are identified by their ability to reconstitute tumor-specific T cell recognition of the transfected cells. The exact T cell epitope is then identified by a combination of molecular subcloning and the use of synthetic peptides based on the predicted amino acid sequence. This approach led to the identification of antigens encoded by genes whose expression is specific for tumors, such as MAGE and of other antigens related to melanocyte differentiation such as tyrosinase (4, 13). In the second approach, naturally occurring peptides associated with MHC molecules on the tumor cells are directly extracted, fractionated by HPLC, and used to reconstitute recognition by melanoma-specific CTL of a nonmelanoma cell expressing appropriate MHC molecules (15). The peptide epitope within a reconstituting peptide fraction is identified and sequenced by tandem mass spectrometry (16, 17). Using this approach, a peptide, YLEPGPVTA, from the protein Pmel-17/gp100, was identified as an epitope for HLA-A2.1–restricted, melanoma-specific CTL from multiple individuals (18).

A different naturally occurring HLA-A2.1–associated peptide, YMDGTMSQV, was identified by mass spectrometry because it was very abundant in peptides extracted from melanoma cells but was absent in lymphoid

The contributions of the first two authors were equivalent and their order should be considered arbitrary.

cells, suggesting that it was derived from a protein of melanocytic lineage (reported incorrectly in reference 19) (18). The sequence of this peptide was identical to a nonamer peptide corresponding to residues 368-376 of tyrosinase, except that aspartic acid (D) was found instead of asparagine (N) at position three.

It is interesting to note that the genetic approach described above had indicated that this region of the gene encoded an epitope recognized by HLA-A2.1–restricted, tyrosinase-specific CTL (14). Here, we show that the naturally occurring tyrosinase peptide recognized by these CTL is in fact the species identified by mass spectrometry, and that it is derived by the posttranslational modification of a genetically encoded asparagine residue to aspartic acid.

Materials and Methods

Cell Lines and Peptides. Melanoma line NA8Mel+tyr was derived from transfection of the tyrosinase negative cell line NA8Mel with the tyrosinase cDNA 123.B2 (13). T2 is a mutant human B lymphoblastoid cell line (20, 21), which was a kind gift from Dr. Peter Cresswell (Yale University, New Haven, CT). All cell lines were maintained in RPMI-1640 medium supplemented with 10% FCS in a humidified atmosphere, with 5% CO_2 at 37°C. Synthetic peptides were made by standard Fmoc chemistry using a peptide synthesizer (model AMS422; Gilson Co. Inc., Middleton, WI). All peptides were purified to >98% by reverse-phase HPLC on a C-4 column (VYDAC, Hesperia, CA) with 0.05% TFA/water and an acetonitrile gradient.

CTL Clone IVSB and Cytotoxic Assays. The tyrosinase-specific CTL clone IVSB was produced by limiting dilution from responders of autologous mixed lymphocyte tumor cell cultures and maintained in long-term culture as described previously (13, 14). Standard ^{51}Cr release assays were performed to determine CTL recognition of tyrosinase peptides. Indicated concentrations of synthetic tyrosinase peptides were incubated with 2×10^3 ^{51}Cr-labeled HLA-A2.1$^+$ T2 target cells for 1 h at 37°C. CTL were added at an E/T ratio of 20:1 and chromium release was measured after a 4-h incubation of 37°C. ^{51}Cr-labeled T2 target cells were also incubated with 20 μl of culture supernatant containing anti-HLA-A2 mAb MA2.1 (22) for 1 h before incubation with indicated concentrations of synthetic peptides.

Class I Binding Assay. The ability of the tyrosinase peptides to compete with the radiolabeled standard peptide FLPSDYFPSV for binding to purified HLA-A2.1 molecules was measured with an equilibrium binding assay as described (23, 24). Indicated concentrations of tyrosinase peptides were incubated with iodinated hepatitis B virus core peptide FLPSDYFPSV (5–10 nM, specific activity $5–10 \times 10^{17}$ cpm/mol), purified class I molecules (10–50 nM) (23), and 1 μM human β2-microglobulin (Calbiochem-Novabiochem Corp., La Jolla, CA) at room temperature in PBS, pH 7.0, 0.05% NP-40, 1 mM PMSF, 1.3 mM 1,10-phenanthroline, 73 μM pepstatin A, 8 mM EDTA, and 200 μM tosyllysylchloromethyl ketone (TLCK). After 48 h, class I–peptide complexes and free peptides were separated by gel filtration using a Sephadex G-50 column (2-ml bed volume) eluted with PBS, pH 7.0, 0.5% NP40, and 0.1% NaN_3. Class I bound and free radioactivity were measured and peptide concentrations inducing 50% inhibition of binding of the standard peptide (IC_{50}) determined. Peptides were tested in three independent experiments.

HLA-A2.1–associated Peptide Isolation and Microcapillary Reverse-Phase HPLC Coelution. Peptides were acid eluted from affinity-purified HLA-A2.1 molecules as described (15, 19). Briefly, cells were washed three times in cold PBS and detergent solubilized in 1% CHAPS, 174 μg/ml PMSF, 5 mg/ml aprotinin, 10 mg/ml leupeptin, 16 mg/ml pepstatin A, 33 mg/ml iodoacetamide, 0.2% sodium azide, and 0.03 mg/ml EDTA for 1 h at 4°C. After centrifugation at 100,000 *g* for 1 h at 4°C, the supernatant was filtered (0.22 μm) and passed over a protein A–Sepharose column containing the mAb BB7.2. HLA-A2.1 molecules and associated peptides were eluted with 0.2 N acetic acid, pH 2.7, and peptides were dissociated at pH 2.1 by bringing the solution to 10% acetic acid followed by boiling for 5 min. Finally, peptides were centrifuged through Ultrafree-CL 5000-kD filters (Millipore Corp., Bedford, MA) at 2,500 *g* for 5 h. Filtrates were concentrated using vacuum centrifugation and stored at −80°C.

HLA-A2.1–associated peptides were also isolated from T2 cells pulsed with synthetic peptides. T2 cells (10^9) were preincubated with 0.5 mM synthetic peptide for 12 h at 37°C before immunoaffinity purification of HLA-A2.1 molecules and acid elution of peptides as described above. Isolated peptides were loaded onto a C18 microcapillary column (75 μm i.d. × 12 cm) and gradient eluted using acetonitrile and 0.1 M acetic acid with the concentration of acetonitrile increasing at 2%/min into a triple quadrupole mass spectrometer (model MAT TSQ-7000; Finnigan, San Jose, CA) equipped with an electrospray ion source. Scans were acquired every 1.5 s over a mass range m/z 300–1,400 and then plotted with intensities for mass-to-charge ratio (m/z) 1,031 to 1,032.

Mass Spectrometric Peptide Sequencing. Collision activated dissociation (CAD)[1] mass spectra were recorded on peptide methyl esters (16, 19). Since the amino acids at position 3 in the two tyrosinase peptides YMNGTMSQV and YMDGTMSQV differ by a single mass unit, confirmation of the sequences was obtained by recording CAD spectra on the corresponding methyl esters, as this derivatization results in a 14 mass unit shift for each carboxylate group, including the COOH terminus.

Results

The naturally occurring peptide YMDGTMSQV was tested for recognition by the tyrosinase-specific CTL clone IVSB, which had been used to identify the genetically encoded peptide YMNGTMSQV (14). It is interesting to note that YMDGTMSQV sensitized target cells for lysis at a 100-fold lower concentration than did YMNGTMSQV (half-maximal lysis with 0.1 and 10 mM, respectively) (Fig. 1 *A*). Even when target cells were pretreated with the mAb MA2.1 to facilitate exogenous peptide binding to HLA-A2.1 (14, 22), the concentration of YMNGTMSQV required to give half-maximal target cell lysis was >1 μM. This is significantly higher than that observed for numerous other peptide epitopes (17, 18, 25–28).

To determine whether differences in target cell sensitizing activity were due to differences in the ability of the individual peptides to bind to HLA-A2.1 molecules, the binding affinity of the two peptides was measured using a

[1] *Abbreviations used in this paper:* CAD, collision-activated dissociation; ER, endoplasmic reticulum; PNGase, peptide:*N*-glycanase; TAP, transporter associated with antigen processing.

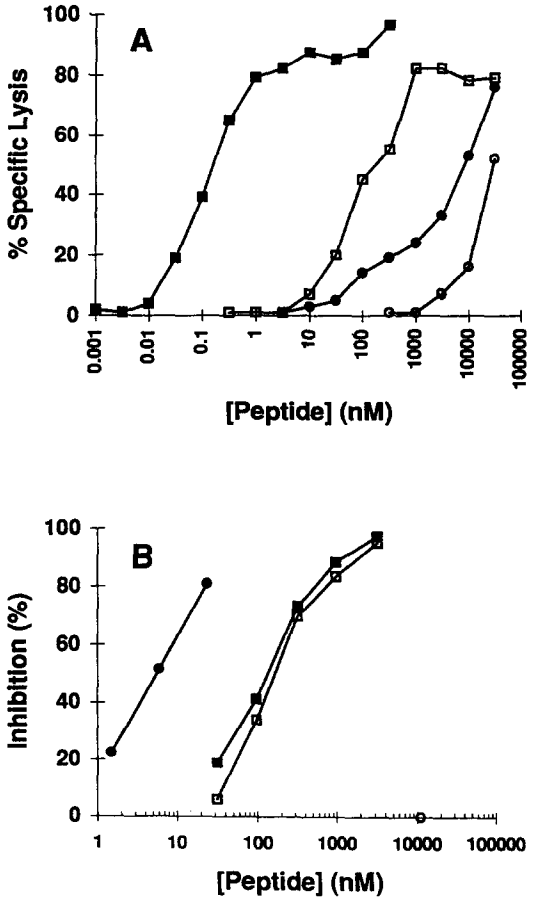

Figure 1. (*A*) Recognition of tyrosinase peptides by melanoma-specific CTL IVSB. Indicated concentrations of synthetic tyrosinase peptides were incubated with 2×10^3 ^{51}Cr-labeled HLA-A2.1$^+$ T2 target cells for 1 h at 37°C. T2 is a mutant human B lymphoblastoid cell line (20, 21). IVSB CTL were added at an E/T ratio of 20:1 and chromium release was measured after a 4-h incubation at 37°C. ^{51}Cr-labeled T2 target cells were also incubated with anti-HLA-A2 mAb MA2.1 for 1 h before incubation with indicated concentrations of synthetic peptides. Targets were T2 cells pulsed with YMNGTMSQV (○) and YMDGTMSQV (□), or MA2.1-treated T2 cells pulsed with YMNGTMSQV (●) and YMDGTMSQV (■). (*B*) Binding of synthetic tyrosinase peptides to HLA-A2.1. The ability of the tyrosinase peptides to compete with the radiolabeled standard peptide FLPSDYFPSV for binding to purified HLA-A2.1 molecules was measured with an equilibrium binding assay as described (23, 24). (■) YMDGTMSQV; (□) YMNGTMSQV; (●) ALWGFFPVL, another endogenous peptide isolated from HLA-A2.1 (17); (○) APRTVALTA, an endogenous peptide isolated from HLA-B7 that does not bind to HLA-A2.1 (48).

quantitative binding assay (23, 24). Inhibition of the binding of a standard peptide to purified HLA-A2.1 molecules was observed at similar concentrations of YMDGTMSQV and YMNGTMSQV (Fig. 1 *B*). Thus, the asparagine and aspartic acid residues at position three of these peptides have either a similar or no influence on peptide binding to HLA-A2.1; differences in binding affinity do not account for the difference in peptide recognition by the CTL.

Preferential CTL recognition of the naturally processed YMDGTMSQV suggests that the TCR expressed by the tyrosinase-specific CTL clone IVSB has a greater affinity for this species. One possible explanation of these observations is that this aspartic acid–containing peptide is derived from a previously undescribed allelic variant of tyrosinase or a mutated tyrosinase gene. Although asparagine has been found at this position (residue 371 of the precursor protein) in published sequences of human tyrosinase (13, 29, 30), an allelic form containing a threonine substitution at this position has been reported in oculocutaneous albino patients (31). The naturally processed peptide YMDGTMSQV was identified in HLA-A2.1–associated peptides extracted from two melanoma cell lines, DM6 and DM93 (our unpublished data) and the tyrosinase genes in these cell lines have not been sequenced. Another possibility is that this peptide originates from a gene distinct from tyrosinase. Finally, the naturally processed peptide could arise from the genetically encoded tyrosinase sequence through posttranslational modification.

To distinguish among these hypotheses, mass spectrometry was used to analyze the HLA-A2.1–associated peptides extracted from the melanoma cell line NA8Mel and NA8Mel+

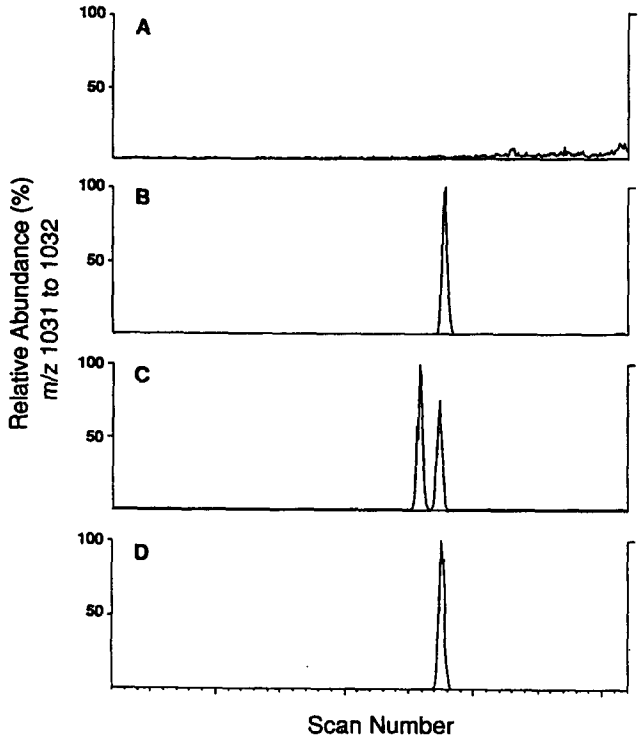

Figure 2. Ion chromatograms recorded on HLA-A2.1–associated peptides from NA8Mel and NA8Mel+tyr transfectant. Isolated peptides were loaded onto a C18 microcapillary column (75 μm i.d. × 12 cm) and gradient eluted into a Finnigan-MAT TSQ-7000 triple quadrupole mass spectrometer equipped with an electrospray ion source. Scans were acquired every 1.5 s over a mass range m/z 300–1,400 and then plotted with intensities for m/z 1031–1032 (*A*) Ion chromatogram for m/z 1031–1032 from NA8Mel material equivalent to 1.3×10^8 cells. (*B*) Ion chromatogram for m/z 1,031–1,032 from NA8Mel+tyr material equivalent to 4×10^7 cells. (*C*) Ion chromatogram for m/z 1,031–1,032 from NA8Mel+tyr material equivalent to 4×10^7 cells with synthetic YMNGTMSQV peptide added. (*D*) Ion chromatogram for m/z 1,031–1,032 from NA8Mel+tyr material equivalent to 4×10^7 cells with synthetic YMDGTMSQV peptide added.

tyr. NA8mel does not express a tyrosinase gene, whereas NA8Mel+tyr was transfected with a tyrosinase gene encoding asparagine at position 371 (13). PCR amplification and sequencing of tyrosinase RNA from this cell line confirmed that no mutation had occurred in this sequence after transfection (data not shown). Peptides YMDGTMSQV and YMNGTMSQV have molecular masses of 1,032 and 1,031 daltons, respectively. Analysis of the mixture of naturally processed peptides extracted from NA8Mel by microcapillary reversed-phase HPLC failed to detect any species of mass 1,031–1,032 at >0.17 fmol/3 × 10^7 cells (Fig. 2 *A*). However, a single major peak, corresponding to peptide(s) in the mass window 1031–1032, was detected at the level of 200 fmol/3 × 10^7 cells among the peptides extracted from NA8Mel+tyr (Fig. 2 *B*). This result confirms that an HLA-A2.1–associated peptide of this mass was derived from the tyrosinase gene product. When synthetic YMNGTMSQV was added to the peptide extract, two distinct peaks were detected in this mass range (Fig. 2 *C*), indicating that the naturally processed peptide did not have this sequence. Furthermore, no signal above background was discernible at the elution position of YMNGTMSQV in normal extracts of NA8Mel+tyr (Fig. 2 *B* and our unpublished data), indicating that this tyrosinase gene encoded peptide is not present among HLA-A2.1–associated peptides. By contrast, the synthetic peptide YMDGTMSQV did coelute with the naturally occurring tyrosinase peptide from NA8Mel+tyr (Fig. 2 *D*), suggesting that these peptides were identical. Proof for this conclusion was provided by obtaining sequence information on the naturally occurring tyrosinase peptide. CAD mass spectra were recorded on $(M + H)^+$ ions of the corresponding peptide methyl esters. The CAD spectrum representing the peptide extracted from NA8Mel+tyr (Fig. 3 *A*) was identical to that of the synthetic tyrosinase peptide YMDGTMSQV (Fig. 3 *B*), and distinct from that of YMNGTMSQV (Fig. 3 *C*). This establishes that the peptide encoded by the tyrosinase gene has been posttranslationally modified before its presentation by HLA-A2.1 on the surface of these cells.

Creation of the naturally occurring peptide YMDGTMSQV from the genetically encoded sequence involves deamidation of asparagine to aspartic acid. Nonenzymatic deamidation of unmodified asparagine residues has been documented for a variety of proteins and is frequently associated with the presence of an asparagine-glycine sequence as occurs at positions 371-372 in tyrosinase (32). However, although the half-lives of deamidation in these proteins and peptides depend on both their sequence and structure, they

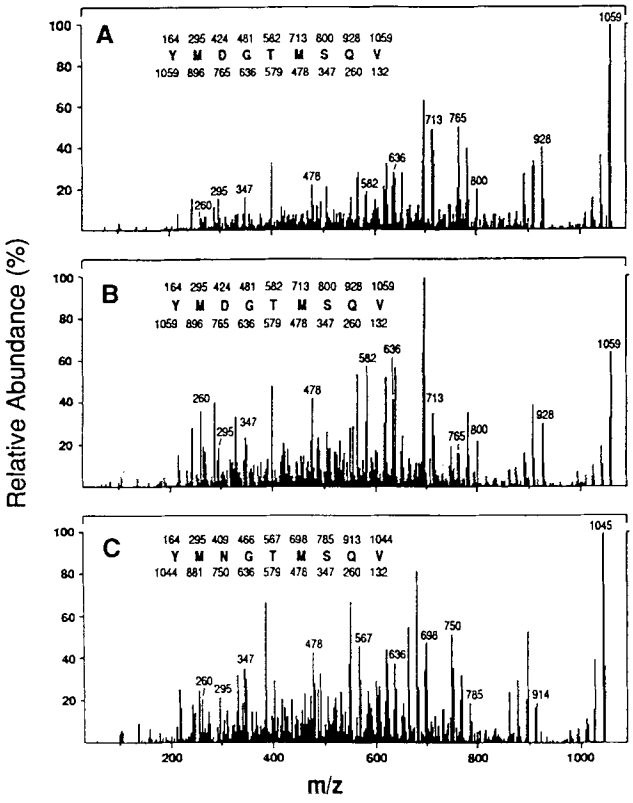

Figure 3. CAD mass spectra recorded on peptide methyl esters. The derivatization of peptides YMNGTMSQV and YMDGTMSQV results in a 14 mass unit shift for each carboxylate group, including the COOH terminus. CAD mass spectrum of $(M+H)^+$ ions at m/z: (*A*) 1,059 from A2.1-associated peptides isolated from NA8Mel+tyr; (*B*) 1,059 from synthetic YMDGTMSQV; (*C*) 1,045 from synthetic YMNGTMSQV.

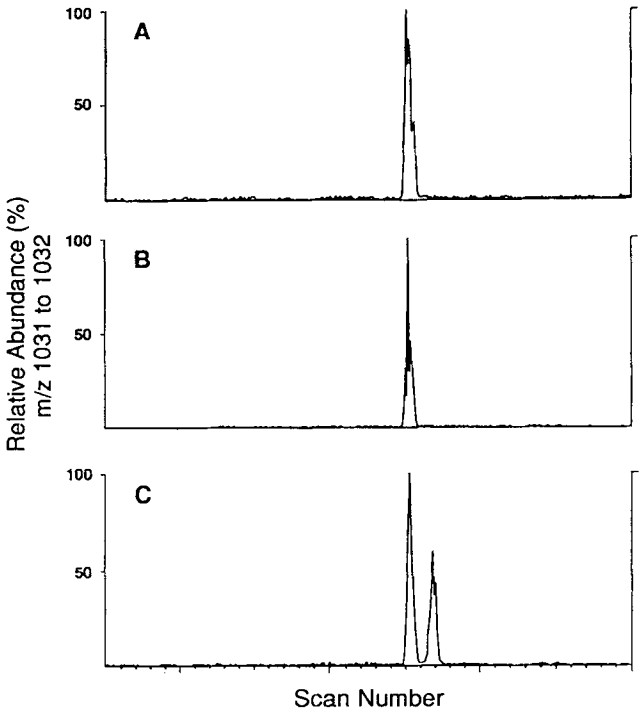

Figure 4. Ion chromatograms of peptides isolated from T2 cells pulsed with YMNGTMSQV. (*A*) T2 cells (10^9) were preincubated with 0.5 mM synthetic YMNGTMSQV for 12 h at 37°C before extraction and immunoaffinity purification of HLA-A2.1 molecules. Peptides were extracted and material equivalent to 5 × 10^7 cells was analyzed as described in the legend to Fig. 2. (*B*) Synthetic YMNGTMSQV was added to an aliquot of peptide extract equivalent to that analyzed in *A*. (*C*) Synthetic YMDGTMSQV was added to an aliquot of peptide extract equivalent to that analyzed in *A*.

are generally estimated to be of the order of days to years. Nonetheless, to ensure that this conversion had not occurred during MHC purification and peptide extraction, the antigen-processing mutant cell line T2 (20, 21) was pulsed with exogenous YMNGTMSQV, and the HLA-A2.1–associated peptides were extracted. Analysis of this extract revealed a single peak in the mass range 1031–1032, corresponding to 5.1 fmol/1.4 × 10^7 cells (Fig. 4 *A*), which was not detected among peptides extracted from unpulsed T2 cells (our unpublished data). Microcapillary HPLC demonstrated that this peptide coeluted with synthetic YMNGTMSQV (Fig. 4 *B*). No peak of >0.05 fmol/1.4 × 10^7 cells was observed at the elution position of synthetic YMDGTMSQV (compare Fig. 4, *A* and *C*). Thus, the conversion of asparagine to aspartic acid in the naturally processed peptide did not occur during the peptide extraction procedure, or while it was associated with HLA-A2 at the cell surface.

A second experiment was performed to establish whether spontaneous deamidation had occurred during the time that tyrosinase or tyrosinase-related peptides resided inside the cell. Whereas enzymatic deamidation of asparagine residues in peptides results in the production of α-linked aspartic acid, spontaneous deamidation of asparagine-glycine sequences occurs by the β-aspartyl shift mechanism, producing a mixture of α- and β-linked asparagine residues with an approximate ratio of 1:3 (32). Microcapillary HPLC analysis demonstrated that a tyrosinase peptide containing β-linked aspartic acid [YM(β-D)GTMSQV] could be resolved from YMDGTMSQV (Fig. 5). However, all of the naturally occurring tyrosinase peptide from NA8Mel+tyr cells coeluted with YMDGTMSQV, and none was detectable at the elution position of the β-linked form (Fig. 5). Thus, the naturally occurring peptide was not produced by a spontaneous deamidation mechanism.

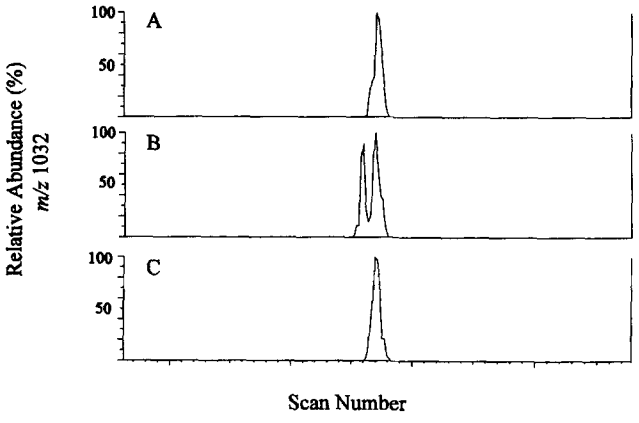

Figure 5. Ion chromatograms of peptides isolated from NA8Mel+tyr cells analyzed as described for Fig. 2. (*A*) Ion chromatogram for m/z 1,031–1,032 from NA8Mel+tyr material equivalent to 4 × 10^7 cells. (*B*) Ion chromatogram for m/z 1,031–1,032 from NA8Mel+tyr material equivalent to 4 × 10^7 cells with synthetic YM(β-D)GTMSQV peptide added. (*D*) Ion chromatogram for m/z 1,031–1,032 from NA8Mel+tyr material equivalent to 4 × 10^7 cells with synthetic YMNGTMSQV peptide added.

Discussion

Our results establish that the naturally occurring peptide corresponding to a tyrosinase epitope is distinct from that deduced from the gene. This modified peptide is recognized by tyrosinase-specific human CTL more effectively than the direct translation product, and is the only one of these two peptides to be presented by HLA-A2.1 molecules on the cell surface. Since the two peptides have a similar binding affinity for HLA-A2.1, it would have been predicted that both would be found among the naturally processed peptides with this MHC molecule. Consequently, the failure to detect any significant amount of YMNGTMSQV while the quantity of YMDGTMSQV was 1,000 times greater than background, suggests that spontaneous deamidation cannot account for the generation of this peptide epitope. The only explanation for the presence of the naturally processed species is that it arises via an enzymatically posttranslational modification that results in the conversion of asparagine to aspartic acid.

Enzymatic deamidation of asparagine to aspartate could occur through the action of peptide:*N*-glycanase (PNGase). This enzyme generates an aspartate through hydrolysis of the linkage between complex-type or high-mannose glycans and asparagine during degradation of N-linked glycoproteins (33, 34). A less likely possibility is glycoasparaginase which has similar activity but acts preferentially on free glycoasparagine compared to peptide-bound forms (35–37). Tyrosinase is a glycoprotein that contains six potential N-glycosylation sites (38, 39). One of these includes the asparagine residue at position 371 which has been shown in the present report to undergo posttranslational modification to aspartic acid. Given the strong probability that the N residue in YMNGTMSQV is glycosylated (40), it seems most likely that this mechanism accounts for the presentation of YMDGTMSQV in association with HLA-A2.1. Additionally, it should be pointed out that N-glycosylation of the asparagine residue would protect it from nonenzymatic deamidation. Furthermore, the attachment of a large carbohydrate side chain would more than likely interfere with binding to HLA-A2.1, since this residue acts as a secondary anchor for peptide binding (23). In either case, these factors would result in the absence of the asparagine-containing form of this peptide on the cell surface.

It is interesting to consider how this postulated mechanism for posttranslational conversion of this residue would fit into the pathway for processing and presentation of class I–associated peptides. The conventional pathway involves the production of peptides from proteins expressed in the cytosol and subsequent transport into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) (41, 42). Proteins that are membrane associated or secreted contain signal sequences that cause them to be cotranslationally transferred into the ER from

membrane-bound ribosomes. Such proteins would thus be protected from the action of cytoplasmic proteases. Since peptide epitopes do arise from such proteins, it has been assumed that the proteolysis to generate these epitopes occurs after the proteins have been aberrantly translated on cytoplasmic ribosomes (1, 41). However, the generation of an aspartic acid–containing peptide by the action of PNGase or glycosylasparaginase would necessitate synthesis of tyrosinase on ER-associated ribosomes for it to become N-glycosylated. The sequence and location of further processing steps is unknown. PNGase is a soluble protein whose cellular location has not yet been determined, although it has virtually no activity at lysosomal pH (33, 34). On the other hand, glycoasparaginase is located exclusively in lysosomes (36); and N-glycosylated forms of tyrosinase have been observed in this compartment (40). In any case, tyrosinase would need to move from the ER to the subcellular location containing one of these enzymes to allow generation of the deglycosylated form of the peptide. Given this scenario, it also remains uncertain whether such peptides would be dependent upon the TAP complex for transport into the ER. It is interesting that a peptide epitope derived from the HIV-1 envelope protein was shown to be generated independently of the TAP complex, whereas generation of epitopes from the transmembrane fusion protein of measles virus were dependent upon TAP (43, 44). Further investigation into the N-glycosylation/N-deglycosylation and the catabolism of tyrosine should reveal the processes involved in formation of this naturally occurring posttranslationally modified tyrosinase epitope and their significance to class I antigen presentation. Such a mechanism may be generally applicable to the presentation of both glycosylated and nonglycosylated proteins from membrane-bound and -secreted proteins.

The identification of the naturally occurring tyrosinase epitope YMDGTMSQV involved two alternative strategies. These complementary techniques reveal that this naturally occurring peptide undergoes a posttranslational modification that is relevant to the identification and prediction of other peptide antigens. The results presented here indicate that the peptide structures presented by MHC molecules may not be directly predictable from a DNA coding sequence. Other peptides associated with class II molecules have been previously shown to be posttranslationally modified by the attachment of carbohydrate side chains (45, 46), and the extent of carbohydrate attachment has been shown to affect T cell recognition of peptide structures (46, 47). However, a posttranslational modification that results in an alteration in the primary amino acid sequence of the peptide has not been previously reported. Our results also suggest that changes in the regulation of posttranslational modification in different cells could lead to the generation of new antigens. Such antigens could be relevant both to autoimmunity and to tumor rejection.

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References

1. Townsend, A., and H. Bodmer. 1989. Antigen recognition by class I–restricted T lymphocytes. *Annu. Rev. Immunol.* 7: 601–624.
2. Morrison, L.A., A.E. Lukacher, V.L. Braciale, D.P. Fan, and T.J. Braciale. 1986. Differences in antigen presentation to MHC class I– and class II–restricted influenza virus specific cytolytic T lymphocyte clones. *J. Exp. Med.* 163:903–921.
3. Moore, M.W., F.R. Carbone, and M.J. Bevan. 1988. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell.* 54:777–785.
4. van der Bruggen, P., C. Traversari, P. Chomez, C. Lurquin, E. De Plaen, B. Van den Eynde, A. Knuth, and T. Boon. 1991. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science (Wash. DC).* 254:1643–1647.
5. Traversari, C., P. van der Bruggen, I.F. Luescher, C. Lurquin, P. Chomez, A. Van Pel, E. De Plaen, A. Amar-Costesec, and T. Boon. 1992. A nonapeptide encoded by human MAGE-1 is recognized on HLA-A1 by cytolytic T lymphocytes directed against tumor antigen MZ2-E. *J. Exp. Med.* 176:1453–1457.
6. Gaugler, B., B. Van den Eynde, P. van der Bruggen, P. Romero, J.J. Gaforio, E. De Plaen, B. Lethe, F. Brasseur, and T. Boon. 1994. Human gene MAGE-3 codes for an antigen recognized on melanoma by autologous cytolytic T lymphocytes. *J. Exp. Med.* 179:921–930.
7. Boon, T., J.C. Cerottini, A. Van Pel, and P. van der Bruggen. 1994. Tumor antigens recognized by T lymphocytes. *Annu. Rev. Immunol.* 12:337–365.
8. Bakker, A.B.H., W.J. Schreurs, A.J. de Boer, Y. Kawakami,

S.A. Rosenberg, G.J. Adema, and C.G. Figdor. 1994. Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes. *J. Exp. Med.* 179:1005–1009.

9. Kawakami, Y., S. Eliyahu, C.H. Delgado, P.F. Robbins, K. Sakaguchi, E. Appella, J.R. Yannelli, G.J. Adema, T. Miki, and S.A. Rosenberg. 1994. Identification of a human melanoma antigen recognized by tumor infiltrating lymphocytes associated with in vivo tumor rejection. *Proc. Natl. Acad. Sci. USA.* 91:6458–6462.
10. Kawakami, Y., S. Eliyahu, C.H. Delgado, P.F. Robbins, L. Rivoltini, S.L. Topalian, T. Miki, and S.A. Rosenberg. 1994. Cloning of the gene for a shared melanoma antigen recognized by autologous T cells infiltrating into tumor. *Proc. Natl. Acad. Sci. USA.* 91:3515–3519.
11. Coulie, P.G., V. Brichard, A. Van Pel, T. Wolfel, J. Schneider, C. Traversari, S. Mattei, E. De Plaen, C. Lurquin, J.-P. Szikora, J.C. Renauld, and T. Boon. 1994. A new gene coding for a differentiation antigen recognized by autologous cytolytic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med.* 180:35–42.
12. Kawakami Y., S. Eliyahu, K. Sakaguchi, P.F. Robbins, L. Rivoltini, J.R. Yannelli, E. Appella, and S.A. Rosenberg. 1994. Identification of the immunodominant peptides of the MART-1 human melanoma antigen recognized by the majority of HLA-A2 restricted tumor infiltrating lymphocytes. *J. Exp. Med.* 180:347–352.
13. Brichard, V., A. Van Pel, T. Wolfel, C. Wolfel, E. De Plaen, B. Lethe, P. Coulie, and T. Boon. 1993. The tyrosinase gene encodes for an antigen recognized by autologous cytotoxic T lymphocytes on HLA-A2 melanomas. *J. Exp. Med.* 178:489–495.
14. Wolfel, T., A. Van Pel, V. Brichard, J. Schneider, B. Seliger, K.H.M. zum Buschenfelde, and T. Boon. 1994. Two tyrosinase nonapeptides recognized on HLA-A2 melanomas by autologous cytolytic T lymphocytes. *Eur. J. Immunol.* 24:759–764.
15. Slingluff, C.L., Jr., A.L. Cox, R.A. Henderson, D.F. Hunt, and V.H. Engelhard. 1993. Recognition of human melanoma cells by HLA-A2.1–restricted CTL is mediated by at least six shared peptide epitopes. *J. Immunol.* 150:2955–2963.
16. Hunt, D.F., R.A. Henderson, J. Shabanowitz, K. Sakaguchi, H. Michel, N. Sevilir, A. Cox, E. Appella, and V.H. Engelhard. 1992. Characterization of peptides bound to the class I MHC molecule HLA-A2.1 by mass spectrometry. *Science (Wash. DC).* 255:1261–1263.
17. Henderson, R.A., A.L. Cox, K. Sakaguchi, E. Appella, J. Shabanowitz, D.F. Hunt, and V.H. Engelhard. 1993. Direct identification of an endogenous peptide recognized by multiple HLA-A2.1 specific cytotoxic T cells. *Proc. Natl. Acad. Sci. USA.* 90:10275–10279.
18. Cox, A.L., J. Skipper, Y. Chen, R.A. Henderson, T.L. Darrow, J. Shabanowitz, V.H. Engelhard, D.F. Hunt, and C.L. Slingluff. 1994. Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. *Science (Wash. DC).* 264:716–719.
19. Engelhard, V.H., E. Appella, D.C. Benjamin, W.M. Bodnar, A.L. Cox, Y. Chen, R.A. Henderson, E.L. Huczko, H. Michel, K. Sakaguchi, et al. 1993. Mass spectrometric analysis of peptides associated with the human class I MHC molecules HLA-A2.1 and HLA-B7 and identification of structural features that determine binding. *Chem. Immunol.* 57:39–62.
20. Salter, R.D., D.N. Howell, and P. Cresswell. 1985. Genes regulating HLA class I antigen expression in T-B lymphoblast hybrids. *Immunogenetics.* 21:235–246.
21. Henderson, R.A., H. Michel, K. Sakaguchi, J. Shabanowitz, E. Appella, D.F. Hunt, and V.H. Engelhard. 1992. HLA-A2.1 associated peptides from a mutant cell line: a second pathway of antigen presentation. *Science (Wash. DC).* 255: 1264–1266.
22. Bodmer, H., G. Ogg, F. Gotch, and A. McMichael. 1989. Anti-HLA-A2 antibody-enhancement of peptide association with HLA-A2 as detected by cytotoxic T lymphocytes. *Nature (Lond.).* 342:443–446.
23. Chen, Y., J. Sidney, S. Southwood, A.L. Cox, K. Sakaguchi, R. Henderson, E. Appella, D.F. Hunt, A. Sette, and V.H. Engelhard. 1994. Naturally processed peptides longer than nine amino acid residues bind to the class I MHC molecule HLA-A2.1 with high affinity and in different conformations. *J. Immunol.* 152:2874–2881.
24. Ruppert, J., J. Sidney, E. Celis, R.T. Kubo, H.M. Grey, and A. Sette. 1993. Prominent role of secondary anchor residues in peptide binding to A2.1 molecules. *Cell.* 74:929–937.
25. den Haan, J.H.H., N.E. Sherman, E. Blokland, E.L. Huczko, F. Koning, J.W. Drijfhout, J. Skipper, J. Shabanowitz, D.F. Hunt, V.H. Engelhard, and E. Goulmy. 1995. Identification of a graft-versus-host disease associated human minor histocompatibility antigen. *Science (Wash. DC).* 268:1476–1480.
26. Bertoletti, A., F.V. Chisari, A. Penna, S. Guilhot, L. Galati, G. Missale, P. Fowler, H. Schlicht, A. Vitiello, R.C. Chesnut, et al. 1993. Definition of a minimal optimal cytotoxic T-cell epitope within the hepatitis B virus nucleocapsid protein. *J. Virol.* 67:2376–2380.
27. Utz, U., S. Koenig, J.E. Coligan, and W.E. Biddison. 1992. Presentation of three different viral peptides, HTLV-1 Tax, HCMV gB, and influenza virus M1, is determined by common structural features of the HLA-A2.1 molecule. *J. Immunol.* 149:214–221.
28. Bednarek, M.A., S.Y. Sauma, M.C. Gammon, G. Porter, S. Tamhankar, A.R. Williamson, and H.J. Zweerink. 1991. The minimum peptide epitope from the influenza virus matrix protein. Extracellular and intracellular loading of HLA-A2. J. Immunol. 147:4047–4053.
29. Kwon, B.S., A.K. Haq, S.H. Pomerantz, and R. Halaban. 1987. Isolation and sequence of a cDNA for human tyrosinase that maps at the mouse c-albino locus. *Proc. Natl. Acad. Sci. USA.* 84:7473–7477.
30. Bouchard, B., B.B. Fuller, S. Vijayasaradhi, and A.N. Houghton. 1989. Induction of pigmentation in mouse fibroblasts by expression of human tyrosinase cDNA. *J. Exp. Med.* 169:2029–2042.
31. Oetting, W.S., H.Y. Handoko, M.M. Mentink, A.S. Paller, J.G. White, and R.A. King. 1991. Molecular analysis of an extended family with type IA (tyrosinase negative) oculocutaneous albinism. *J. Invest. Dermatol.* 97:15–19.
32. Wright, H.T. 1991. Nonenzymatic deamidation of asparaginyl and glutaminyl residues in proteins. *Crit. Rev. Biochem. Mol. Biol.* 26:1–52.
33. Suzuki, T., A. Seko, K. Kitajima, Y. Inoue, and S. Inoue. 1993. Identification of peptide:N-glycanase activity in mammalian-derived cultured cells. *Biochem. Biophys. Res. Commun.* 194:1124–1130.
34. Suzuki, T., A. Seko, K. Kitajima, Y. Inoue, and S. Inoue. 1994. Purification and enzymatic properties of peptide:N-glycanase from C3H mouse–derived L-929 fibroblast cells. *J. Biol. Chem.* 269:17611–17618.

35. Aronson, N.N., and M.J. Kuranda. 1989. Lysosomal degradation of Asn-linked glycoproteins. *FASEB (Fed. Am. Soc. Exp. Biol.).* 3:2615–2622.
36. Kaartinen, V., T. Mononen, R. Laatkainen, and I. Mononen. 1992. Substrate specificity and reaction mechanism of human glycoasparaginase. *J. Biol. Chem.* 267:6855–6858.
37. Mononen, I., K.J. Fisher, V. Kaartinen, and N.N. Aronson. 1993. Aspartylglycosaminuria: protein chemistry and molecular biology of the most common lysosomal storage disorder of glycoprotein degradation. *FASEB (Fed. Am. Soc. Exp. Biol.).* 7:1247–1256.
38. Hearing, V.J., and M. Jimenez. 1987. The critical regulatory control point in melanocyte pigmentation. *Int. J. Biochem.* 19: 1141–1147.
39. Hearing, V.J., and M. Jimenez. 1989. Analysis of mammalian pigmentation at the molecular level. *Pigm. Cell Res.* 2:75–85.
40. Ohkura, T., K. Yamashita, Y. Mishima, and A. Kobata. 1984. Purification of hamster melanoma tyrosinase and structural studies of their asparagine-linked sugar chains. *Arch. Biochem. Biophys.* 235:63–77.
41. Yewdell, J.W., and J.R. Bennink. 1992. Cell biology of antigen processing and presentation to MHC class I molecule restricted T lymphocytes. *Adv. Immunol.* 52:1–123.
42. Germain, R.N., and D.H. Margulies. 1993. The biochemistry and cell biology of antigen processing and presentation. *Annu. Rev. Immunol.* 11:403–450.
43. Hammond, S.A., R.C. Bollinger, T.W. Toberty, and R.F. Siliciano. 1993. Transport-independent processing of HIV-1 envelope protein for recognition by $CD8^+$ T cells. *Nature (Lond.).* 364:158–161.
44. van Binnendijk, R.S., C.A. van Baalen, M.C. Poelen, P. de Vries, J. Boes, V. Cerundolo, A.D. Osterhaus, and F.G. Uytdehaag. 1992. Measles virus transmembrane fusion protein synthesized de novo or presented in immunostimulating complexes is endogenously processed for HLA class I– and class II–restricted cytotoxic T cell recognition. *J. Exp. Med.* 176:119–128.
45. Chicz, R.M., R.G. Urban, J.C. Gorga, D.A. Vignali, W.S. Lane, and J.L. Strominger. 1993. Specificity and promiscuity among naturally processed peptides bound to HLA-DR alleles. *J. Exp. Med.* 178:27–47.
46. Michaelsson, E., V. Malmstrom, S. Reis, A. Engstrom. H. Burkhardt, and R. Holmdahl. 1994. T cell recognition of carbohydrates on type II collagen. *J. Exp. Med.* 180:745–749.
47. Jerome, K.R., N. Domenech, and O.J. Finn. 1993. Tumor-specific cytotoxic T cell clones from patients with breast and pancreatic adenocarcinoma recognize EBV-immortalized B cells transfected with polymorphic epithelial mucin complementary DNA. *J. Immunol.* 151:1654–1662.
48. Huczko, E.L., W.M. Bodnar, D. Benjamin, K. Sakaguchi, N.Z. Zhu, J. Shabanowitz, R.A. Henderson, E. Appella, D.F. Hunt, and V.H. Engelhard. 1993. Characteristics of endogenous peptides eluted from the class I MHC molecule HLA-B7 determined by mass spectrometry and computer modeling. *J. Immunol.* 151:2572–2587.