The Class I Antigen-processing Pathway for the Membrane Protein Tyrosinase Involves Translation in the Endoplasmic Reticulum and Processing in the Cytosol

By Claudio A. Mosse,* Leslie Meadows,‡ Chance J. Luckey,* David J. Kittlesen,§ Eric L. Huczko,∗ Craig L. Slingluff, Jr.,§ Jeffrey Shabanowitz,‡ Donald F. Hunt,‡ and Victor H. Engelhard∗

From the ∗Department of Microbiology and the Beirne Carter Center for Immunology Research; †the Department of Chemistry; ‡the Department of Surgery; and §the Department of Pathology, University of Virginia, Charlottesville, Virginia 22904

Summary

Formation of major histocompatibility complex class I–associated peptides from membrane proteins has not been thoroughly investigated. We examined the processing of an HLA-A*0201–associated epitope, YM
DGTMSQV, that is derived from the membrane protein tyrosinase by posttranslational conversion of the sequence YMN
GTMSQV. Only YM
DGTMSQV and not YMN
GTMSQV was presented by HLA-A*0201 on cells expressing full-length tyrosinase, although both peptides have similar affinities for HLA-A*0201 and are transported by TAP. In contrast, translation of YMN
GTMSQV in the cytosol, as a minigene or a larger fragment of tyrosinase, led to the presentation of the unconverted YMN
GTMSQV. This was not due to overexpression leading to saturation of the processing/conversion machinery, since presentation of the converted peptide, YM
DGTMSQV, was low or undetectable. Thus, presentation of unconverted peptide was associated with translation in the cytosol, suggesting that processing of the full-length tyrosinase occurs after translation in the endoplasmic reticulum. Nevertheless, presentation of YMDGTMSQV in cells expressing full-length tyrosinase was TAP (transporter associated with antigen processing) and proteasome dependent. After inhibition of proteasome activity, tyrosinase species could be detected in the cytosol. We propose that processing of tyrosinase involves translation in the endoplasmic reticulum, export of full-length tyrosinase to the cytosol, and retransport of converted peptides by TAP for association with HLA-A*0201.

CD8+ T cells recognize peptides in association with class I MHC proteins on the surface of cells. In general, these MHC class I–associated peptides are derived from intracellular proteins (1). In the classical pathway for processing of class I–associated peptides, cytosolic proteases such as the proteasome degrade proteins to generate peptides that are transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP; 2–6). Upon entry into the ER, the peptides are bound to “empty” or peptide-free MHC class I molecules that are associated with TAP (7, 8) via an intermediary protein, tapasin (9). After binding peptide, the MHC class I heterotrimer dissociates from TAP and proceeds through the ER, Golgi, and exocytic pathway to the cell surface (10). The peptides in association with the MHC class I molecule are then available for recognition on the cell surface by CTLs.

Because membrane and secreted proteins are normally cotranslationally translocated into the ER, they would appear to bypass the cytosolic proteases of the classical pathway. Nevertheless, a number of MHC class I–associated peptides that originate from membrane proteins have been identified, and the pathways by which they are produced have been the object of several recent studies. Peptides from the signal sequences of IP-30, HLA-E, Signal Sequence Receptor Protein–α (SSR-α), and calreticulin (11, 12), as well as peptides from more internal sequences of the HIV env (13) and Epstein-Barr virus Latent Membrane Protein 2 (LMP2) proteins (14), and a peptide epitope of uncertain location (15) are presented by HLA-A*0201 in cells that lack expression of TAP. Independence of TAP indicates that the source proteins for these peptides are produced in the ER, and that complete proteolytic processing occurs in the ER or distal vesicular compartments, and not the cytosol. Although the signal peptide is likely to be involved in the generation of signal sequence–derived peptides, it is unlikely to account for the production of peptides from more unknown location.
internal sequences. In addition, none of the peptides derived from signal sequences is full-length, raising the possibility that additional proteases are involved in secondary proteolytic events. In support of this possibility, it has been shown that the production of some, but not all, of these peptides is sensitive to high concentrations of the protease inhibitor LLnL (16). In addition, several vaccinia virus constructs containing peptide epitopes embedded in a larger sequence that is in turn linked to a signal sequence can be processed for presentation in a TAP-independent manner, presumably via ER resident proteases (17–20).

An alternative pathway for the processing of membrane protein–derived epitopes has been suggested by the observation that the presentation of peptides from the measles virus transmembrane (21) and the HIV env (22) proteins as well as peptides from the signal sequence of some MHC class I molecules (23, 24) and the LCMV gp33 protein (25) are dependent on TAP function. R. Wells et al. (26) demonstrated that in vitro, peptides transported into the ER that are too long to bind to class I MHC molecules could be exported to the cytosol for further processing, and the products then retransported to the ER by TAP. Although a similar mechanism has not been demonstrated in vivo, partial proteolysis in the ER followed by final proteolysis in the cytosol could account for the TAP-dependent presentation of these epitopes. Alternatively, their presentation may occur after mistranslation of the source protein in the cytosol (27). This could occur either as the result of incomplete translocation blockade by signal sequences on cytosolic ribosomes, or by the use of an alternate start codon internal to the signal sequence, as has been shown to occur for several class I–associated epitopes (28–34). Support for such a cytosolic mistranslation mechanism has been provided by observations with an HLA-B*3501-restricted HIV env epitope. This epitope contains a site for N-linked glycosylation that is modified during cotranslational translocation of the full-length protein into the ER (35). However, the epitope presented by HLA-B*3501 has not undergone either glycosylation or deglycosylation (22, 36). Thus, it appears that HIV env protein that gives rise to this epitope has been mistranslated in the cytosol and processed there.

A final possible explanation for the TAP-dependent presentation of peptides derived from membrane proteins is that the source protein itself is exported from the ER for proteolysis in the cytosol. Recently, the reverse translocation of several apparently full-length membrane proteins from the ER to the cytosol has been reported (37–48). Visualization of these proteins has generally been possible only after inhibition by protease inhibitors, in most cases those which are effective against the proteasome (38–48). However, no evidence supporting the involvement of this pathway in the production of MHC class I–associated peptide antigens has been presented. Thus, at this point in time, the only evidence available for the processing of membrane proteins for class I presentation support a cytosolic mistranslation mechanism.

Recently, we described an epitope from the membrane-associated protein tyrosinase that is presented by HLA-A*0201 to CTL reactive with human melanomas (49). The sequence of this peptide, YM\textbf{D} GTM SQV, differed from the corresponding primary sequence of the tyrosinase protein, YM\textbf{N} GTM SQV, by the substitution of aspartic acid for asparagine at position 3. This was shown to be due to a posttranslational conversion, and not to spontaneous deamidation nor R NA editing. The asparagine in YM\textbf{N} GTM SQV is part of an N-linked glycosylation site, a mammalian enzyme, peptide-N\textsuperscript{4}-(N acetyl-\beta-glucoamyl) asparagine amidase (PNGase), has been isolated, which removes the N-linked oligosaccharide side chains from glycopeptides. This process converts the modified asparagine residues to aspartic acid (50). Our working hypothesis is that glycosylation in the ER and subsequent deglycosylation at an unknown site are responsible for the conversion of YM\textbf{N} GTM SQV to YM\textbf{D} GTM SQV. Regardless of whether this hypothesis is correct, it focused our attention on how tyrosinase is processed. Since tyrosinase is a melanosomal membrane protein, mistranslation of tyrosinase in the cytosol could lead to proteolysis of the unconverted protein, generating peptides that would be transported by TAP into the ER and converted before or after binding to HLA-A*0201. Alternatively, conversion could occur during normal cotranslational translocation into the ER and subsequent degradation either in the ER, after export of partially proteolysed fragments, or in the cytosol after reverse translocation of the source protein. In this paper, we have examined the processing and conversion of this epitope in detail.

**Materials and Methods**

**Cell Lines.** N8 Mel + Tyr was derived by transfection of the tyrosinase negative melanoma N8 Mel with the full-length tyrosinase gene and was a gift from Vincent Brichard and Thierry Boon (Ludwig Institute for Cancer, Université Catholique de Louvain, Brussels, Belgium). N8 Mel + T3.1 was derived from transfection of N8 Mel with the truncated tyrosinase cdNA T3.1 (51). JY is a human HLA-A*0201 cell line. T2 is an HLA-A*0201 human B lymphoblastoid cell line with a deletion in the MHC including the genes for TAP1, TAP2, LMP2, and LMP7 (52, 53). All cell lines were maintained in RPMI 1640 supplemented with 5% FCS/SerXtend (Irvine Scientific, Santa Ana, CA) in a humidified 5% CO\textsubscript{2} atmosphere at 37°C.

**Peptides.** Synthetic peptides were made by standard Fmoc chemistry using a model AM S422 peptide synthesizer (Gibson Co. Inc., Middleton, WI). All peptides were purified to >98% purity by reverse-phase HPLC on a C-8 column (VYDAC, Hesperia, CA). Purity and identity were established using a triple quadrupole mass spectrometer (model TSQ-7000; Finnigan, San Jose, CA).

**RecombinantVaccinia Viruses.** The minigene vaccinia was constructed using synthetic oligonucleotides (5’ GTA-CCACCATGTATATGAATGGAAACAATGGCCAGTATA 3’ and 5’ AGCTTATACCTGGGACATTGTTCCATTCA-TATACATGGTG 3’ for peptide (M)YM\textbf{D} GTM SQV and 5’ GTACCAACCATGTATATGAATGGAAACAATGGCCAGTATA 3’ and 5’ AGCTTATACCTGGGACATTGTTCCATTCA-TCCATATACATGGTG 3’ for peptide (M)YM\textbf{N} GTM SQV) ligated directionally into the plasmid pSC11.3 (54) at the Acc65I and HindIII sites. In addition to appropriate overhang sequences for
ligation, the synthetic nucleotide sequences contained a favorable Kozac sequence for translation initiation, a methionine start codon, the nucleotides encoding the T cell epitope, and a transla-
tional stop signal. The full-length tyrosinase was excised from the pcDNA A1/amp-123.b2 vector using HindIII and XbaI and sub-
cloned into pS CII.3 at the HindIII and Spel sites. R recombinant
vaccinia viruses were produced from these vectors using standard
methods (55). Purified vaccinia stocks were titered and tested for
proper expression of tyrosinase or minigenes using specific murine
HLA-A2-restricted CTL. Vaccinia viruses encoding the TAP1 and
TAP2 genes were a gift from Drs. Jon Yewdell and Jack Ben-
nink (Laboratory of Viral Diseases, National Institutes of Allergy
and Infectious Diseases, Bethesda, MD).

CTL Lines and Cytotoxicity. CTL lines were generated by in-
traperitoneal injection of 5 × 10^6 PFU's of recombinant vaccinia en-
coding either YM DGTMSQV or YM NGTM SQV into C57BLJ/6
mice expressing a chimeric MHC class I with the α1 and α2 do-
 mains from HLA-A2.1 and the α3 domain from K(6) (56). 3 wk after
priming, splenocytes were removed and stimulated with autolo-
gous irradiated splenocytes that had been pulsed with YM D-
GTMSQV or YM NGTM SQV peptide. After the first week in
culture, autologous irradiated peptide-pulsed splenocytes and 10
U/ml IL-2 were added. IL-2 was also added on day 4 of every
weekly stimulation. Standard 51Cr-release assays were performed
to determine CTL recognition of tyrosinase peptides. For peptide
dose response assays, T2 cells were 3H-labeled in the presence of
10 μCi/ml MA2.1, and then incubated with the indicated concen-
trations of synthetic tyrosinase peptides for 3 h at 37°C. For vac-
cinia-infected target cells, 2 × 10^5 PFU's vaccinia were added to
10^5 targets in HBSS for 1 h. RPMI 10% FCS, 15 mM
Hepes, 50
M
b-mercaptoethanol, 2 mM glutamine, and essen-
tial and nonessential amino acids was then added to the infected
cells for 9 h to allow for expression. Targets were labeled in 100
μCi N a2CrO4 for the final 2 h. CTLs were added at an effector:
target ratio of 10:1 unless otherwise noted.

HLA-A*0201-associated Peptide Isolation. Peptides were acid
eluted from affinity-purified HLA-A*0201 molecules as previ-
ously described (49, 57). The peptide extracts from 2 × 10^9 NA8
melanoma cells were separated on a narrow bore reverse phase column (RP-18 Spheri-5, 2.1 × 3 mm), Buffer A was 0.1%
TFA in water and buffer B was 0.085% TFA in 80% acetonitrile. The gradient consisted of 100% buffer A (0–20
min), 0–15% buffer B (20–25 min) and 15–67% buffer B (25–80
min) at a flow rate of 200 μl/min. The synthetic peptides
YM DGTMSQV and YM NGTM SQV were separated on the same column under identical conditions immediately after the ex-
tracts, and their elution positions were used to identify fractions
from the extracts that contained the naturally processed forms of
these peptides. These fractions were loaded onto a 18 cm
microcapillary column (75 µm intradimensionally × 12 cm) and eluted
using a 2%/min increasing gradient of acetonitrile in 0.1 M acetic
acid into a triple quadrupole mass spectrometer (model TSTQ-
7000; Finnigan) equipped with an electrospray ion source. Scans
were acquired every 1.5 s over a mass range m/z (mass charge ra-
tio) 300:1400 and then plotted with intensities for m/z 1,031:
1,032. For the NA8 mel + Tyr peptide extract, 10% of the sam-
ple was analyzed by mass spectrometry. For the NA8 mel + T31 peptide extract, 20% of the sample was analyzed by mass spectrom-
etry. The loaded sample amounts were standardized using an
unrelated peptide of m/z 541 found in fractions 28, 29, and 30.
We determined the cell surface copy numbers of YM DGTMSQV
peptides using synthetic YM DGTMSQV peptides as stan-

Inhibitors. Lactacystin (gift of Dr. S. O'mura of the Kitasato
Institute, Tokyo, Japan) is a Streptomyces metabolite which irre-
versibly inhibits proteasomes via covalent binding to the active
sites of the catalytically active β subunits (58). LLNl, also known
as Calpain Inhibitor I, was purchased from Calbiochem (La Jolla,
CA). LLNl reversibly inhibits proteasomes as well as several other
classes of proteases, including cysteine proteases, calpain, and cathe-
pain B (59).

Epitope Recognition Assay. Tumor targets (1–2 × 10^7) were
grown in RPMI 1640 supplemented with 5% FCS/SerXtend and
then centrifuged. The pellet was gently resuspended in 500 μl of
300 mM glycine, pH 2.5, 1% (wt/vol) BSA and incubated for 3
min at 37°C. The suspension was diluted with 40 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were aliquoted into 1 ml of RPMI
1640 medium supplemented with 5% FCS/SerXtend and then
centrifuged. Cells (2 × 10^6) were ali...
To detect either the YM\_D\_GTMSQV or the YM\_N\_GTMSQV epitopes, we developed CTLs specific for each peptide from transgenic mice expressing a chimeric HLA-A*0201/H2Dd molecule. CTLs generated against the YM\_D\_GTMSQV peptide (D-specific CTLs) required 5–6 logs less of the YM\_D\_GTMSQV peptide than the YM\_N\_GTMSQV peptide to give equivalent lysis of peptide-pulsed target cells (Fig. 1A). Conversely, CTLs generated against the YM\_N\_GTMSQV peptide (N-specific CTLs) required 5–6 logs less YM\_N\_GTMSQV than YM\_D\_GTMSQV for recognition (Fig. 1B). Since YM\_NGTM SQV and YM\_D\_GTMSQV bind to HLA-A2.1 with similar affinities (49), these differences reflect the ability of the CTLs to discriminate between these two peptides. Nevertheless, HLA-A*0201+ melanomas expressing tyrosinase were lysed by D-specific CTLs but not N-specific CTLs (Fig. 2). This result demonstrates that the YM\_NGT SQV peptide produced in the cytosol can be expressed in association with HLA-A*0201 molecules at the cell surface. In addition, this expression is dependent upon the expression of TAP by the target cell (data not shown). Interestingly, however, expression of this minigene-encoded form of the YM\_NGT SQV sequence did not result in conversion and presentation of the YM\_D\_GTMSQV peptide at the surface. This contrasts to the presentation of only the YM\_D\_GTMSQV peptide in cells expressing full-length tyrosinase.

One possible explanation for the exclusive production of YM\_D\_GTMSQV from full-length tyrosinase and of YM\_N\_GTMSQV from the minigene is that the former is translated in the ER, where it undergoes conversion, whereas the latter is translated in the cytosol, where it does not. However, the minigene product was preprocessed to the ideal length to bind to HLA-A*0201, while the full-length tyrosinase requires proteolysis before binding. Therefore, it was also possible that immediate binding of the minigene product to HLA-A*0201 after entry into the ER prevented conversion, whereas longer peptides derived from full-length tyrosinase were converted before being trimmed to the optimal length for binding. To distinguish between these two possibilities, we transfected N\_A8 Mel, a tyrosinase-negative melanoma cell line, with a truncated tyrosinase gene fragment called T3.1 (51). The translation product of T3.1 spans residues 143–377 of the full-length tyrosinase sequence (Fig. 4). Because it lacks the NH\_2-terminal end of full-length tyrosinase, including the signal sequence, it should be translated in the cytosol, as is the minigene, rather than the ER. However, the YM\_NGTM SQV epitope sequence...
in T3.1 (residues 368–376) is bounded by 225 residues at its NH₂ terminus and 1 residue at its COOH terminus. To be presented by HLA-A*0201, it requires additional processing as compared to the minigene product. If the failure of the minigene product to undergo conversion were due to its expression in the cytosol, regardless of length, then we would expect that cells expressing T3.1 would also express the unconverted peptide epitope. This was found to be the case (Fig. 5 A). We conclude from this that the production of the unconverted epitope is associated with translation in the cytosol, and that its absence from cells expressing full-length tyrosinase indicates that the converted epitope is produced from this protein after it is translated into the ER, rather than mistranslated in the cytosol. Although the presence of the unconverted epitope was clearly associated with the translation of protein in the cytosol, the absence of the converted epitope was not. Surprisingly, we found that cells expressing T3.1 presented the converted epitope as well as the unconverted form (Fig. 5 B). This low but reproducible level of lysis by the D-specific CTL indicates that cytosolic proteins can give rise to epitopes that have undergone conversion.

One possible caveat to the conclusion that the presence of the unconverted epitope was associated with cytosolic translation was the possibility that the amount of T3.1 protein in the cells, or its inability to fold appropriately, led to the accumulation of a much higher level of the YM NGTMSQV peptide or a precursor than would be produced in cells expressing full-length tyrosinase. This could lead to a saturation of the processing and conversion machinery, resulting in a failure to convert all of the peptides produced in the T3.1 transfectant to YM DGTMSQV. However, if this were so, then the T3.1 transfectant should express an amount of the converted YM DGTMSQV peptide equal to or higher than that found on the full-length transfectant. Consequently, we immunoaffinity purified the HLA-A2.1 molecules from comparable amounts of NA8 Mel + T3.1 and NA8 Mel + Tyr, and extracted the associated peptides. After HPLC separation, we used tandem mass spectrometry to determine the quantity of the YM DGTMSQV peptide in these extracts (Fig. 6). A coeluting species at m/z 541 was found in both extracts and was used to normalize the amount of cell extract injected into the mass spectrometer.
By using known amounts of synthetic peptide as a standard, we determined the quantity of this peptide in each extract, and normalized to the number of cell equivalents of material injected. The density of YM\textsubscript{DGTM}SQV on the full-length tyrosinase transfectant was determined to be 1,400 peptides per cell, in good agreement with a previously reported value of 1,200 copies per cell (49). However, the density of this peptide on the T3.1 transfectant was only 50 copies per cell, or 4% of that seen on the NA8 + Tyr cells. Although detected by N-specific CTL, the density of YM\textsubscript{NGTM}SQV on the NA8 Mel + T3.1 transfectant was too low to be detected by mass spectrometry with the amount of cell extract analyzed (<25 copies per cell). These results demonstrate that the T3.1 transfectant generated less YM\textsubscript{DGTM}SQV than did the full-length tyrosinase transfectant, and indicate that the presence of the unconverted peptide in the T3.1 transfectant was not due to saturation of the processing and conversion machinery. We conclude that the processing of the T3.1 translation product and full-length tyrosinase must differ in some respect. Since T3.1 has no signal sequence or transmembrane domain, it should be produced in the cytosol and processed by the classical antigen-processing pathway. Since full-length tyrosinase does not give rise to the unconverted peptide that is characteristic of cytosolic translation of both T3.1 and the minigene, it must therefore be translated in the ER, and undergo processing in a distinct manner.

Several epitopes from membrane proteins are generated by a pathway that is independent of the TAP protein, suggesting that after translation in the ER, their processing can be completed in this compartment (37, 63–65). We were interested in whether the formation of YM\textsubscript{DGTM}SQV from full-length tyrosinase, which involves both translation in the ER and conversion at an unknown site, was also TAP-independent. T2 cells, which are HLA-A*0201 but have deleted the genes encoding TAP1 and TAP2, were infected with a vaccinia vector encoding full-length tyrosinase and screened with the D-specific CTLs. There was no lysis of the vaccinia-tyrosinase–infected T2 cells. However, when T2 cells were infected with a mixture of recombinant vaccinia viruses expressing tyrosinase and the genes encoding TAP1 and TAP2, the YM\textsubscript{DGTM}SQV epitope was restored on the cell surface (Fig. 7). These results demonstrate that the production of the YM\textsubscript{DGTM}SQV epitope is TAP-dependent, and indicates that if, or a precursor that contains the epitope, is in the cytosol before binding HLA-A2.1 in the ER.

Because of the TAP dependence of the YM\textsubscript{DGTM}SQV epitope, we were interested in determining whether the proteasome is involved in epitope formation. Proteasome-dependent processing was evaluated by brief exposure of target cells to pH 2.5 medium to denature all cell surface MHC class I molecules, followed by incubation to allow reexpression of the epitope in the presence or absence of lactacystin or LLnL as described elsewhere (Luckey, C.J., G.M. King, J.A. Marto, B.F. Maier, V.L. Crother, T.A. Colella, J. Shabanowitz, D.F. Hunt, and V.H. Engelhard, manuscript submitted for publication). CTL recognition of acid-treated target cells was abolished, but recovered to control levels if the targets were incubated for 5 h at 37°C (Fig. 8). This recovery of epitope expression was inhibited by >50% in the presence of BFA, which blocks the egress...
targets were then washed and used in a 4-h 51Cr-release assay in the presence of any inhibitor (BFA (squares), 20 μM lactacystin (triangles) or 20 μM LLNL (circles). All targets were then washed and used in a 4-h 51Cr-release assay in the presence of 10 μg/ml BFA to block any further expression of newly synthesized peptide-HLA-A*0201 complexes. These results are representative of four independent experiments.

Figure 8. Recognition of the melanoma line DM93 by D Tyr CTL. DM93 was left untreated (crosses) or acid-treated as described in Materials and Methods and then incubated at 37°C for 5 h either in the absence of any inhibitor (diamonds) or in the presence of one of the following: 10 μg/ml BFA (squares), 20 μM lactacystin (triangles) or 20 μM LLNL (circles). All targets were then washed and used in a 4-h 51Cr-release assay in the presence of 10 μg/ml BFA to block any further expression of newly synthesized peptide-HLA-A*0201 complexes. These results are representative of four independent experiments.

of newly synthesized peptide MHC class I complexes from the ER (66–68). Although the failure of BFA to completely inhibit reexpression of the epitope is not understood, the difference in recognition between the acid-washed, untreated cells and the acid-washed, BFA-treated cells allowed us to determine whether protease inhibitors also blocked the generation of the YM DGTMSQV epitope. Incubation of acid-washed targets with 20 μM LLNL, a concentration known to selectively inhibit the proteasome (58), inhibited reexpression of the YM DGTMSQV epitope on the cell surface to the same extent as BFA (Fig. 8). In addition, treatment of the acid-washed cells with lactacystin, a highly specific inhibitor of the proteasome (58), also blocked reexpression of the YM DGTMSQV epitope. It has been shown elsewhere that the effects of these proteasome inhibitors are not due to nonspecific effects on class I biosynthesis or susceptibility of target cells to CTL-mediated lysis (Luckey, C.M.J., G.M. King, J.A. Marto, B.F. Maier, V.L. Crotzer, T.A. Colella, J. Shabanowitz, D.F. Hunt, and V.H. Engelhard, manuscript submitted for publication). Together these results indicate that the processing of intact tyrosinase to generate the YM DGTMSQV epitope is dependent upon the proteasome.

The results obtained thus far suggested that initial translation of tyrosinase in the ER was followed by the export of the full-length protein or a fragment to the cytosol for processing by the proteasome. To investigate the nature of the tyrosinase species exported from the ER to the cytosol, we used lactacystin to inhibit proteasome activity in DM93, a tyrosinase-expressing melanoma, and N A8 Mel, a tyrosinase-negative melanoma. After 5 h the cells were divided into cytosolic and membrane fractions, separated by SDS-PAGE, and immunoblotted for tyrosinase (Fig. 9). In untreated DM93 cells, we observed a strong doublet of 70–75 kD that was located exclusively in the membrane fraction. This doublet represents two major glycosylated species of tyrosinase, and correlate well with those previously identified by others (61, 69). In DM93 cells that had been treated with lactacystin, three additional bands were observed at 56–58 kD, similar in size to the unglycosylated full-length tyrosinase. The band of lowest mobility among this group was located exclusively in the membrane fraction, whereas the other two were found only in the cytosol. To further identify the potential nature of these lower molecular weight bands, the membrane fraction from DM93 cells was treated with PNGase F to deglycosylate asparagine-linked glycosylations (Fig. 10). Human tyrosinase contains seven potential glycosylation sites (70, 71) yet it appears that only four or five sites are used in DM93 cells. Although deglycosylation was not complete after 12 h, the fastest migrating species comigrated with the cytosolic 57-kD band, which appears after lactacystin treatment of DM93 cells. One additional species with an apparent molecular weight of 85–90 kD was found in the cytosol of untreated cells, but increased in intensity upon lactacystin treatment. The size of this band is too high to be attributed to glycosylation, but is consistent with the attachment of four ubiquitin chains to the unglycosylated full-length tyrosinase polypeptide chain. After long exposure times, two additional low intensity bands of 61 and 65 kD were also found in the cytosol. One of these bands was observed in extracts of N A8 M el (Fig. 9), whereas a similar pattern was observed in N A8 M el + Tyr cells (data not shown), indicating that all of the species are derived from tyrosinase. These results are consistent with the hypothesis that the export of a full-length or almost full-length tyrosinase from the ER for degradation by the proteasome provides source material for the production of the YM DGTMSQV peptide epitope.

Discussion

In a previous paper, we demonstrated that the peptide YM DGTMSQV is formed by the processing and post-translational modification of the sequence YM NGTMSQV located in the full-length tyrosinase protein sequence (49). Since the proposed posttranslational modification responsible for this conversion involved glycosylation in the endoplasmic reticulum, and since tyrosinase is a membrane protein, the objective of this study was to determine whether processing to produce this epitope occurred after normal translation of tyrosinase into the endoplasmic reticulum, or after aberrant translation in the cytosol. Using either a vaccinia-encoded minigene or a transfected fragment of tyrosinase that is missing the signal sequence, we have demonstrated that translation of the sequence YM NGTMSQV in the cytosol results in detectable quantities of this unconverted peptide on the cell surface in association with HLA-A*0201. In contrast, this peptide is not observed in cells that express the full-length form of tyrosinase. This suggests that the routes by which the full-length and truncated protein forms are processed are different, and that the full-
length form is not processed after mistranslation in the cytosol.

It is not immediately clear why expression of the unconverted YMNGTM SQV peptide should be a marker of protein translation in the cytosol. It is also unclear why this is the only form detectable on the surface of cells expressing the peptide epitope as a vaccinia minigene, whereas in cells expressing the T3.1 fragment of tyrosinase, both this and the posttranslationally converted epitopes are detected. We believe that the most likely explanation lies in the length and heterogeneity of the epitope-containing fragments produced from these two translation products. The vaccinia minigene product contains the unconverted YMNGTM SQS peptide in “preprocessed” form with the exception of an NH_2-terminal methionine residue that is likely to be rapidly removed by cytosolic aminopeptidases (72). As a result, the epitope-containing peptides that are located in the cytosol and transported into the ER by TAP are of the optimal size to bind to newly synthesized HLA-A^*0201 molecules. Androlewicz has demonstrated TAP-dependent transport of the YMNGTM SQV peptide from the cytosol to the ER, and has further shown that a fraction of these HLA-A^*0201–associated peptides become glycosylated (62). However, we hypothesize that rapid peptide binding prevents further posttranslational conversion of asparagine to aspartic acid by deglycosylation or another mechanism, either because of steric hindrance or because trafficking of class I–peptide complexes does not bring them into contact with all of the enzymes necessary to mediate this process. As a result, the converted form of the peptide is not expressed at the cell surface.

In contrast, the epitope-containing fragments produced after translation and the initial processing of the T3.1 protein could contain an additional COOH-terminal residue and up to 225 additional residues at the NH_2 terminus. Based on the size selectivity of TAP (73), it is likely that only those epitope-containing peptides of 9–16 amino acids will be efficiently transported into the ER. As with the minigene-encoded peptides, those 9mers with the sequence YMNGTM SQV would immediately bind to HLA-A^*0201 and be protected from posttranslational conversion. However, those peptides of 10–16 residues would be unable to bind to HLA-A^*0201 because the dominant motif residues are no longer positioned appropriately with respect to the peptide termini. These peptides would be substrates for enzymes that mediate the conversion of asparagine to aspartic acid. After additional proteolytic processing, they would give rise to the YMNGTM SQV peptides associated with HLA-A^*0201.

Regardless of whether the above hypothesis is correct in all aspects, the absence of the unconverted peptide and the exclusive presence of the posttranslationally converted form in cells expressing full-length tyrosinase indicates that the unconverted 9mer peptide is not produced at all, or at least is not produced in a compartment that allows it to bind to HLA-A^*0201 before conversion. One possibility is that the normal translation of full-length tyrosinase in the ER results in quantitative glycosylation of the asparagine residue in the context of the intact protein, thus precluding the possibility of producing the unconverted peptide. Deglycosylation before, after, or during proteolysis would lead to

| NA8 Mel | DM93 |
|---------|------|
| Control | Lactacystin |
| Mem Cyt Whole | Mem Cyt Whole |
| Mem Cyt Whole | Mem Cyt Whole |

Figure 9. Tyrosinase can be detected in the cytosol of lactacystin treated melanoma. NA8 Mel (lanes 1–6), a tyrosinase-negative melanoma, and DM93 (lanes 7–12), a tyrosinase-expressing melanoma, were left untreated (lanes 1–3, 7–9) or treated (lanes 4–6, 10–12) with 10 μg/ml lactacystin for 5 h, after which time they were separated into membrane (lanes 1, 4, 7, 10) or cytosolic (lanes 2, 5, 8, 11) fractions or used as whole cell lysates (lanes 3, 6, 9, 12) as described in Materials and Methods. Lysate equivalent to 10^6 cells was run in each lane of a 10% SDS-PAGE denaturing gel. The blot from this gel was probed with T311, a tyrosinase specific mAb. These results are representative of four independent experiments.

Figure 10. Deglycosylation of membrane tyrosinase produces a band which comigrates or nearly comigrates with the tyrosinase species found in the cytosol. Lane 1 contains the membrane fraction from 5 × 10^6 cells mock treated with PNGase F. Lanes 2–5 contain equivalent DM93 membrane fractions treated with 1.5 mU PNGase for 2, 4, 8 and 12 h, respectively. Lane 6 contains the cytosolic fraction of 5 × 10^6 DM93 cells treated with lactacystin for 5 h.

The Class I Antigen Processing of a Membrane Protein
conversion of asparagine to aspartic acid, and result in the production of the converted YM**D**GT M SQV peptide. Alternatively, if glycosylation is incomplete, the unconverted N residue may be a site for proteolysis in the ER, resulting in epitope destruction. In this scenario, the production of the unconverted peptide from T3.1 would be a result of incomplete glycosylation or incomplete proteolysis in the ER, presumably as a result of association with HLA-A*0201. Thus, the differential expression of unconverted and converted forms of this tyrosinase-derived peptide is most likely to be due to the site of translation, the extent of glycosylation, the length of the fragments produced during initial proteolytic processing, and protection from further processing by association with HLA-A*0201.

Given the initial translation of full-length tyrosinase in the ER, a second important result of this study is that the expression of the converted peptide epitope in association with HLA-A*0201 is dependent on TAP and the proteasome. Although it was formally possible that this peptide is fully processed in the ER, and that the role of TAP was to enable efficient peptide loading by interaction with an HLA-A*0201–tapasin complex (64), the inhibition of epitope formation by the specific proteasomal inhibitor lactacystin provides strong evidence that processing occurs at least in part in the cytosol. In fact, in the presence of this inhibitor, tyrosinase species of molecular weights that were consistent with a full-length deglycosylated polypeptide chain were detected in the cytosol. These results are consistent with observations in several other laboratories of a reverse translocation mechanism for the export of misfolded membrane proteins from the ER for degradation by the proteasome (37–48). In addition, tyrosinase expressed in amelanotic melanomas was recently shown to be trapped in the ER and then degraded by a lactacystin-dependent pathway (74). Our results do not allow us to completely exclude a mechanism in which tyrosinase is partially degraded by proteasomes in the ER, followed by export of the fragments to the cytosol for processing by the proteasome. Nonetheless, we believe that the most likely mechanism involves the reverse translocation of the full-length protein into the cytosol, accompanied by deglycosylation, and followed by proteasomal processing. Previous studies have provided suggestive evidence for two mechanisms of membrane protein processing that involve partial or complete proteolysis in the ER (11–20), or mistranslation of the protein in the cytosol (21–25, 27–35). The results of this study provide strong evidence for a third pathway that involves reverse translocation of the intact protein from the ER to the cytosol for proteolysis. If, as seems likely, this is a normal pathway for the degradation of misfolded membrane and secreted proteins, then we think it likely that it will also be the major pathway for processing and presentation of the epitopes from these proteins as well.
13. Hammond, S.A., R.C. Bollinger, T.W. Tobeity, and R.F. Siliciano. 1993. Transport-independent processing of HIV-1 envelope protein for recognition by CD8+ T cells. Nature. 364:158–161.

14. Lee, S.P., W.A. Thomas, N.W. Blake, and A.B. Rickinson. 1996. Transporter (TAP)-independent processing of a multiple membrane-spanning protein, the Epstein-Barr virus latent membrane protein 2. Eur. J. Immunol. 26:1875–1883.

15. Hendersen, 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.

16. Hughes, E.A., B. Ottemann, M. Surman, and P. Cresswell. 1996. The protease inhibitor, N-acetyl-l-leucyl-l-leucyl-l-norleucinal, decreases the pool of major histocompatibility complex class I-binding peptides and inhibits peptide trimming in the endoplasmic reticulum. J. Exp. Med. 183:1569–1578.

17. Elliott, T., A. Willis, V. Cernudolo, and A. Townsend. 1995. Processing of major histocompatibility class I–restricted antigens in the endoplasmic reticulum. J. Exp. Med. 181:1481–1491.

18. Back, I., J.H. Cox, R. Anderson, J.W. Yewdell, and J.R. Bennink. 1994. TAP (transporter associated with antigen processing)–independent presentation of endogenously synthesized peptides is enhanced by endoplasmic reticulum insertion sequences located at the amino- but not carboxyl-terminus of the peptide. J. Immunol. 152:381–387.

19. Anderson, K., P. Cresswell, M. Gammon, J. Hermes, A. Wiliamson, and H. Zweerink. 1991. Endogenously synthesized peptide with an endoplasmic reticulum signal sequence sensitizes antigen processing mutant cells to class I–restricted cell-mediated lysis. J. Exp. Med. 174:489–492.

20. Snyder, H.L., J.W. Yewdell, and J.R. Bennink. 1994. Trimming of antigenic peptides in an early secretory compartment. J. Exp. Med. 180:2389–2394.

21. van Binnendijk, R.S., C.A. van Baalen, M.C. Poelen, P. de Vries, J. Boes, V. Cernudolo, A.D. Osterhaus, and F.G. Uyt. 1996. Translocation of HLA-A2.1-specific cytotoxic T cells. Nature. 381:443–446.

22. Scott, D.M., I.E. Ehrmann, P.S. Ellis, E. Simpson, A.I. Aguilnik, C. Bish, and M.J. Mitchell. 1995. Identification of a mouse male-specific transplantation antigen, H-Y. Nature. 376:695–698.

23. Wang, R.F., M.E. Parkhurst, Y. Kawakami, P.F. Robbins and S.A. Rosenberg. 1996. Utilization of an alternative open reading frame of a normal gene in generating a novel human cancer antigen. J. Exp. Med. 183:1131–1140.

24. Bullock, T.N.J., and L.C. Eisenlohr. 1996. Ribosomal scanning past the primary initiation codon as a mechanism for expression of CTL epitopes encoded in alternative reading frames. J. Exp. Med. 184:1319–1329.

25. Leonard, C.K., M.W. Spellman, L. Riddle, R.J. Harris, J.N. Thomas, and T.J. Gregory. 1990. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. J. Biol. Chem. 265:10373–10382.

26. Ferris, R.L., C. Buck, S.A. Hammond, A.S. Woods, R.J. Cotter, M. Takiguchi, Y. Igarashi, Y. Ichikawa, and R.F. Siliciano. 1996. Class I–restricted presentation of an immunodominant cytotoxic T lymphocyte epitope in the signal sequence of a virus protein. J. Exp. Med. 182:1615–1619.

27. Olsen, J., M. Gromme, F. Momburg, G. Hammerling, and J. Neefjes. 1994. Trimming of TAP-translocated peptides in the endoplasmic reticulum and in the cytosol during recycling. J. Exp. Med. 180:1591–1597.

28. Yewdell, J.W., and J.R. Bennink. 1992. Cell biology of antigen processing and presentation to major histocompatibility complex class I molecule–restricted T lymphocytes. Adv. Immunol. 52:1–123.

29. Boon, T., and A. Van Pel. 1989. T cell–recognized antigenic peptides derived from the cellular genome are not protein degradation products but can be generated directly by transcription and translation of short subgenomic regions. A hypothesis [see comments]. Immunogenetics. 29:75–79.

30. Sibille, C., P. Chomez, C. Wildmann, A. Van Pel, E. De Plaen, J.L. Maryanski, V. de Bergeyck, and T. Boon. 1990. Structure of the gene of tumour transplantation antigen P91A: a point mutation generates a new antigenic peptide. J. Exp. Med. 172:35–45.

31. Chomez, P., E. De Plaen, A. Van Pel, C. Smet, J.P. Sizi-kora, C. Lurquin, A.M. Lebacq-Verheyden, and T. Boon. 1992. Efficient expression of tumour antigen P91A by transfected subgenomic fragments. Immunogenetic. 35:241–252.

32. Scott, D.M., I.E. Ehrmann, P.S. Ellis, E. Simpson, A.I. Aguilnik, C. Bish, and M.J. Mitchell. 1995. Identification of a mouse male-specific transplantation antigen, H-Y. Nature. 376:695–698.

33. Wang, R.F., M.E. Parkhurst, Y. Kawakami, P.F. Robbins and S.A. Rosenberg. 1996. Utilization of an alternative open reading frame of a normal gene in generating a novel human cancer antigen. J. Exp. Med. 183:1131–1140.

34. Bullock, T.N.J., and L.C. Eisenlohr. 1996. Ribosomal scanning past the primary initiation codon as a mechanism for expression of CTL epitopes encoded in alternative reading frames. J. Exp. Med. 184:1319–1329.

35. Leonard, C.K., M.W. Spellman, L. Riddle, R.J. Harris, J.N. Thomas, and T.J. Gregory. 1990. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. J. Biol. Chem. 265:10373–10382.

36. Ferris, R.L., C. Buck, S.A. Hammond, A.S. Woods, R.J. Cotter, M. Takiguchi, Y. Igarashi, Y. Ichikawa, and R.F. Siliciano. 1996. Class I–restricted presentation of an immunodominant cytotoxic T lymphocyte epitope in the signal sequence of a virus protein. J. Exp. Med. 182:1615–1619.

37. Olsen, J., M. Gromme, F. Momburg, G. Hammerling, and J. Neefjes. 1994. Trimming of TAP-translocated peptides in the endoplasmic reticulum and in the cytosol during recycling. J. Exp. Med. 180:1591–1597.

38. Yewdell, J.W., and J.R. Bennink. 1992. Cell biology of antigen processing and presentation to major histocompatibility complex class I molecule–restricted T lymphocytes. Adv. Immunol. 52:1–123.

39. Boon, T., and A. Van Pel. 1989. T cell–recognized antigenic peptides derived from the cellular genome are not protein degradation products but can be generated directly by transcription and translation of short subgenomic regions. A hypothesis [see comments]. Immunogenetics. 29:75–79.

40. Sibille, C., P. Chomez, C. Wildmann, A. Van Pel, E. De Plaen, J.L. Maryanski, V. de Bergeyck, and T. Boon. 1990. Structure of the gene of tumour transplantation antigen P91A: a point mutation generates a new antigenic peptide. J. Exp. Med. 172:35–45.

41. Chomez, P., E. De Plaen, A. Van Pel, C. Smet, J.P. Sizi-kora, C. Lurquin, A.M. Lebacq-Verheyden, and T. Boon. 1992. Efficient expression of tumour antigen P91A by transfected subgenomic fragments. Immunogenetic. 35:241–252.

42. Scott, D.M., I.E. Ehrmann, P.S. Ellis, E. Simpson, A.I. Aguilnik, C. Bish, and M.J. Mitchell. 1995. Identification of a mouse male-specific transplantation antigen, H-Y. Nature. 376:695–698.
ubiquitin-proteasome pathway. Science. 273:1725–1728.

Jensen, T.J., M.A. Loo, S. Pind, D.B. Williams, A.L. Goldberg, and J.R. Riordan. 1995. M multipe proteolytic systems, including the proteasome, contribute to CFTR processing. Cell. 83:129–135.

Ward, C.L., S. O’mura, and R.R. Kopito. 1995. Degradation of CFTR by the ubiquitin-proteasome pathway. Cell. 83:121–127.

Hughes, E.A., C. Hammond, and P. Cresswell. 1997. Misfolded major histocompatibility complex class I heavy chains are translocated into the cytoplasm and degraded by the proteasome. Proc. Natl. Acad. Sci. U.S.A. 94:1896–1901.

Hupp, J.B., and H.L. Ploegh. 1997. The alpha chain of the T cell antigen receptor is degraded in the cytosol. Immunity. 7:113–122.

Kopito, R.R. 1997. ER quality control: the cytoplasmic con-

47 Mosse et al.

55. Mackett, M., G.L. Smith, and B. Moss. 1984. General method for product on and selection of infectious vaccinia virus recombinants expressing foreign genes. J. Virol. 49:857–

64. Sadasivan, B., P.J. Lehner, B. Ortmann, T. Spies, and P.

69. Jimenez, M., W.L. Maloy, and V.J. Hearing. 1989. Specific immunodominant influenza hemagglutinin site by cytotoxic T lymphocyte. Recognition of an

65. Lee, S.P., W.A. Thomas, N.W. Blake, and A.B. Rickinson. 1994. Inhibitors of the proteasome block the degradation of most cell proteins and the generation of peptides presented on MHC class I molecules. Cell 78:761–771.

66. Sadasivan, B., P.J. Lehner, B. Ortmann, T. Spies, and P.

70. 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.

71. Bouchard, B., B.B. Fuller, S. Vijayasaradhi, and A.N. Hough-
ton. 1989. Induction of pigmentation in mouse fibroblasts by expression of human tyrosinase cDNA. J. Exp. Med. 169: 2029–2042.

72. Arfin, S.M., R.L. Kendall, L. Hall, L.H. Weaver, A.E. Stewart, B.W. Matthews, and R.A. Bradshaw. 1995. Eukaryotic methionyl aminopeptidases: two classes of cobalt-dependent enzymes. Proc. Natl. Acad. Sci. USA. 92:7714–7718.

73. Schumacher, T.N., D.V. Kantesaria, M.T. Heemels, P.G. Ashton-Rickardt, J.C. Shepherd, K. Fruh, Y. Yang, P.A. Peterson, S. Tonegawa, and H.L. Ploegh. 1994. Peptide length and sequence specificity of the mouse TAP1/TAP2 translocator. J. Exp. Med. 179:533–540.

74. Halaban, R., E. Cheng, Y. Zhang, G. Moellmann, D. Hanlon, M. Michalak, V. Setaluri, and D.N. Hebert. 1997. Aberrant retention of tyrosinase in the endoplasmic reticulum mediates accelerated degradation of the enzyme and contributes to the dedifferentiated phenotype of amelanotic melanoma cells. Proc. Natl. Acad. Sci. USA. 94:6210–6215.