Subcellular Localization of the Melanoma-associated Protein Melan-A<sup>MART-1</sup> Influences the Processing of Its HLA-A2-restricted Epitope*  

Donata Rimoldi†, Katja Muehlethaler, Suzanne Salvi, Danila Valmori‡, Pedro Romero§, Jean-Charles Cerottini, and Frédéric Lévy¶

From the Ludwig Institute for Cancer Research, Lausanne Branch, University of Lausanne, CH-1066 Epalinges, and the Division of Clinical Onco-Immunology, Ludwig Institute for Cancer Research, University Hospital, CH-1011 Lausanne, Switzerland

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The peptide derived from the melanoma-associated protein Melan-A (Melan-A<sub>26–35</sub>/HLA-A2) is an attractive candidate for tumor immunotherapy but little is known about the intracellular processing of this antigen. Here we show that Melan-A is a single-pass membrane protein with an N terminal exposed to the lumen of the exocytic compartment. In transfected melanoma cells, Melan-A accumulates in the Golgi region. Inversion of the membrane topology leads to the retention of Melan-A in the endoplasmic reticulum. Most strikingly, melanoma cells expressing this form of Melan-A are more effectively recognized by specific CTL than those expressing either Melan-A in its native membrane orientation or Melan-A artificially localized in the cytosol. Our data are compatible with the notion that proteins retained in the endoplasmic reticulum are more efficiently degraded and produce more antigenic peptides.

One promising development in designing effective anti-melanoma immunotherapy is the identification and characterization of melanoma-specific cytolytic T lymphocytes (CTL).<sup>1</sup> These cells recognize short peptides derived from melanoma-associated proteins and presented by HLA class I molecules. Several CTL-defined peptide melanoma antigens have been molecularly identified. They derive from proteins localized in specific cellular organelles, as may be the case for melanocytic lineage (both transformed, i.e. melanoma, and normal cells). They therefore constitute lineage specific tumor antigens.

Melan-A<sup>MART-1</sup> (called hereafter Melan-A) expression is restricted to normal and transformed melanocytes and retinal pigment epithelium, and thus also belongs to the family of melanocyte lineage-specific proteins (6). Melan-A is a relatively small polypeptide of 118 amino acids, with an apparent molecular mass of 22–24 kDa and is devoid of Asn-linked glycosylation sites. The sequence contains a putative transmembrane domain (residues 27–48) and subcellular fractionation analysis has suggested that Melan-A is present in melanosomes and the endoplasmic reticulum (ER) (7). The cDNA coding for Melan-A was originally isolated by functional screening of expression libraries using melanoma-specific CTL (6, 8). In contrast to the known HLA-A2-restricted antigenic peptides derived from tyrosinase, TRP-1 and gp100, which are located in the extracytosolic domain of these proteins, the immunodominant Melan-A CTL-defined epitope (Melan-A<sub>26–35</sub>) is embedded within the putative transmembrane domain, except for the first amino acid (Glu<sup>26</sup>).

The generation of HLA class I-restricted antigenic peptides, including peptide tumor antigens, usually takes place in the cytosol and involves degradation of the proteins by the proteasome and further trimming by other peptidases (9). A fraction of these peptides is transported into the ER via the TAP1/2 transporters associated with antigen processing complex where the binding to HLA class I molecules occurs. Recent evidence indicates that proteins destined to the exocytic pathway or located in specific cellular organelles, as may be the case for Melan-A and other melanosomal proteins, gain access to the processing machinery through a retrograde transport mechanism from the ER to the cytosol (10). According to this mechanism, proteins misfolded in the ER would be transported back to the cytosol via the translocation pore complex (Sec61 complex) and subsequently degraded by the proteasome (11, 12). Several examples of cellular and viral proteins degraded via this ER degradation mechanism exist (10). Notably, retrograde transport and subsequent degradation has been shown to be involved in the processing of tyrosinase<sub>369–377</sub>/HLA-A2 (13). An elevated amount of misfolded, ER-retained membrane proteins containing CTL-defined epitopes may therefore result in a more efficient generation and presentation of such epitopes at the surface of cells.

In the present study, we have characterized the membrane topology of Melan-A. In the course of this analysis, we noticed that a small fraction of Melan-A was inserted into the membrane in an inverted orientation. Moreover, the subcellular...
localization of Melan-A was dramatically altered, when inserted in the inverted orientation. Whereas the natural Melan-A protein co-localized with Golgi markers in transfected melanoma cells, the protein with an inverted membrane topology exclusively co-localized with ER markers. We therefore investigated whether the altered subcellular localization of Melan-A influenced the presentation of Melan-A\(_{\text{NVI}}\)/HLA-A2 in melanoma cells. The results indicated that cells expressing the mislocalized Melan-A were more efficiently recognized by the COOH-terminal glycosylation site, the sequence AMMNVTGS was one-letter code, was inserted between residue 6 and 7 of Melan-A. For immunofluorescence detection, the following second antibodies were used: Alexa-Fluor 488 goat anti-mouse IgG antibody (Molecular Probes), Cy3-conjugated donkey anti-rabbit IgG antibody, Cy3-conjugated streptavidin, and Cy3-conjugated sheep anti-mouse IgG antibody (all from Jackson ImmunoResearch Laboratories).

**EXPERIMENTAL PROCEDURES**

**Construction of Plasmids**—All DNA constructs used in this study were based on the plasmid pGFP/Ub described earlier (14) and were inserted in-frame to the 3’ end of the ubiquitin sequence (except for the constructs described in Fig. 2, see below). Constructs I and II have been described previously (15). Construct III was obtained by 2 rounds of PCR amplification. First, oligonucleotides carrying the appropriate mutations (i.e. codons for three Arg replacing Glu\(^{59}\), and for three Glu replacing Arg\(^{49-51}\)) were used to amplify Melan-A fragments coding for amino acids 1-44 and 38-118, respectively, using construct I as template. The fragments were purified using the PCR purification kit (Qiagen AG, Basel, Switzerland). A second round of PCR using the oligonucleotides matching the 5’ and 3’ end of the Melan-A coding sequence and containing appropriate restriction sites was performed on the fragments obtained from the first round. The latter amplification was digested with SacII and AccI and introduced into the SacII-AccI site of pGFP/Ub plasmid. Construct IV was generated by inserting an extra 9 base pairs coding for 3 Lys residues between codons 38 and 39 of COOH-terminal glycosylated construct II (see below), using the QuickChange kit (Stratagene) and following the manufacturer’s protocol. Constructs VI and VII were obtained by introducing a termination codon at the 3’ end of codon 35 of construct II and IV, respectively. This was achieved by using the QuickChange kit (Stratagene) and following the manufacturer’s protocol. All sequences were confirmed by DNA sequencing.

The glycosylated versions of Melan-A shown in Fig. 2 were produced by replacing the GFP/Ub ORF with the one coding for Melan-A. In these plasmids, the Melan-A sequence was cloned into the NheI-AvaI sites. The rationale for this was that the role of the GFP/Ub moiety in the targeting of Melan-A to the ER was unknown. Later experiments showed that glycosylation did not affect the targeting of the N-terminus of Melan-A (Stratagene) and following the manufacturer’s protocol. All sequences were confirmed by DNA sequencing.

The COOH-terminal glycosylated versions of constructs II and IV shown in Fig. 3 were obtained by PCR, introducing the sequence within the oligonucleotide used for the PCR reaction. In these constructs the Melan-A sequence was extended at the COOH terminus by the amino acid sequence, in one-letter code, AMMNVTGS.

**Cell Culture**—Human embryonic kidney cells 293 were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum. NA8-MEL and SK-Mel-37 melanoma cells (a gift from F. Jotereau, Nantes, France, and E. Stockert, Ludwig Institute for Cancer Research, New York Branch, respectively) were cultured in RPMI 1640, 10% fetal calf serum medium. These cells do not express Melan-A protein, as assessed by Western blot analysis, although SK-Mel-37 cells express low levels of Melan-A mRNA as detected by RT-PCR (not shown). Both cell lines express HLA-A2. For transient transfections, cells were plated 1 day in advance and transfected either with LipofectAMINE (Life Technologies, Inc.) (Figs. 2, 3, and 6A) or with FuGENE reagent (Roche Molecular Biochemicals), according to the manufacturer’s instructions. The Melan-A-specific CTL used in this study, clone 17, were derived from TILs of patient LAU 203 by limiting dilution as described (16).

**Antibodies**—The anti-Melan-A A103 mouse monoclonal antibody was a gift from E. Stockert (17). This antibody recognizes an epitope located at the COOH-terminal end of the protein (amino acids 100-110), as revealed by competitive enzyme-linked immunosorbent assay using peptides synthesized according to the predicted Melan-A sequence. For double immunofluorescence experiments, A103 was used in a biotinylated form. Anti-calnexin rabbit polyclonal antibody was a gift from R. F. Pettersson, Ludwig Institute for Cancer Research, Sweden. Melan-A monoclonal GI/53, specific for ERGIC-53, was a gift from H.-P. Hauri (Basel, Switzerland) (18). Anti-golgin 97 (mouse monoclonal 4F4, 150 ng/ml) was from Boehringer Mannheim, Germany. Anti-HA monoclonal antibody was from Berkeley Antibodies Co. For immunofluorescence detection, the following second antibodies were used: Alexa-Fluor 488 goat anti-mouse IgG antibody (Molecular Probes), Cy3-conjugated donkey anti-rabbit IgG antibody, Cy3-conjugated streptavidin, and Cy3-conjugated sheep anti-mouse IgG antibody (all from Jackson ImmunoResearch Laboratories).

**Immunofluorescence**—Cells were plated in 4-well glass chamber slides (Nunc, Life Technologies) pre-coated with 5 µg/ml fibronectin (Sigma) to enhance cell adherence. Twenty-four to 48 h after transfection, cells were rinsed with serum-free medium and fixed in acetone for 5 min at −20 °C. Slides were incubated with 0.5% bovine serum albumin/phosphate-buffered saline for 30 min at room temperature, then incubated with primary antibodies diluted in 0.2% bovine serum albumin, 0.1% human serum in phosphate-buffered saline for 1 h at room temperature. After washing in 0.5% bovine serum albumin/phosphate-buffered saline, fluorescent secondary antibodies were applied for 30 min at room temperature. For double labelings with two mouse antibodies, anti-Melan-A was used in a biotinylated form. In this case, incubation with unlabeled primary antibody followed by the respective secondary antibody before staining with A103 was followed by incubation with the streptavidin conjugate. At the end, slides were mounted with 24 mg/ml 1,4-diazabicyclo-2,2-octane in phosphate-buffered saline/glycerol (pH 7.5). Confocal analysis was performed with a Zeiss Axiovert 100 microscope (Zeiss Laser Scanning Microscope 410) with a x83 Plan Apochromat objective (1.4 oil). Control cells stained with single antibodies and secondary reagents only were routinely performed. For CTL assays, transfection efficiency was as-
sessed in parallel by immunofluorescence with either the anti-Melan-A or anti-HA antibodies.

**CTL Recognition**—Recognition of transiently transfected melanoma cells by anti-Melan-A-specific CTL was performed by measuring the release of IFN-γ. Approximately 24 h after transfection, CTL were added (at a ratio of 10:1) in CTL medium. Medium was collected 24 h later and aliquots analyzed for IFN-γ content by enzyme-linked immunosorbent assay (BIOSOURCE Europe, Fleurus, Belgium). As positive control, mock-transfected melanoma cells incubated with 1 μm antigenic peptide were included in each assay. For the recognition of peptide-pulsed NA8-MEL, cells were plated in fibronectin-coated 96-well plates (2.5 × 10⁴ cells/well). The following day, cells were incubated in the presence of the indicated concentrations of peptide and 3 μg/ml human β₂-microglobulin for 1 h at 37 °C, rinsed, and incubated for 24 h with CTL (10:1 ratio) before collection of medium for IFN-γ assay. To inhibit proteasome activity, lactacystin (BIOMOL, Plymouth Meeting, PA) was added to melanoma cells at 50 μM 6 h after starting transfection. Cells were incubated with the drug overnight and washed once before addition of CTL.

**RESULTS**

**Melan-A Is a Signal-anchor Membrane Protein with a Cytoplasmic COOH Terminus**—Analysis of the primary sequence of the melanoma-associated protein Melan-A revealed the presence of a hydrophobic amino acid stretch between residues 27 and 48, flanked, on the NH₂-terminal side, by two acidic amino acids and, on the COOH-terminal side, by three basic amino acids (Fig. 1, construct I). This sequence arrangement suggested that Melan-A may be a type III signal-anchor protein. Type III proteins are characterized by the presence of a hydrophobic domain, which functions simultaneously as an ER targeting sequence and as a transmembrane domain, and by a cytosolically exposed COOH terminus (20). To test this hypothesis, we performed two sets of experiments. First, we confirmed that Melan-A is inserted into membranes. Human embryonic kidney (HEK 293) cells were transfected with the UPR-based plasmid encoding GFPha-Ub-Melan-A (Fig. 1, construct I). As previously described, this plasmid allows the production of equimolar amounts of two physically distinct proteins, GFPha-Ub and Melan-A, from a single mRNA (14, 21). Transfected cells were metabolically labeled, lysed in a buffer containing Triton X-114, and the lysates separated into a detergent-rich fraction (enriched in membrane-embedded proteins) and a detergent-poor fraction (enriched in water-soluble proteins) (19). The presence of Melan-A and GFPha-Ub in these fractions was monitored by immunoprecipitation using anti-Melan-A and anti-HA tag monoclonal antibodies, respectively. In accordance with previously published data (7), Melan-A was enriched in the detergent-rich fraction (Fig. 2A) while the soluble GFPha-Ub was found exclusively in the detergent-poor fraction. Melan-A had an identical distribution in a melanoma cell line naturally expressing Melan-A (data not shown).

Second, we determined the membrane orientation of Melan-A by artificially introducing an Asn-linked glycosylation site either at the extreme NH₂ or COOH terminus of the protein (see “Experimental Procedures” for details). Asn-linked oligosaccharides are exclusively added to polypeptide chains whose glycosylation sites are exposed to the lumen of the ER. Expression of these modified Melan-A proteins in HEK 293 cells demonstrated that Melan-A carrying an NH₂-terminal glycosylation site was glycosylated, whereas Melan-A carrying a COOH-terminal glycosylation site was not (Fig. 2B). The Asn-linked glycan could be removed by endoglycosidase H (Endo H) treatment.

Close analysis of the autoradiography led us to detect a radioactive band corresponding to the glycosylated form of Melan-A in cells expressing Melan-A with the COOH-terminal glycosylation site (Fig. 2B, lane I). This band disappeared after Endo H treatment and was not observed in the absence of a complete glycosylation signal (Melan-A-NVV). Reciprocally, a band corresponding to the unglycosylated form of Melan-A was detected in cells expressing Melan-A with an NH₂-terminal glycosylation site (Fig. 2B, lane 5). These results suggested that the translocation process may lead to the integration of a fraction of the Melan-A polypeptide in a “wrong” orientation. We therefore studied the biological consequences of such an inverted membrane integration by favoring this process. To this end and based on reports that membrane topology depends in 5% of the charge distribution of the amino acids neighboring the transmembrane domain (22), we undertook to invert this charge distribution.

**Charge Inversion of the Amino Acids Bordering the Hydrophobic Amino Acid Stretch of Melan-A Leads to an Inversion of Its Membrane Topology**—The cDNA encoding Melan-A was mutated so as to replace Glu²⁶ by three consecutive Arg and the
FIG. 2. Localization and membrane topology of Melan-A. A, HEK 293 cells were transiently transfected with the UPR-based construct I, labeled for 20 min with [35S]Met/Cys and lysed in buffer containing 1% Triton X-114 at 4 °C. The detergent-rich fraction containing membrane-embedded proteins was separated from the detergent-poor fraction by repeated cycles of cooling and warming (see “Experimental Procedures” for details). Each fraction was subsequently immunoprecipitated with a mixture of saturating amounts of anti-HA tag and anti-Melan-A monoclonal antibodies, separated by SDS-12% PAGE and exposed for autoradiography. The positions of GFPα-UB and Melan-A are indicated by a gray and a black arrow, respectively. As control, mock-transfected cells were analyzed under identical conditions. B, to monitor the topology of Melan-A within the membrane, Asn-glycosylation sites were artificially introduced either at the extreme NH2 terminus (NVT-Melan-A) or COOH terminus (Melan-A-NVT) of construct I. As control an incomplete glycosylation sequence was inserted at the extreme COOH terminus (Melan-A-NVV). Cells were transiently transfected with each construct, labeled as above, and lysed in buffer containing 1% Triton X-100. Solubilized material of each transfectant was split into two equal parts and subjected to immunoprecipitation with the monoclonal anti-Melan-A antibody. One aliquot of each precipitate was digested with Endo H before separation on SDS-12% PAGE (lanes 2, 4, and 6). The other aliquot was incubated in the same conditions save for the absence of Endo H (lanes 1, 3, and 5). The position of glycosylated Melan-A is marked by a black arrow and the one of unglycosylated Melan-A by a gray arrow. Note the Endo H-sensitive band in lane 1, which migrates at the same position as glycosylated NVT-Melan-A (lane 5).

three Arg (Arg19–21) of the original sequence by three Glu (Fig. 1, constructs III and IV). According to the charge distribution, this should result in a protein with reversed membrane topology, i.e. a cytosolic NH2 terminus. To test this prediction, construct IV was engineered so as to carry Asn glycosylation sites at the COOH terminus. As predicted, the mutated protein was efficiently glycosylated (Fig. 3), confirming the exposure of the COOH terminus to the ER lumen. In contrast, construct II carrying a COOH-terminal Asn glycosylation site remained unglycosylated. Note that, as in Fig. 2B, the autoradiogram revealed a band corresponding to the size of glycosylated construct II. Hence, constructs III and IV encoding a variant of Melan-A will be termed inverted Melan-A (Melan-Ainv), as opposed to Melan-A in the wild-type orientation (Melan-Awt).

We then asked whether the inversion of membrane topology affects the subcellular localization of Melan-A. To this end, plasmids encoding the wild-type or mutated forms of Melan-A were transiently transfected into Melan-A-negative amelanotic SK-Mel-37 melanoma cells. These cells were selected because of their spreading properties that facilitate the discrimination of cytoplasmic organelles. Cells were then analyzed by immunofluorescence with an anti-Melan-A monoclonal antibody and by confocal microscopy (Fig. 4). Melan-Awt was mainly located in a juxtanuclear area, reminiscent of the Golgi region, and, to a lesser extent, to punctate structures in the cytoplasm (Fig. 4, a and b). Accumulation of Melan-Ainv in the Golgi complex was confirmed by double immunofluorescence analysis with antibodies to an ER-Golgi intermediate compartment (ERGIC) marker (p53 and a Golgi marker (Fig. 4, c and e, and not shown) (23). Little co-localization with the ER marker calnexin was observed (Fig. 4, d and f). A more detailed analysis of Melan-A localization in melanotic melanoma cells will be reported elsewhere. In contrast, Melan-Ainv was absent from the Golgi area and present almost exclusively in a reticular network, with a staining pattern similar to that of the ER marker calnexin (Fig. 4, g–l). Similar localization of Melan-Ainv and Melan-Awt was obtained upon transfection of another Melan-A negative cell line, NA8-MEL (not shown). Furthermore, the differential localization of Melan-Ainv and Melan-Awt was not affected by the presence of artificial glycosylation signals such as those described above nor by mutations introduced within the transmembrane region to create an enhanced CTL epitope (see below). Thus, Melan-Ainv was retained in the ER, either because of a putative transport signal being inaccessible or, more likely, because of misfolding of the protein.

Processing Efficiency of Melan-A Is Affected by Its Membrane Topology—We next asked whether the change in Melan-A localization would affect cell recognition by specific CTL. The Melan-A protein contains an immunodominant HLA-A2-restricted epitope (EAAGIGILTV, residues 26–35) (6, 24). We have previously shown that mutations in this sequence, in particular A27L, lead to the production of epitopes that are better recognized by specific CTL and induce stronger CTL responses (15, 25). In this series of experiments we used both UPR-based constructs encoding Melan-A with wild-type sequence, i.e. Melan-Awt and Melan-Ainv (Fig. 1, constructs I and III), as well as constructs encoding the enhanced Melan-A epitope, i.e. Melan-A27L and Melan-Ainv27L (Fig. 1, constructs II and IV). Using a similar transient expression system, we have previously shown that cells expressing Melan-A27L efficiently induce TNF release by Melan-A specific CTL, while cells expressing the wild-type epitope hardly induce TNF release (15). HLA-A2+ Melan-A negative NA8-MEL melanoma cells were transiently transfected with the different cDNAs and tested for
Fig. 4. Melan-A<sub>wt</sub> and Melan-A<sub>inv</sub> have different cellular localization. SK-Mel-37 cells were transiently transfected with plasmids encoding Melan-A<sub>wt</sub> and Melan-A<sub>inv</sub>. Double immunofluorescence analysis of Melan-A/ERGIC-53 and Melan-A/calnexin followed by confocal microscopy was performed as described under “Experimental Procedures.” Single plane confocal images were collected for: a, b, g, and h, Melan-A (green); c and i, ERGIC-53 (red); d and j, calnexin (red). Images e, f, k, and l show the computer-generated overlap of red and green images. Yellow indicates overlapping of signals. The inset in h, j, and l shows an enlarged detail of the cytoplasmic staining.

Fig. 5. Cells expressing Melan-A<sub>inv</sub> protein are better recognized by Melan-A-specific CTL. A and B, HLA-A2−NA8-MEL cells were transiently transfected with the indicated constructs and tested for recognition by a Melan-A-specific CTL clone using a 24-h IFN-γ release assay, as described under “Experimental Procedures.” C− and C+ indicate mock-transfected NA8-MEL cells incubated with CTL in the absence or presence of the enhanced antigenic peptide (ELAGIGILTV, 1 μM), respectively. These experiments are representative of 10 (A) and 3 (B) independent experiments performed. C, the localization of constructs II (lanes 1 and 2) and V (lanes 3 and 4) was assessed by lysing transfected cells as in Fig. 2A. Samples from the detergent-rich (D) and detergent-poor (S) fractions were immunoprecipitated with the monoclonal anti-Melan-A antibody and separated by SDS-12% PAGE. The detection of Melan-A<sub>inv</sub> in the detergent-poor fraction (S) and the absence of a glycosylated species confirms the cytosolic localization of construct V. D, recognition of NA8-MEL cells pulsed with the indicated concentrations of antigenic peptide (ELAGIGILTV) by the Melan-A-specific CTL clone.

...due to the presence of Arg residues immediately preceding the epitope, we engineered two NH<sub>2</sub>-terminal extended minigenes, encompassing amino acids 1–35, and containing the amino acid sequence of Melan-A<sub>A27L</sub> and Melan-A<sub>inv</sub>, respectively (Fig. 1, constructs VI and VII). As shown in Fig. 5A, the recognition of cells expressing either of these constructs was similar. Thus, the more efficient recognition of cells expressing Melan-A<sub>inv</sub> cannot be ascribed to the different amino acids immediately preceding the NH<sub>2</sub> terminus of the CTL-defined epitope.

We also considered the possibility that Melan-A<sub>A27L</sub> may not be inserted into the membrane as efficiently as Melan-A<sub>inv</sub> and a fraction of the protein may remain in the cytosol, leading to a more easily processed form. Therefore, we tested whether the recognition of cells transfected by a plasmid coding for a soluble, cytosolic form of Melan-A, Melan-A<sub>A27L</sub>sol (Fig. 1, construct V), was more efficient than with either Melan-A<sub>A27L</sub> or Melan-A<sub>inv</sub>. Melan-A<sub>inv</sub> contains 3 Lys residues within the transmembrane domain, downstream of the enhanced Melan-A<sub>26−35</sub> epitope. As shown in Fig. 5B, the CTL recognition of melanoma cells expressing Melan-A<sub>A27L</sub> was only moderately higher than that of cells expressing Melan-A<sub>inv</sub> and lower than that of cells expressing Melan-A<sub>inv</sub>sol. Cytosolic localization of Melan-A<sub>inv</sub> was confirmed both by its presence in Triton X-114 soluble fractions and by the absence of glycosylation, indicating the lack of ER translocation of Melan-A<sub>A27L</sub> (Fig. 5C). (Melan-A<sub>A27L</sub> was engineered to contain a COOH-terminal Asn glycosylation site, akin to the construct used in Fig. 2B.) We conclude from these experiments that the more efficient recognition of cells expressing Melan-A<sub>inv</sub> can neither be explained by the amino acid modifications upstream of the CTL-defined epitope nor by the possible presence of untranslated protein within the cytosol.

It is difficult to precisely quantify how much more efficient the presentation of the antigenic peptide from the inverted protein is compared with that of the normal form. However, in an attempt to estimate the extent of this effect, we investigated the correlation between cytokine release and antigenic peptide concentration. Using the same CTL clone as the one described above and NA8-MEL cells pulsed with different concentrations of antigenic peptide (Melan-A<sub>A27L</sub>), we could show that a 4-fold increase in cytokine release corresponded to a 10–100-fold increase in peptide amount (Fig. 5D).

Melan-A<sub>inv</sub> Is Degraded More Rapidly Than Melan-A<sub>wt</sub> and the Presentation of Melan-A<sub>26−35</sub> Depends on the Activities of the Proteasome—Since the cells expressing Melan-A<sub>inv</sub> were...
presentation of peptides derived from both Melan-A<sup>inv</sup> and degradation represents an essential proteolytic pathway for the added antigenic peptide. Consequently, proteasome-dependent effect on the recognition of tumor cells presenting exogenously added antigenic peptide. The role of proteasome in the recognition of their antigenic peptide by specific CTL, as shown in IFN-γ release assays (Fig. 6B). However, lactacystin treatment had no significant effect on the recognition of tumor cells presenting exogenously added peptide. Consequently, proteasome-dependent degradation represents an essential proteolytic pathway for the presentation of peptides derived from both Melan-A<sup>inv</sup> and Melan-A<sup>wt</sup>, irrespective of the protein localization and membrane topology. Moreover, the rate of degradation seems to have a direct impact on the efficiency by which an antigen presenting cell will be recognized by specific CTL.

**DISCUSSION**

The major finding reported in this work is that the subcellular localization of the melanoma-associated protein Melan-A affects the efficiency by which melanoma cells are recognized by Melan-A<sub>26-35</sub>-specific CTL. Immunofluorescence analysis of cells expressing various Melan-A constructs revealed that the inversion of membrane topology resulted in the relocalization of the Melan-A protein from a predominant Golgi/post-Golgi area to the ER. Therefore, we postulate that the enhanced recognition of cells expressing Melan-A<sub>inv</sub> is mainly caused by its retention in the ER.

We have demonstrated that Melan-A is a bona fide type III membrane protein (20). It contains a signal-anchor domain flanked at the NH<sub>2</sub>-terminus by 2 acidic amino acids and at the COOH terminus by 3 basic amino acids. In agreement with the distribution of the charged amino acids around the transmembrane domain, we have shown that the COOH terminus is exposed to the cytosol and the NH<sub>2</sub>-terminal domain is facing the lumen of the exocytic compartment (22). The rules that determine the orientation of type III proteins in the membrane are not well characterized. It appears that the folding state of the NH<sub>2</sub>-terminal domain (preceding the transmembrane domain), the net charge difference between the amino acids bordering the membrane-spanning domain, and the amino acid composition and length of the transmembrane domain can influence the final membrane topology of type III polypeptides (20). It has been shown that small reduction in the length of the hydrophobic amino acid stretch of the microsomal epoxide hydrolase, a type III protein, resulted in a fraction of the polypeptide inserting in the opposite membrane topology to that of the wild-type protein (29). In another study, the substitution of the natural amino acids forming the transmembrane domain of an NH<sub>2</sub>-terminal truncated asialoglycoprotein receptor subunit I by a stretch of 19 Leu residues again resulted in a mixture of correctly and incorrectly oriented polypeptides (30). Based on these reports and on our results (Fig. 2), it is possible that a small fraction of newly synthesized Melan-A does not translocate properly and is therefore inserted in the wrong orientation. Such a phenomenon may have gone undetected in cells constitutively expressing Melan-A because the protein does not contain any natural Asn-glycosylation site.

In cells of the melanocytic lineage, the precise subcellular localization of Melan-A has not been reported yet. Although Melan-A has been shown to be enriched in melanosomes by membrane fractionation techniques (7), analysis of pigmented melanoma cells by immunofluorescence and electron microscopy indicated that the protein is mainly found in the Golgi/trans-Golgi network area and only a fraction of the protein co-localizes with melanosomal markers. In transfected cells, Melan-A<sub>inv</sub> was detected by immunofluorescence in a juxtanuclear area of the cell, suggesting accumulation in the Golgi area (Fig. 4 and data not shown). Thus, the subcellular localization of Melan-A transiently expressed in amelanotic melanoma cells appears similar to the naturally expressed protein. In contrast, Melan-A<sub>inv</sub> had a strikingly different subcellular localization and was almost exclusively located in the ER. The cause for the ER retention is not known. It is possible that a cytosolic sorting signal may become inaccessible due to its different membrane topology. More likely, the inverted membrane topology results in the misfolding and ER retention of the protein.

**Fig. 6. Degradation kinetics of Melan-A<sub>wt</sub> and Melan-A<sub>inv</sub> and the role of proteasome in the recognition of their antigenic peptide.** A, HEK 293 cells were transiently transfected with constructs II (filled diamonds) and IV (open squares), pulsed for 15 min with [35S]methionine/cysteine for 15 min and chased for 60, 120, and 240 min. After lysis, proteins were immunoprecipitated with the monoclonal anti-Melan-A and separated by SDS-12% PAGE. The radioactive bands corresponding to Melan-A were quantified by PhosphorImager and expressed as percentage of Melan-A present at the end of the pulse. B, NA8-MEL cells were transiently transfected with the same plasmids, incubated with lactacystin for 16 h before addition of Melan-A<sub>specific</sub> CTL. Control melanoma cells (C+) treated with lactacystin and incubated with CTL in the presence of antigenic peptide (ELAGIGILTV, 1 μM) were used to monitor the toxicity of lactacystin.

Several misfolded membrane-embedded proteins have been shown to be degraded by the proteasome (26). Previous reports have demonstrated that purified proteasome is able to produce the antigenic peptide Melan-A<sub>26-35</sub> from an extended precursor peptide and that presentation of the epitope is drastically reduced upon proteasome inhibition (27, 28). The involvement of the proteasome activity in the intracellular processing of Melan-A<sub>inv</sub> was therefore assessed. Addition of the proteasome inhibitor lactacystin to NA8-MEL melanoma cells transiently transfected with plasmids encoding either Melan-A<sub>27L</sub> or Melan-A<sub>27L</sub> (Fig. 1, constructs II and IV, respectively) dramatically reduced the recognition by specific CTL, as shown in IFN-γ release assays (Fig. 6B). However, lactacystin treatment had no significant effect on the recognition of tumor cells presenting exogenously added antigenic peptide. Consequently, proteasome-dependent degradation represents an essential proteolytic pathway for the presentation of peptides derived from both Melan-A<sub>inv</sub> and Melan-A<sub>wt</sub>, irrespective of the protein localization and membrane topology. Moreover, the rate of degradation seems to have a
The extreme localizations of Melan-A<sub>wt</sub>, Melan-A<sub>inv</sub>, and Melan-A<sub>inv</sub> obtained in our experimental setup allowed us to clearly delineate the impact of altered protein localization on the generation of a defined CTL-defined epitope. The significant increase in IFNγ released by specific CTL exposed to cells expressing Melan-A<sub>inv</sub> clearly demonstrated that epitopes derived from this species were more efficiently produced and presented. Using an indirect assay, we estimated that Melan-A<sub>inv</sub> could produce up to 100 times more antigenic peptides than Melan-A<sub>wt</sub> (Fig. 5B). Several explanations may account for the increased presentation of Melan-A<sub>inv</sub>. The immunodominant CTL-defined epitope (Melan-A<sub>26-35/HLA-A2</sub>) has the particularity of being almost completely embedded within the lipid environment of the membrane, except for the first amino acid (Glu<sup>26</sup>), which is exposed to the lumen of the ER. Since the production of this epitope depends on the activity of the proteasome, which is located on the cytosolic side of the ER membrane (Fig. 6), this stretch of amino acids must therefore be exposed at some point in the cytosol. Inversion of the membrane topology could indirectly enhance the presentation efficiency because the epitope would already be facing the cytosol, thus facilitating processing. Alternatively, the amino acid substitutions introduced to engineer Melan-A<sub>inv</sub> could themselves affect the generation of the epitope. However, neither explanation seems plausible, because minigene-encoded, hydrophilic peptides carrying NH₂-terminal extended sequences matching those of Melan-A<sub>wt</sub> or Melan-A<sub>inv</sub> were presented with similar efficiency (Fig. 5A). Rather, the increased degradation kinetics of Melan-A<sub>inv</sub> compared with Melan-A<sub>wt</sub> is more likely to explain the more efficient presentation of Melan-A<sub>26-35</sub>. A similar observation was made in cells infected with recombinant vaccinia virus coding for influenza nucleoprotein (31). In this case, a more rapidly degraded form of NP led to a more efficient recognition of infected cells by specific CTL. However, this effect was observed in a situation where the antigenic peptide precursor was already localized in the cytosol. Here, we have shown that the processing of the antigenic peptide Melan-A<sub>26-35</sub> was the most efficient when Melan-A was artificially retained in the ER. Cells expressing a soluble, cytosolic form of Melan-A were recognized only slightly more efficiently by CTL than those expressing Melan-A<sub>wt</sub>. These surprising results contrast with those reported by Toborey and Siliciano (32) on the increased recognition of cells expressing a cytosolic mutant of the HIV-1 envelope protein and indicate that the membrane insertion of Melan-A<sub>inv</sub> plays a significant role in the processing of its antigenic peptide. Although the molecular basis for this phenomenon remains to be elucidated, it is possible that a membrane-proximal degradation of Melan-A<sub>inv</sub> by the proteasome increases the likelihood of antigenic peptides to be in close proximity of the TAP transporters. Taken together, these results provide, to our knowledge, the first demonstration that the altered organelle localization of a full-length protein impacts on the amount of HLA class I-restricted epitopes presented at the cell surface. It is likely that the retention of Melan-A<sub>inv</sub> in the ER targets the polypeptide more rapidly to the ER-associated degradation pathway.

The ER-associated degradation pathway has been recently identified as a major degradation pathway for proteins of the early secretory pathway (26). This pathway is restricted to the ER, probably because of the exclusive presence of the translocation pore complex Sec61. Indeed, this pore appears to be necessary not only for the translocation of newly synthesized polypeptides across the ER membrane but also for the retrograde transport of misfolded proteins from the ER to the cytosol. These misfolded proteins include newly translocated polypeptides, which associate with luminal chaperones and fail to reach their proper conformation. They are eventually re-exported to the cytosol where they are degraded by the ubiquitin/proteasome pathway. Indeed, the inhibition of recognition of cells treated with lactacystin by specific CTL (Fig. 6) indicates that this pathway plays an important role in the generation of Melan-A<sub>26-35/HLA-A2</sub>. However, we cannot exclude the possibility that a fraction of Melan-A<sub>wt</sub> is degraded by other proteolytic systems such as those of the lysosome.

A recent report has demonstrated the existence of a cotranslational, ubiquitin-dependent, degradation pathway targeting up to 30% of the newly translated polypeptides (33, 34). This degradation pathway could constitute an important source of major histocompatibility complex class I-restricted antigenic peptides. Support for this hypothesis was provided by the findings that defective ribosomal products and newly synthesized proteins are major substrates of the TAP transporters (35, 36). Since most antigenic peptides are transported by the latter, it has been inferred that major histocompatibility complex class I ligands are essentially derived from this pool of polypeptides. Whereas this may be the case for some polypeptides, it does not seem to apply to the Melan-A<sub>26-35</sub> epitope. Since the CTL-defined epitope of Melan-A<sub>wt</sub> and Melan-A<sub>inv</sub> is identical and at the same position within the polypeptide and under the assumption that the translation rate of both constructs is identical (the codon bias of both constructs is highly similar), one would expect similar recognition of all transfected cells if the majority of antigenic peptides were indeed produced cotranslationally. In addition, cells expressing Melan-A<sub>inv</sub> should be much more efficiently recognized, as the protein is already localized in the cytosol. Rather, our results suggest that the different organellar distribution of Melan-A<sub>wt</sub> and Melan-A<sub>inv</sub> has a stronger impact on the production of the epitope. Incidentally, studies on the presentation of tyrosinase<sub>369-377/HLA-A2</sub> also showed that the protein from which the epitope is derived is normally translocated into the ER and exposed to the glycosylating enzyme of the ER lumen before being re-exported into the cytosol and degraded by the proteasome (13). It will be interesting to analyze other membrane proteins to investigate whether the defective ribosomal product hypothesis applies only to soluble proteins present in the cytosol/nucleus.

Subcellular localization of tumor-associated antigens may be clinically relevant. In melanoma patients, CTL response against epitopes derived from melanosomal proteins are frequently observed. Melanoma cells from metastatic lesions, in contrast to their normal cells or cells from primary tumors, are often hypomelanotic, due to still poorly defined defects in melanosome formation and function. To this regard, it has recently been shown that the melanosome-associated protein tyrosinase, the first and rate-limiting enzyme in the pathway of melamin synthesis, accumulates aberrantly in the ER of amelanotic melanoma cells (37). It is not known whether this abnormal localization of tyrosinase, which leads to an accelerated degradation, has an impact on the generation of antigenic peptides and recognition by specific CTL. Based on the results presented in this work, the prediction is that this will be the case. Preliminary results analyzing a panel of melanoma cell lines indicate that the subcellular localization of Melan-A may also vary between different lines. If confirmed, these results may be relevant for the design of proteins with modified localization for the generation of CTL responses in melanoma patients.

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3 K. Muehlethaler and D. Rimoldi, unpublished data.
