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### Research Article

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# $\beta_2$ -Microglobulin Mutations, HLA Class I Antigen Loss, and Tumor Progression in Melanoma

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## Abstract

The potential negative impact of HLA class I antigen abnormalities on the outcome of T cell-based immunotherapy of melanoma has prompted us to investigate the mechanisms underlying lack of HLA class I antigen expression by melanoma cell lines Me18105, Me9923, and Me1386. Distinct mutations in the  $\beta_2$ -microglobulin ( $\beta_2m$ ) gene were identified in each cell line which result in loss of functional  $\beta_2m$ . In Me18105 cells, an aberrant splicing mechanism caused by an A→G point mutation in the splice acceptor site of intron 1 of the  $\beta_2m$  gene, deletes 11 bp from the  $\beta_2m$  mRNA creating a shift in the reading frame. In Me9923 cells a 14-bp deletion in exon 2 and in Me1386 cells a CT deletion in exon 1 of the  $\beta_2m$  gene produce a frameshift mutation. The  $\beta_2m$  gene mutations identified in Me18105, Me9923, and Me1386 cells were also detected in the surgically removed melanoma lesions from which the cell lines originated. Transfection of each melanoma cell line with a wild-type  $\beta_2m$  gene restored HLA class I antigen expression and, in Me18105 cells, recognition by Melan-A/MART-1-specific, HLA-A2-restricted cytotoxic T lymphocytes. Interestingly, the  $\beta_2m$  mutation present in Me9923 cells that were derived from a metastatic lesion was also found in the Me9923P cell line that originated from the autologous primary lesion. These data suggest that  $\beta_2m$  mutations in melanoma cells may be an early event in progression to the malignant phenotype. (*J. Clin. Invest.* 1998, 101:2720–2729.) Key words: CTL • HLA loci • immunosurveillance • metastasis • splicing

## Introduction

Malignant transformation of melanocytes and tumor progression are frequently associated with loss of HLA class I antigens. A recent review of the literature (1) has reported that ~ 15% and 55% of surgically removed primary and metastatic melanoma lesions, respectively, are not stained in immunohistochemical reactions by mAb to monomorphic determinants of HLA class I antigens. Loss or reduced HLA class I antigen expression may enable melanoma cells to evade the host's im-

mune response, since recognition and eventually destruction of melanoma cells by cytotoxic T lymphocytes (CTL)<sup>1</sup> requires presentation by HLA class I antigens of peptides derived from melanoma associated antigens (MAA) (2). This possibility is supported by the in vitro resistance of HLA class I antigen-negative melanoma cells to lysis by HLA class I-restricted, MAA-specific CTL (3–5) and by the poor prognosis associated with downregulation of HLA class I antigens in metastases in patients with malignant melanoma (6).

The emphasis on the application of T cell-based immunotherapy for the treatment of malignant melanoma (7) has stimulated interest in the characterization of the molecular lesions underlying HLA class I antigen loss by melanoma cells. This information will contribute to our understanding of the molecular mechanism(s) utilized by melanoma cells to escape from immune surveillance and may eventually suggest approaches to restore HLA class I antigen expression by melanoma cells. To the best of our knowledge, only two human melanoma cell lines have been characterized with respect to the molecular lesions underlying the complete loss of HLA class I antigens. In both cell lines, mutations in the  $\beta_2$ -microglobulin ( $\beta_2m$ ) gene have been identified. In the cell line FO-1, the  $\beta_2m$  gene is not transcribed because of a deletion spanning the first exon and a segment of the first intron (8). In the cell line SK-MEL-33, a point mutation in the second exon of the  $\beta_2m$  gene results in a frameshift and synthesis of a truncated, nonfunctional  $\beta_2m$  polypeptide (9). In the present study, we have characterized the molecular abnormalities underlying HLA class I antigen loss by the melanoma cell lines Me18105, Me9923, and Me1386, established from surgically removed metastatic lesions. Furthermore, we have analyzed the effects of the loss and restoration of HLA class I antigen expression by melanoma cells on their interactions with HLA class I-restricted, MAA-specific CTL. Lastly, we have compared the molecular defects underlying the loss of HLA class I antigen expression by the melanoma cell line Me9923 and by the autologous cell line Me9923P, originated from the primary melanoma lesion. The latter information will contribute to define the stage of the disease when molecular defects leading to HLA class I antigen loss by melanoma cells occur and may define the role of HLA class I antigen loss in the metastatic potential of melanoma cells.

## Methods

**Cell lines.** Melanoma cell lines Me18105 and Me1386 were generated at the Istituto Nazionale dei Tumori in Milan, Italy from metastatic lesions of two patients with the HLA phenotypes HLA-A2, -B35, -B41, -Cw4 and HLA-A1, -A31, -B8, -B51, -Cw7, respectively. Mela-

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1. **Abbreviations used in this paper:**  $\beta_2m$ , beta-2-microglobulin; CTL, cytotoxic T lymphocyte; MAA, melanoma associated antigen; NK, natural killer; RT-PCR, reverse transcriptase PCR.

noma cell lines Me9923 and Me9923P were generated at the Istituto Nazionale dei Tumori in Milan, Italy from a metastatic and a primary lesion, respectively, from a patient with the HLA phenotype HLA-A2, -B41, -B50, -Cw2, -Cw6.

Cultured human melanoma cells A375, Colo38, FO-1, MeFE, MeVAR, Me18105, and Me1386, and cultured lymphoid cells T2 were maintained in RPMI-1640 medium (Gibco BRL, Grand Island, NY) supplemented with 10% Serum Plus (Hazelton Biologics Inc., Lenexa, KS) and 2 mM L-glutamine (Gibco BRL). Cultured human melanoma cells Me9923 and Me9923P were seeded onto fibronectin coated dishes and maintained in DMEM medium (Gibco BRL) supplemented with 15% heat-inactivated fetal bovine serum (HyClone Laboratories, Lexington, KY), 10% bovine pituitary extract (Sigma Chemical Co., St. Louis, MO) and 2 mM L-glutamine. Cells were cultured at 37°C in a 5% CO<sub>2</sub> atmosphere and routinely passaged by trypsin-EDTA treatment.

The CD8-positive clone A42, which recognizes the Melan-A/MART-1<sub>27-35</sub> peptide AAGIGILTV in the context of HLA-A2 antigens, was established from a bulk tumor-infiltrating lymphocyte culture by limiting dilution (10). The antiinfluenza virus CTL line was established from an HLA-A0201 melanoma patient by *in vitro* stimulation with the immunodominant M<sub>1</sub>-Flu<sub>58-66</sub> nonamer peptide GILG-FVFTL (11). CTL were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated human serum, 2 mM L-glutamine and IL-2 (1,000 U/ml).

*Interferon-γ, restriction endonucleases, and peptides.* Recombinant human IFN-γ was obtained from Hoffmann-La Roche, Inc. (Nutley, NJ). Restriction enzymes HindIII and XbaI were purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). The Melan-A/MART-1<sub>27-35</sub> (AAGIGILTV) and the matrix M<sub>1</sub>-Flu<sub>58-66</sub> (GILG-FVFTL) peptides were purchased from American Peptide Company (Sunnyvale, CA). Peptide preparations were at least 95% pure.

*mAb and conventional antisera.* mAb W6/32 to a monomorphic determinant expressed on β<sub>2</sub>m-associated HLA-A, -B and -C heavy chains (12), mAb TP25.99 to a monomorphic determinant expressed on β<sub>2</sub>m-associated and β<sub>2</sub>m-free HLA-B and -C heavy chains (reference 8, unpublished results), mAb HC-10 to a determinant preferentially expressed on β<sub>2</sub>m-free HLA-B and -C heavy chains (13), anti-β<sub>2</sub>m mAb NAMB-1 (14), mAb LGIII-220.6 to a determinant expressed on β<sub>2</sub>m-associated HLA-A heavy chains (Ferrone, S., unpublished results), mAb 4E to a determinant expressed on β<sub>2</sub>m-associated HLA-B and -C heavy chains (15), anti-ICAM-1 mAb CL203.4 (16), and anti-Melan-A/MART-1 mAb M2-7C10 (17) were characterized as described. Rabbit anti-human β<sub>2</sub>m xenoserum and FITC-conjugated goat anti-mouse IgG F(ab')<sub>2</sub> antibodies were purchased from Sigma Chemical Co. and Boehringer Mannheim Biochemicals, respectively.

*Synthetic oligonucleotide primers and human β<sub>2</sub>m plasmids.* Forward primers 744M, 5'CTCTAACCTGGCACTG CGTCGC3' (742-763); 261M, 5'CCTGAAGCTGACAGCATTCG3' (856-875); 491M, 5'CTG GCAATATTAATGTGTCTTTCC3' (1302-1325); B2PH, 5'CCAAGCTTCTAACCTGGCACTGCGTCG3' (742-762); and reverse primers 468M, 5'TTGAGAAGGAAGTCACGGAGCG3' (1025-1004), 462M, 5'TCATACACAACCTTTCAGCAGC3' (1662-1642), 262M, 5'ACCTCCATGATGCTGCTT ACA3' (2264-2244), B2PX, 5'GTTCTAGAACCTCCATGATGCTGCTTACA3' (2264-2244) were synthesized on a BioSearch DNA synthesizer (MilliGen/BioSearch, Burlington, MA). Nucleotide positions of each primer are indicated in brackets and correspond to the human β<sub>2</sub>m gene sequence described previously (18). Primers specific for amplification of GAPDH cDNA generating a 452-bp fragment were purchased from Clontech Laboratories (Palo Alto, CA). The plasmid pb2m13 which carries a functional human β<sub>2</sub>m gene has been described elsewhere (18). The plasmid phB2m was constructed by reverse transcriptase (RT)-PCR utilizing mRNA from melanoma cells Colo38 and primers B2PH and B2PX which introduce a 5' HindIII site and a 3' XbaI site, respectively. The RT-PCR product was digested with enzymes HindIII and XbaI and ligated into the expression vector pcDNA3 (Invitrogen Corp., San Diego, CA) to generate the phB2m

construct. The phB2m sequence and reading frame was verified by sequencing of both strands using T7 and Sp6 primers (Invitrogen Corp.).

*Flow cytometry analysis.* HLA class I antigen cell surface expression by melanoma cells was analyzed utilizing cells harvested from subconfluent cultures grown in 100-mm plates, washed in ice-cold PBS, and detached with PBS/EDTA. Cells (1 × 10<sup>6</sup>) were incubated for 1 h on ice with an excess of mAb in PBS containing 1% BSA, 0.02% sodium azide (flow buffer). After two washings with flow buffer, cells were incubated for 30 min on ice with an appropriate amount of FITC-conjugated goat anti-mouse IgG (Fab')<sub>2</sub> antibodies diluted in flow buffer. After two additional washings with flow buffer, cells were analyzed on an Epics Elite (Coulter Corp., Hialeah, FL) flow cytometer. Dead cells and debris were eliminated from the analysis on the basis of forward and sideways light scatter. Melan-A/MART-1 protein expression in melanoma cells was evaluated by flow cytometry of permeabilized cells using the method described previously (17).

*Immunochemical assays.* Metabolic labeling of cells, indirect immunoprecipitation, SDS-PAGE, and Western blotting assays were performed utilizing the methodology described elsewhere (19).

*RT-PCR analysis.* Total RNA was extracted from melanoma cells by the method described previously (20). Poly (A)<sup>+</sup> RNA was purified using oligo (dT) cellulose (Invitrogen Corp.) according to the manufacturer's instructions. mRNA was reverse transcribed in a buffer containing 25 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl<sub>2</sub>, 10 mM DTT, 0.5 mM each of dCTP, dGTP, dATP, and dTTP, 200 U of reverse transcriptase (SuperScript II; Gibco BRL), and a mixture of oligo (dT) and random hexamer primers in a final volume of 20 μl. The mixture was sequentially incubated for 1 h at 37°C and for 5 min in boiling water and then transferred to ice. PCR was performed on the reverse transcriptase reaction product in a 100-μl reaction mixture containing 10 mM Tris-HCl (pH 8.4), 50 mM KCl, 15 mM MgCl<sub>2</sub>, 20 μg/ml gelatin, 0.2 mM dNTPs, 2.5 U Taq polymerase (AmpliTaq; Perkin-Elmer Cetus, Norwalk, CT) and 50 pmol each of primers 261M and 262M. The mixtures were initially heated for 4 min at 94°C followed by 30 cycles of 1.5 min at 58°C, 2 min at 72°C, and 1 min at 94°C in a Perkin-Elmer Cetus Thermal Cycler (Perkin-Elmer Cetus). PCR products were analyzed on 2% agarose gels and visualized with ethidium bromide staining.

*PCR amplification of genomic DNA.* DNA from melanoma cells was purified using a Qiagen genomic DNA kit (Qiagen Inc., Chatsworth, CA). DNA was prepared from tissue sections of surgically removed melanoma lesions as described previously (9). Extracted DNA was amplified by PCR in a 50-μl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM MgCl<sub>2</sub>, 0.1% NP-40, 100 μg/ml gelatin, 0.2 mM dNTPs, 2 U of AmpliTaq, and 100 mM each of primers 744M and 468M or 491M and 462M. Samples were overlaid with mineral oil and amplified on a thermal cycler programmed for one cycle at 95°C for 5 min followed by 30 cycles at 94°C for 1 min, 58°C for 1 min, and 72°C for 2 min. Amplified samples were analyzed by electrophoresis on 2% agarose gels and visualized by ethidium bromide staining.

*Cloning and sequencing of PCR products.* PCR products were purified by electrophoresis on 2% agarose gels. DNA was recovered using a QIAquick gel extraction kit (Qiagen Inc.) according to the manufacturer's instructions. Double-stranded direct sequencing of PCR products was performed by using a Cyclist sequencing kit (Stratagene Cloning Systems, La Jolla, CA). Alternatively, PCR products were ligated into the plasmid vector pCRII (Invitrogen Corp.) and used to transform bacteria INVαF' according to the manufacturer's instructions. Colonies of transformed bacteria were selected in medium containing ampicillin (100 μg/ml) and expanded for plasmid DNA preparation. Plasmid DNA was extracted and purified using QIAprep plasmid spin columns (Qiagen Inc.). Cloned PCR products were sequenced with a Dye Terminator cycle sequencing kit and automated sequencer, (373A; Applied Biosystems Inc., Foster City, CA), using T7 and Sp6 primers.

**Transfection of melanoma cells with a human wild-type  $\beta_2m$  gene and cDNA.** Transfections of Me18105 and Me1386 cells with plasmid pb2m13 and pSVneo or with pSVneo alone were performed by electroporation as described previously (9). Selection was started 3 d after electroporation in media containing 400  $\mu$ g/ml G418-sulfate (Gibco BRL). After 3 wk of selection, drug-resistant colonies were analyzed by flow cytometry. Positive clones were then expanded in complete media without G418. Me9923 cells were transfected with the pb2m plasmid or pcDNA3 alone by the calcium phosphate-DNA coprecipitation method (21). Transfected Me9923 cells were seeded into 6-well culture plates and grown in complete media for 48 h before testing for antigen expression.

**Transduction of melanoma cells with the Melan-A/MART-1 gene.** The retroviral vector (Aa-CSM) was constructed as described (22) encoding the full-length Melan-A/MART-1 cDNA (23) under the control of the long terminal repeat. Me18105 cells were transduced with Aa-CSM by retroviral transfer as described previously (22).

**IFN- $\gamma$  release assay.** Effector cells ( $1 \times 10^5$ ) were incubated for 24 h at 37°C with target cells ( $1 \times 10^5$ )/well in U-bottomed 96-well plates in a total volume of 200  $\mu$ l of complete medium. Controls consisted of effector and target cells incubated alone or effector cells incubated with target cells pulsed with an unrelated peptide. At the end of the incubation, supernatant was harvested and stored at -70°C until tested for the presence of IFN- $\gamma$  using a commercially available ELISA kit (R & D Systems, Minneapolis, MN).

**Cytotoxicity assay.** The  $^{51}\text{Cr}$ -release cytotoxicity assay was performed as previously described (24). Results are expressed as % lysis = [experimental release (cpm) - spontaneous release (cpm)]/maximum release (cpm) - spontaneous release (cpm)] where spontaneous release was determined by incubating target cells in the absence of lymphocytes and maximum release determined in the presence of 1% NP-40 detergent.

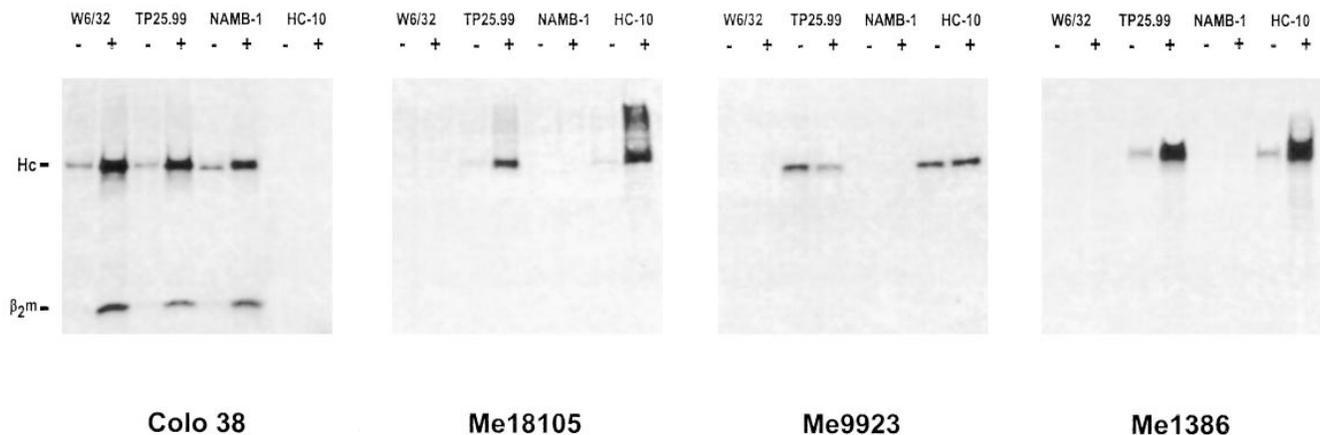
## Results

**Lack of  $\beta_2m$  synthesis by melanoma cells Me18105, Me9923, and Me1386, without detectable HLA class I antigen expression.** The cultured melanoma cells Me18105, Me9923, and Me1386 were not stained in flow cytometry analysis by anti-

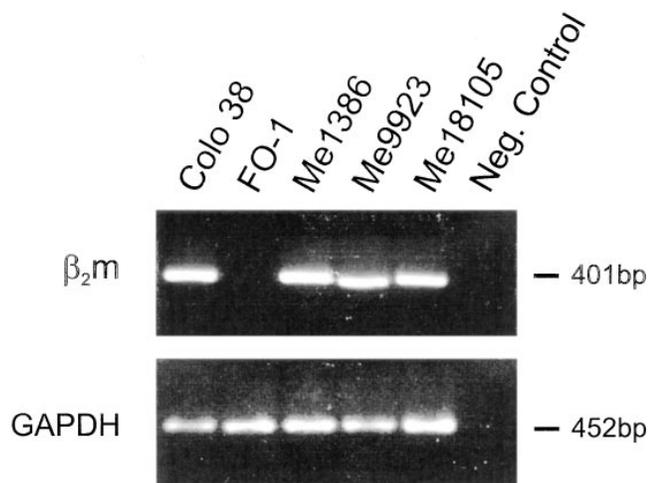
$\beta_2m$  associated HLA class I heavy chain mAb W6/32 or by anti- $\beta_2m$  free and  $\beta_2m$  associated HLA class I heavy chain mAb TP25.99, even following a 48-h incubation at 37°C with IFN- $\gamma$  (data not shown). The three cell lines are susceptible to modulation by IFN- $\gamma$ , since their staining by anti-ICAM-1 mAb CL203.4 was increased following incubation with IFN- $\gamma$ .

The lack of HLA class I antigen cell surface expression by the three melanoma cell lines, reflects lack of association of HLA class I heavy chains with  $\beta_2m$ , since no components were immunoprecipitated from the three  $^{35}\text{S}$ -methionine/cysteine-labeled melanoma cell lines by anti- $\beta_2m$ -associated HLA class I heavy chain mAb W6/32 and by anti- $\beta_2m$  mAb NAMB-1 (Fig. 1). In contrast, HLA class I heavy chains were detected in immunoprecipitates with anti- $\beta_2m$ -free HLA class I heavy chain mAb HC-10 and with anti- $\beta_2m$ -free and  $\beta_2m$ -associated HLA class I heavy chain mAb TP25.99. When IFN- $\gamma$ -treated Me18105 and Me1386 cells were used as an antigen source, the intensity of the components corresponding to HLA class I heavy chains was increased in the precipitates with mAb HC-10 and TP25.99, but no component was detected in the immunoprecipitates with mAb W6/32 and NAMB-1. When IFN- $\gamma$ -treated Me9923 cells were used as an antigen source, no increase was detected in HLA class I heavy chain steady-state levels. These results indicate that the cell lines Me18105, Me9923, and Me1386 do not synthesize  $\beta_2m$  protein and as a result do not assemble HLA class I heavy chain- $\beta_2m$  complexes. Furthermore, a defect in the modulation of HLA class I heavy chains by IFN- $\gamma$  is likely to be present in Me9923 cells.

**Transcription of  $\beta_2m$  genes in Me18105, Me9923, and Me1386 cells.** To determine whether  $\beta_2m$  genes are transcribed in Me18105, Me9923, and Me1386 cells, mRNA isolated from each cell line was reverse transcribed using oligo (dT) primers followed by PCR amplification with primers 261M and 262M corresponding to exon 1 and exon 3 of the  $\beta_2m$  gene. A PCR product with the expected size of 401 bp was obtained from melanoma cells Me1386 and from the control melanoma cell line Colo38 (Fig. 2). The PCR products from



**Figure 1.** Lack of  $\beta_2m$  synthesis by Me18105, Me9923, and Me1386 melanoma cells. After a 48-h incubation in the presence (+) or absence (-) of IFN- $\gamma$  (500 U/ml), cells were labeled with  $^{35}\text{S}$ -methionine/cysteine for 6 h. Detergent cell extracts were prepared, precleared with irrelevant mouse IgG bound to GammaBind-G-Sepharose, and then incubated with anti- $\beta_2m$  associated HLA-A, -B, -C heavy chain mAb W6/32, anti- $\beta_2m$  free and  $\beta_2m$ -associated HLA class I heavy chain mAb TP25.99, anti- $\beta_2m$  mAb NAMB-1, or anti- $\beta_2m$  free HLA-B, -C heavy chain mAb HC-10 for 2 h at 4°C. Immune complexes were precipitated with GammaBind-G-Sepharose, washed, and eluted by boiling in SDS-PAGE sample buffer. Eluted immune complexes were separated on 12% SDS-PAGE gels followed by autoradiography. Position of HLA class I heavy chains (Hc) and  $\beta_2m$  are indicated on the left side of the figure.



**Figure 2.** Transcription of  $\beta_2m$  genes in Me18105, Me9923, and Me1386 melanoma cells. Total RNA was extracted from cells incubated at 37°C for 48 h in the presence of IFN- $\gamma$  (500 U/ml) and purified on oligo (dT) cellulose to obtain mRNA. cDNA was synthesized from mRNA using Superscript II reverse transcriptase and a mixture of oligo (dT) and random hexamer primers. PCR was performed using Taq polymerase and primers 261M and 262M specific for exon 1 and exon 3, respectively, of the human  $\beta_2m$  gene. Negative control reactions were carried out by omitting DNA template from the reaction mixture and using mRNA from FO-1 cells which do not transcribe the  $\beta_2m$  gene. Integrity of RNA for each cell line was verified in control amplifications using primers specific for the GAPDH gene. PCR products were analyzed on 2% agarose gels and visualized with ethidium bromide staining.

Me18105 cells and more so from Me9923 cells appeared to be slightly shorter than the predicted product size of 401 bp. These results demonstrate that  $\beta_2m$  genes are transcribed in Me18105, Me9923, and Me1386 cells and suggest that base deletions may be present in the  $\beta_2m$  mRNA from Me18105 and Me9923 cells.

*Distinct mutations in  $\beta_2m$  genes in Me18105, Me9923, and Me1386 cells.* Sequencing of  $\beta_2m$  cDNAs identified distinct mutations in Me18105, Me9923, and Me1386 cells (Fig. 3). An 11-bp deletion at position 68–78 of  $\beta_2m$  cDNA was found in Me18105 cells. This deletion results in a frameshift and premature termination after 29 missense codons. The possibility of a splice junction defect was investigated as the mechanism underlying the 11-bp deletion, since this mutation was located at a region corresponding to the extreme 5' end of exon 2 of  $\beta_2m$  gene. PCR amplification and sequencing of the  $\beta_2m$  genes from Me18105 cells detected an A→G transition mutation in the dinucleotide AG splice acceptor site of intron 1, which results in a splice acceptor defect immediately 5' of exon 2. This mutation causes the utilization of a downstream cryptic splice site in exon 2 resulting in the deletion of 11 bases in the Me18105 cell  $\beta_2m$  message (Fig. 4). PCR and sequence analysis of genomic DNA from Me18105 cells did not detect the wild-type  $\beta_2m$  gene sequence.

A 14-bp deletion at nucleotide position 235–248 of  $\beta_2m$  cDNA was found in Me9923 cells. This deletion, located in exon 2, causes a frameshift and introduces a premature UGA stop codon at nucleotide position 264 which results in a puta-

tive COOH-terminal truncated protein of 83 amino acids. In addition, a C→G transversion mutation at nucleotide position 258 was identified which would introduce an in-frame premature stop at codon 86 in the  $\beta_2m$  cDNA that does not contain the 14-bp deletion mutation. Analysis of genomic DNA from Me9923 cells by PCR amplification and sequencing did not detect a wild-type  $\beta_2m$  gene sequence.

A CT deletion in an 8-bp CT repeat region of exon 1 of the  $\beta_2m$  gene was found in Me1386 cells. This deletion, located in the signal peptide coding sequence, results in a frameshift mutation and premature termination after 40 missense codons. Sequencing of genomic DNA amplified by PCR from Me1386 cells demonstrated only the mutated  $\beta_2m$  gene sequence.

To determine whether the  $\beta_2m$  gene mutations identified in Me18105, Me9923, and Me1386 cells were present in the surgically removed lesion from which each cell line originated, genomic DNA was extracted from the corresponding lesions and amplified by PCR using primers flanking exon 1 and primers flanking exon 2 which include the 3' region of intron 1. Sequence analysis of the entire amplified fragment on both strands detected in the melanoma lesions the mutations found in the corresponding cell line (Fig. 5).

These results indicate that distinct mutations account for the lack of  $\beta_2m$  gene translation in the melanoma cell lines Me18105, Me9923, and Me1386 and each cell line is homozygous or hemizygous for the identified  $\beta_2m$  gene mutation(s). Furthermore, the  $\beta_2m$  gene mutations identified in Me18105, Me9923, and Me1386 cells are present in the melanoma lesion from which each cell line originated.

*Restoration by transfection with a wild-type  $\beta_2m$  gene or cDNA of HLA class I antigen cell surface expression by Me18105, Me9923, and Me1386 cells.* To determine whether HLA class I antigen expression could be restored, Me18105 and Me1386 cells were stably transfected with a wild-type  $\beta_2m$  gene and Me9923 cells were transiently transfected with a  $\beta_2m$  cDNA. A 12-kD component representing  $\beta_2m$  was detected in  $\beta_2m$ -transfected Me18105, Me9923, and Me1386 cells by Western blotting analysis with anti-human  $\beta_2m$  xenoantisera (Fig. 6 A). This reactivity is specific, since anti-human  $\beta_2m$  xenoantisera recognized no component in the extract from FO-1 melanoma cells which do not synthesize the  $\beta_2m$  polypeptide (8). Flow cytometry analysis showed staining of  $\beta_2m$  transfectant clones of Me18105, Me9923, and Me1386 cell lines by anti-HLA class I mAb W6/32 (Fig. 6 B). Furthermore, mAb W6/32 immunoprecipitated the two characteristic subunits of HLA class I antigen from these  $\beta_2m$  transfectants (data not shown). Restoration of HLA class I antigen expression by transfection with a wild-type  $\beta_2m$  gene indicates that mutations in  $\beta_2m$  genes represent the only molecular lesion underlying the complete loss of HLA class I antigen expression by Me18105, Me9923, and Me1386 cells.

*Recognition of  $\beta_2m$ -transfected Me18105 cells by HLA-A2-restricted CTL recognizing Melan-A/MART-1 or M1-Flu-specific peptides.* To evaluate the functional integrity of HLA class I allotypes expressed after  $\beta_2m$  gene transfection, melanoma cells were tested for their ability to induce cytokine release by HLA class I-restricted, MAA-specific CTL and for their susceptibility to lysis by these CTL. HLA-A2-positive Me18105 cells were selected for these studies, since the MAA-specific CTL available in our laboratory are HLA-A2 restricted. The other HLA-A2-positive melanoma cell line Me9923 was not used in this analysis due to its poor growth in

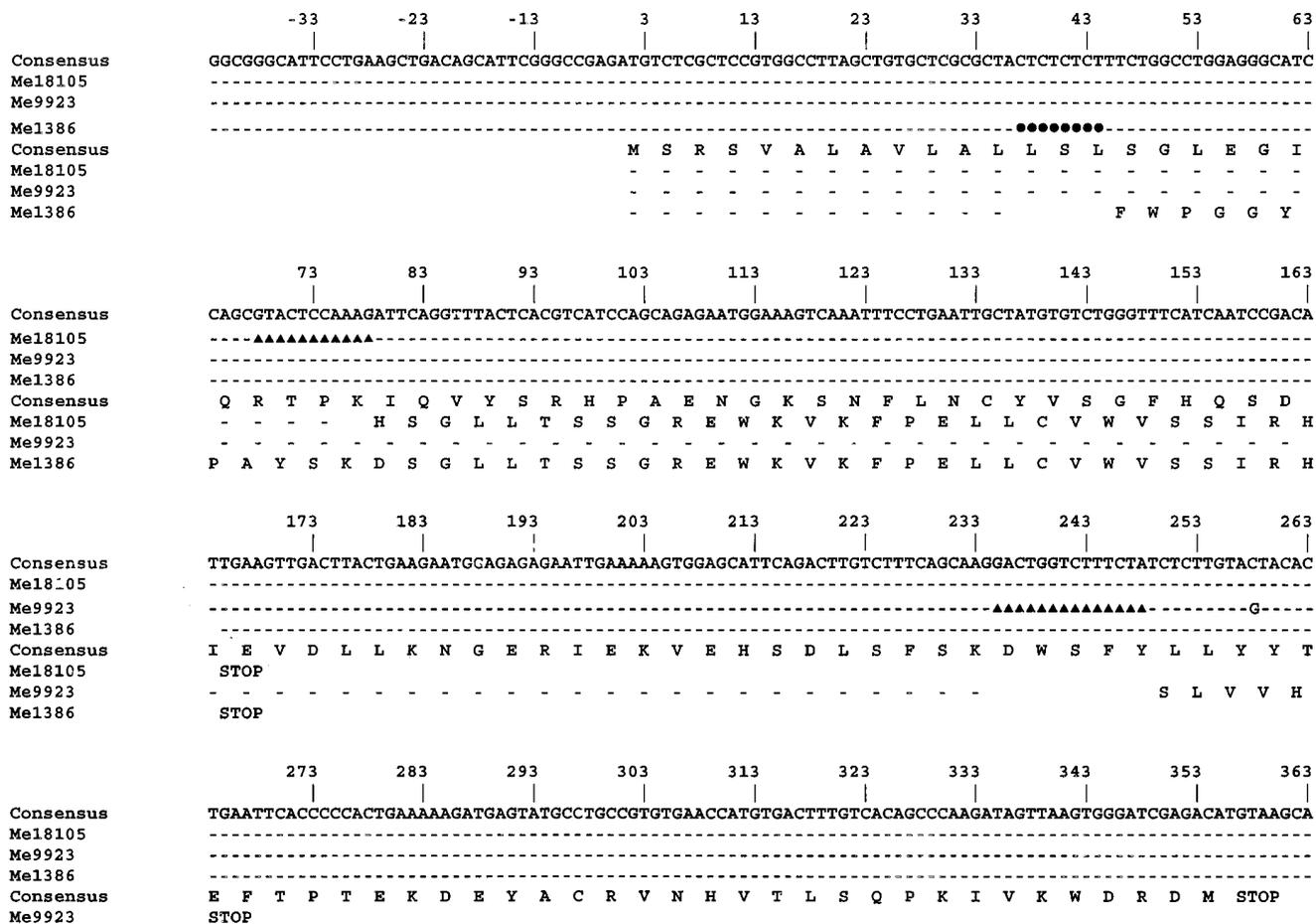


Figure 3. Nucleotide and deduced amino acid sequences of  $\beta_2m$  cDNA from Me18105, Me9923, and Me1386 melanoma cells. Nucleotide and deduced amino acid sequences of  $\beta_2m$  cDNA from Me18105, Me9923, and Me1386 cells are compared to the consensus  $\beta_2m$  nucleotide and amino acid sequence. Nucleotides are numbered in the 5'→3' direction, starting with the first nucleotide of the start codon for the  $\beta_2m$  protein. Amino acids are numbered starting with the NH<sub>2</sub>-terminal Met residue. Conservation of nucleotide and deduced amino acid sequences to that of consensus  $\beta_2m$  sequence are indicated by dashes. Deletion mutations in the nucleotide sequence of Me18105 and Me9923  $\beta_2m$  cDNA are indicated by closed triangles. The 8-bp CT repeat region of the Me1386  $\beta_2m$  cDNA which contains a dinucleotide CT deletion is indicated by closed circles.

culture. As evaluated by RT-PCR analysis, Me18105 cells expressed several melanoma genes encoding MAA, including gp100, tyrosinase, and Melan-A/MART-1, known to be recognized by HLA-A0201-restricted CTL. However, Me18105 cells do not express Melan-A/MART-1 protein, since permeabilized cells were not stained by anti-Melan-A/MART-1 mAb M2-7C10 in flow cytometry (data not shown). Therefore, we tested the ability of CTL to recognize  $\beta_2m$ -transfected pulsing with exogenous peptides and Me18105 cells after transduction with the Melan-A/MART-1 gene. Pulsing for 1 h with MART-1<sub>27-35</sub> or M<sub>1</sub>-Flu<sub>58-66</sub> peptide of IFN- $\gamma$  (2,000 U/ml for 72 h) treated,  $\beta_2m$ -transfected Me18105 cells induced IFN- $\gamma$  release and lysis by the HLA-A2-restricted Melan-A/MART-1-specific, CTL clone A42 and by a HLA-A2-restricted, M<sub>1</sub>-Flu-specific CTL clone, respectively (Table I). Furthermore,  $\beta_2m$ -transfected Me18105 cells transduced with the Melan-A/MART-1 gene also induced IFN- $\gamma$  release by the CTL clone A42 demonstrating that the endogenous antigen processing mechanisms of Me18105 cells are functional (Table II).

*Differential expression of the gene products of HLA class I loci by  $\beta_2m$ -transfected Me18105, Me9923, and Me1386 cells.* To investigate the expression of the gene products of HLA-A, -B and -C loci by  $\beta_2m$ -transfected Me18105, Me9923, and Me1386 cells, clones were stained with HLA-A locus-specific mAb LGIII-220.6 and HLA-B, -C locus-specific mAb 4E and analyzed by flow cytometry. No difference was detected in the intensity of staining of  $\beta_2m$ -transfected Me18105 cells by the two locus-specific mAb (Fig. 7). Furthermore, incubation of Me18105 cells with IFN- $\gamma$  enhanced the intensity of staining by mAb LGIII-220.6 and 4E to a similar extent. The intensity of staining of  $\beta_2m$ -transfected Me9923 cells by anti-HLA-B, -C mAb 4E was stronger than that by anti-HLA-A mAb LGIII-220.6. The intensity of staining by anti-HLA-B, -C mAb 4E was slightly increased after incubation of  $\beta_2m$ -transfected Me9923 cells with IFN- $\gamma$ , while that by anti-HLA-A mAb LGIII-220.6 displayed no detectable change. The intensity of staining of control as well as IFN- $\gamma$ -treated,  $\beta_2m$ -transfected Me1386 cells by HLA-A locus-specific mAb LGIII-220.6 was

Table I. CTL Recognition of  $\beta_2m$ -transfected Me18105 Cells Pulsed with Melan-A/MART-1<sub>27-35</sub> and Flu<sub>58-66</sub> Peptides

CTL specificity	neo-Me18105 cells pulsed with*				$\beta_2m$ -Me18105 cells pulsed with				T2 cells pulsed with				A375 cells <sup>‡</sup> pulsed with			
	MART-1 <sub>27-35</sub>		M <sub>1</sub> -Flu <sub>58-66</sub>		MART-1 <sub>27-35</sub>		M <sub>1</sub> -Flu <sub>58-66</sub>		MART-1 <sub>27-35</sub>		M <sub>1</sub> -Flu <sub>58-66</sub>		MART-1 <sub>27-35</sub>		M <sub>1</sub> -Flu <sub>58-66</sub>	
MART-1 <sub>27-35</sub>	16 <sup>§</sup>	2 <sup>  </sup>	ND <sup>¶</sup>	3	1000	51	16	3	> 1000	70	ND	1	> 1000	65	ND	1
M <sub>1</sub> -Flu <sub>58-66</sub>	ND	8	16	4	ND	7	> 1000	49	16	13	> 1000	66	ND	0	832	72

\*Cells incubated with IFN- $\gamma$  (2,000 U/ml) for 72 h at 37°C were pulsed with Melan-A/MART-1<sub>27-35</sub> peptide (1  $\mu$ M) or with M<sub>1</sub>-Flu peptide (1  $\mu$ M) for 1 h at 37°C. <sup>‡</sup>A375 melanoma cells are HLA-A0201 positive and Melan-A/MART-1 negative. <sup>§</sup>IFN- $\gamma$  (pg/ml) per 1  $\times$  10<sup>8</sup> CTL/24 h. <sup>||</sup><sup>51</sup>Cr-release (%) by target cells. The effector/target ratio was 20:1. <sup>¶</sup>IFN- $\gamma$  < 16 pg/ml.

stronger than that by HLA-B, -C locus-specific mAb 4E. The intensity of staining by the latter mAb of IFN- $\gamma$ -treated transfectants was increased to a level similar to that of control cells by HLA-A locus specific mAb LGIII-220.6. The differential staining patterns with locus-specific mAb suggests differences in the level of HLA-A, -B, and -C antigen expression by Me9923 and Me1386 cells. Furthermore, the lack of upregulation of HLA-A antigens on Me9923 cells incubated with IFN- $\gamma$  suggests additional defects in the mechanisms which regulate HLA-A antigen expression in these cells.

*$\beta_2m$  gene mutations in melanoma cells Me9923 and tumor progression.* To determine whether defects in HLA class I antigen expression in melanoma cells occur at an early or late stage of the disease, we characterized HLA class I antigen loss present by Me9923P cells. This cell line had been established from the primary lesion of the patient from whom the metastatic clone Me9923 had been established. Flow cytometry analysis detected no staining of Me9923P cells by mAb W6/32 and TP25.99, even after a 48-h incubation at 37°C with IFN- $\gamma$  (data not shown). Sequence analysis of the genomic DNA amplified by PCR from Me9923P cells revealed a 14-bp deletion at position 235–248 and a C→G transversion mutation at position 258 (data not shown). Therefore, the same  $\beta_2m$  gene mutations were found in the Me9923P and in the Me9923 cell line that originated from an autologous primary and metastatic lesion, respectively.

## Discussion

Distinct molecular lesions of the  $\beta_2m$  gene have been found in the melanoma cell lines Me18105, Me9923, and Me1386 which do not express HLA class I antigens due to lack of  $\beta_2m$  gene translation. In Me18105 cells, an A→G point mutation at the 3' end of intron 1 of  $\beta_2m$  gene alters the AG splice acceptor site critical for exon-intron splicing (25). The mutant GG splice site sequence results in the utilization of an alternative

downstream AG splice site located in exon 2. Use of this cryptic splice site causes an 11-bp deletion in  $\beta_2m$  mRNA and results in a shift in the reading frame at nucleotide position 67 and a premature stop codon at nucleotide position 165. In Me9923 cells, a 14-bp deletion in exon 2 of  $\beta_2m$  gene results in a frameshift at nucleotide position 249 and introduces a UGA stop codon at nucleotide position 264. The truncation of this putative  $\beta_2m$  protein at the carboxyl terminus to 83 amino acids presumably would cause a dramatic change in its structure. In Me1386 cells, a CT deletion in the 8-bp CT repeat region of exon 1 of  $\beta_2m$  gene produces a shift in the reading frame starting at nucleotide position 45 and introduces a premature UGA stop codon at nucleotide position 165. The deduced  $\beta_2m$  protein would be truncated to 54 amino acids with no significant homology to the native  $\beta_2m$  sequence. It is noteworthy that the molecular defects of  $\beta_2m$  genes identified in the three melanoma cell lines do not represent mutations acquired during in vitro culture of the cell lines, but reflect in vivo abnormalities, since they were also found in the surgically removed lesions from which the cell lines had been originated.

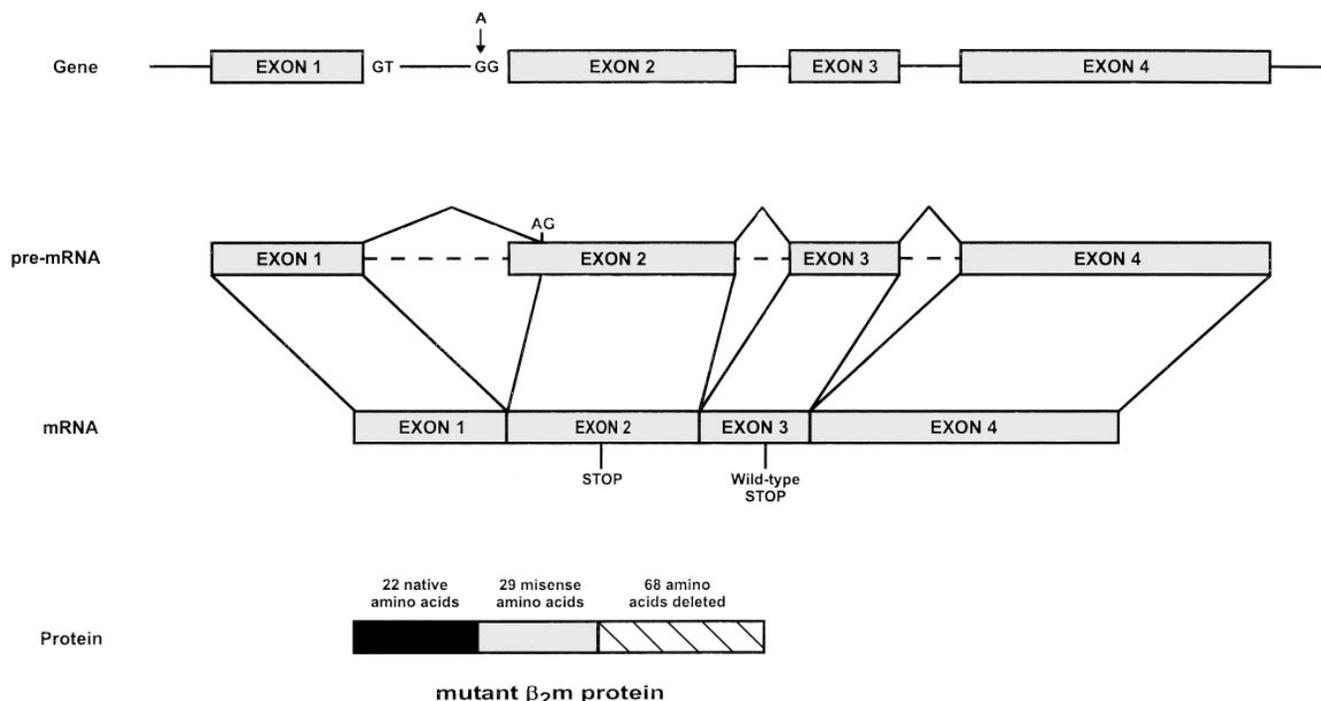
To the best of our knowledge, the  $\beta_2m$  gene mutations we have found in the melanoma cell line Me98923 are novel. In contrast, the lesion found in Me18105 cells is remarkably similar to that found in the human colorectal carcinoma cell line HCT-15 (26). Analysis of  $\beta_2m$  defects in HCT-15 cells showed that one allele of the  $\beta_2m$  gene contained a G→T mutation in the last base of intron 1 which also resulted in a splice acceptor site defect and nonfunctional  $\beta_2m$  transcript (26). Furthermore, the CT deletion found in Me1386 cells is notably similar to CT deletions identified in colorectal carcinoma cells and tissue (27, 28) and in other melanoma cell lines (Margulies, L., D. Hicklin, and S. Ferrone, unpublished results). Apparently, this region of the  $\beta_2m$  gene is highly susceptible to mutation and is frequently the basis for the HLA class I-negative phenotype in malignant cells.

The  $\beta_2m$  gene mutations we have identified are the only

Table II. CTL Recognition of  $\beta_2m$ -transfected and Melan-A/MART-1 Gene Transduced Me18105 Cells

Effector cells	Me18105	MART-1-Me18105	$\beta_2m$ -Me18105	$\beta_2m$ /MART-1-Me18105	Control MeFE <sup>§</sup>	Control MeVAR <sup>  </sup>
<i>n</i>						
5 $\times$ 10 <sup>4</sup>	ND*	ND	2 <sup>‡</sup>	250	250	ND
1 $\times$ 10 <sup>5</sup>	ND	ND	40	> 250	> 250	ND

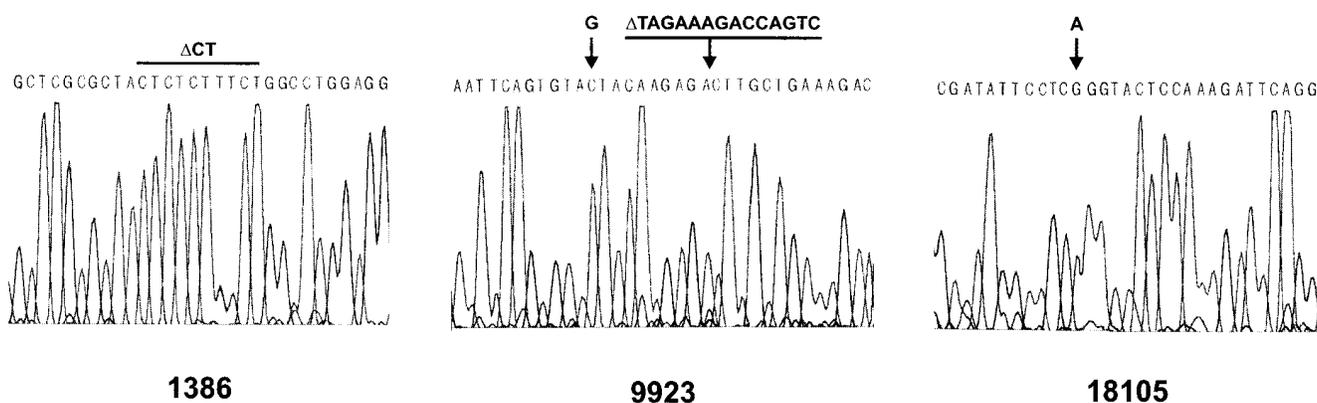
\*IFN- $\gamma$  < 16 pg/ml. <sup>‡</sup>IFN- $\gamma$  (pg/ml) per 1  $\times$  10<sup>8</sup> CTL/24 h. <sup>§</sup>MeFE melanoma cells are HLA-A0201 and Melan-A/MART-1 positive. <sup>||</sup>MeVAR melanoma cells are HLA-A0201 positive and Melan-A/MART-1 negative.



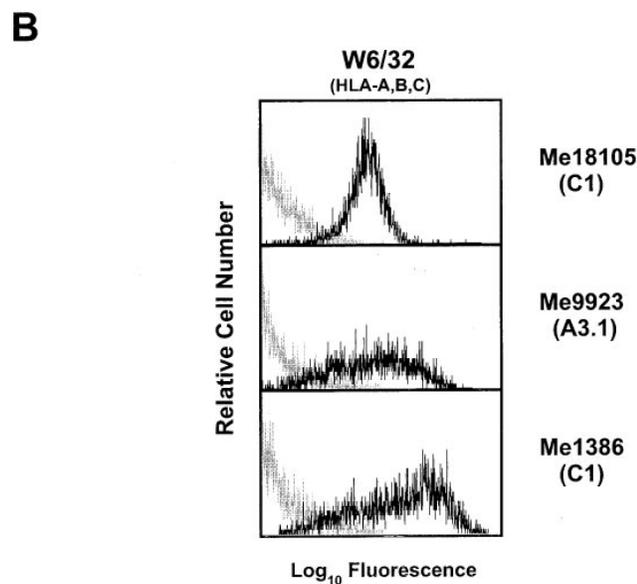
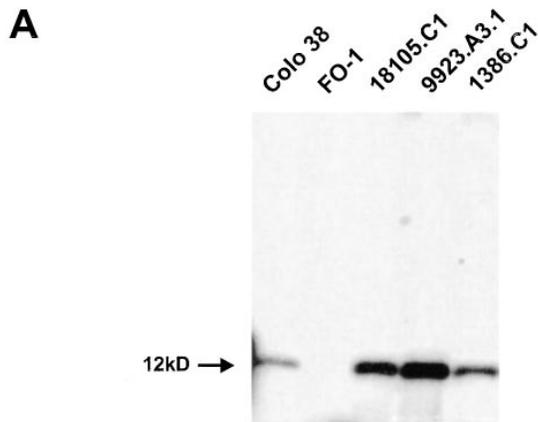
*Figure 4.* Schematic diagram of the synthesis of a mutant  $\beta_2m$  protein resulting from a splice acceptor site defect in the  $\beta_2m$  gene of Me18105 melanoma cells. An A→G mutation in the first base of the dinucleotide AG splice acceptor site at the 3' end of intron 1 of the  $\beta_2m$  gene in Me18105 cells results in the utilization of a downstream cryptic splice site in exon 2. The abnormal splicing results in the loss of 11 bp from the  $\beta_2m$  message producing a frameshift mutation and introducing a premature stop codon. The resulting mutant  $\beta_2m$  protein would contain 22 native amino acids followed by 29 missense amino acids and lack 68 amino acids from the COOH terminus.

defects responsible for the complete HLA class I antigen loss by the melanoma cells Me18105, Me9923, and Me1386, since transfection with a wild-type  $\beta_2m$  gene or cDNA restored HLA class I antigen expression by the three cell lines. Additional defects appear to be present in the cell lines Me9923 and Me1386, since both cell lines display a differential expression of the gene products of HLA-A, -B and -C loci. The level of HLA-A antigens is lower than that of HLA-B and -C antigens

in Me9923, but higher in Me1386 cells. The opposite results obtained by staining Me1386 and Me9923 melanoma cells with locus-specific mAb argue against differences in the association constants of the two mAb as the reason for the differential staining patterns observed. The unbalanced level of HLA-A, -B and -C antigens on the cell surface of Me9923 and Me1386 cells is likely to represent abnormalities in the regulatory mechanisms which control their expression. These abnormali-



*Figure 5.*  $\beta_2m$  gene mutations in surgically removed melanoma lesions from which Me18105, Me9923, and Me1386 cells originated. DNA was extracted from tissue sections of melanoma lesions and amplified by PCR using primers flanking exon 1 (1386) or exon 2 including the 3' region of intron 1 (18105 and 9923) of the  $\beta_2m$  gene. PCR products were cloned into pCRII and analyzed by automated dye terminator cycle sequencing. The sequence shown for 18105 and 1386 lesions corresponds to the sense strand in the 5'→3' orientation. The sequence shown for the 9923 lesion corresponds to the antisense strand in the 5'→3' orientation.



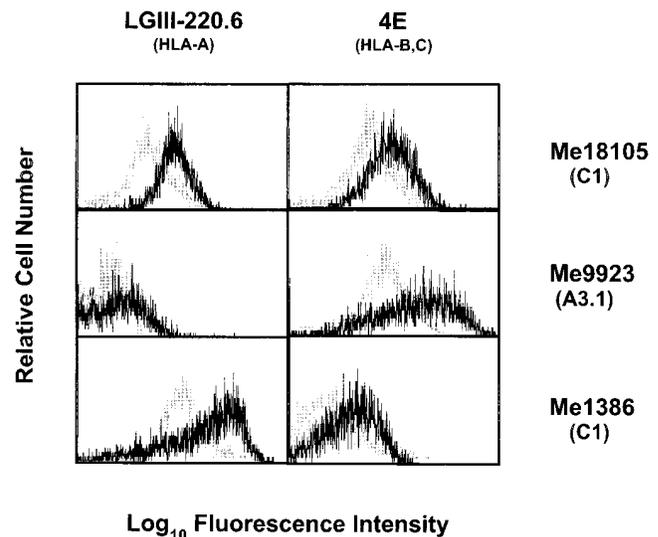
**Figure 6.** Restoration of  $\beta_2m$  protein expression and HLA class I antigen cell surface expression by  $\beta_2m$ -transfected Me18105, Me9923, and Me1386 melanoma cells. In *A*, detergent cell extracts were prepared from  $\beta_2m$ -transfected Me18105, Me9923, and Me1386 cells incubated at 37°C for 48 h in the presence of IFN- $\gamma$  (500 U/ml). Cell extracts equivalent to 30,000 cells were mixed with SDS-PAGE sample buffer and separated on 14% SDS-PAGE gels. Proteins were transferred to an Immobilon-P membrane. After blocking for 2 h at room temperature with 5% nonfat dry milk, membranes were sequentially incubated at room temperature with rabbit anti-human  $\beta_2m$  xenoantiseria for 2 h and with  $^{125}I$ -labeled protein G for 1 h. Membranes were washed, air dried, and exposed to Hyperfilm-MP for 24 h. In *B*, subconfluent cultures of neo-transfected (*shaded lines*) or  $\beta_2m$ -transfected (*dark lines*) Me18105, Me9923, and Me1386 cells were incubated at 37°C for 48 h in the presence of IFN- $\gamma$  (500 U/ml). Cells were detached and sequentially incubated on ice with anti- $\beta_2m$ -associated HLA class I heavy chain mAb W6/32 for 1 h and with FITC-labeled goat anti-mouse IgG (Fab')<sub>2</sub> xenoantibodies for 30 min. Cells were then analyzed by flow cytometry.

ties have not been characterized yet. At any rate, locus-specific downregulation of HLA class I antigens is rather frequent in melanoma cell lines and has been attributed to suppression by *c-myc* and to chromosomal abnormalities (29, 30). Further-

more, the lack of upregulation of HLA-A antigen expression by Me9923 cells suggests structural mutations or defects in the regulatory cascade triggered by IFN- $\gamma$ . These defects are related to cellular events responsible for regulation of HLA-A antigens, since upregulation of ICAM-1 expression in response to IFN- $\gamma$  treatment can be demonstrated in Me9923 cells.

Interestingly, the wild-type  $\beta_2m$  gene sequence was not found in the three melanoma cell lines we have analyzed. Karyotyping demonstrated that at least two copies of chromosome 15, which carries the  $\beta_2m$  gene (31), are present in each melanoma cell line (Minoletti, F., G. Sozzi, and G. Parmiani, unpublished results). Furthermore, abnormalities in chromosome 15, i.e., derivative chromosomes, translocations, and deletions involving chromosome 15, were identified in each cell line. It is unlikely that the  $\beta_2m$  mutations we have identified occurred independently on both  $\beta_2m$  alleles in each cell line. A more likely explanation is represented by the loss of the wild-type  $\beta_2m$  allele through a mitotic recombination event or by nondisjunction and duplication of the chromosome containing the mutated  $\beta_2m$  allele. Whatever the reason for homozygosity, clearly there has been selection by each of the melanoma cell lines for the  $\beta_2m$ -deficient phenotype and subsequent loss of HLA class I antigen expression.

Several studies have demonstrated a markedly higher frequency of HLA class I antigen downregulation in metastases than in primary melanoma lesions (for review, see reference 1). There is only limited and conflicting information about the role of HLA class I antigen loss in the metastatic potential of melanoma cells and about the stage of tumor progression when HLA class I antigen loss takes place. Cordon-Cardo et al. (32) have provided immunohistochemical evidence in support



**Figure 7.** Differential expression of the gene products of HLA class I loci by  $\beta_2m$ -transfected Me9923 and Me1386 melanoma cells. Subconfluent cultures of  $\beta_2m$ -transfected Me18105, Me9923, and Me1386 cells were incubated at 37°C for 48 h in the presence (*dark lines*) or absence (*shaded lines*) of IFN- $\gamma$  (500 U/ml). Cells were detached and sequentially incubated on ice with anti-HLA-A mAb LGIII-220.6, anti-HLA-B, -C mAb 4E, or isotype matched control mAb for 1 h and with FITC-labeled anti-mouse IgG (Fab')<sub>2</sub> xenoantibodies for 30 min. Cells were then analyzed by flow cytometry.

of the possibility that HLA class I antigen loss transpires early in tumor progression and that increased exposure to tumor vasculature results in selection of HLA class I negative tumor cells. These investigators demonstrated that intravascular clusters of human breast carcinoma cells were predominantly HLA class I antigen nonexpressors. Furthermore, they showed that perivascular tumor cells were strongly stained by anti-HLA class I mAb and that the staining intensity decreased as a function of the distance from the blood vessel. In contrast, Restifo et al. (33) reported loss of functional  $\beta_2m$  expression in five melanoma cell lines established from patients who suffered from recurrence of the disease after a favorable clinical response to T cell-based immunotherapy or cytokine gene therapy. These investigators have suggested that loss of functional  $\beta_2m$  in melanoma cells in these patients may have been responsible for the recurrence of their disease and poor response to immunotherapy. Moreover, they have suggested that immunotherapy in these patients may have provided additional immunoselective pressure resulting in melanoma cells with defects in  $\beta_2m$  expression and HLA class I antigen loss. Our study provides for the first time structural evidence in support of the possibility that mutations in the  $\beta_2m$  gene in melanoma cells may occur at an early stage of the disease process. We found that the cell line Me9923P, originated from a primary melanoma lesion, carries the same  $\beta_2m$  mutations as the autologous cell line Me9923 originated from a metastasis. Defects in  $\beta_2m$  expression and subsequent HLA class I antigen loss are likely to provide a selective advantage for the metastatic phenotype from immune recognition and conceivably a poor prognosis for the patient. Indeed, the malignant disease was aggressive in patient 9923 with metastatic progression to several organs.

HLA class I antigens expressed by melanoma cells Me18105 after transfection of a wild-type  $\beta_2m$  gene are functional, since the transfected cells could present exogenously provided and endogenously derived peptides to Melan-A/MART-1 or M<sub>1</sub>-Flu peptide-specific CTL. The ability of Me18105 cells to process endogenous proteins was in keeping with our finding that these cells constitutively express LMP2, LMP7, TAP1, and TAP2 (unpublished results). In contrast, constitutive expression of TAP1 and TAP2 was not detected in Me9923 cells and TAP2 was not detected in Me1386 cells. Therefore, the latter two cell lines have additional defects in their antigen processing mechanisms and may therefore utilize more than one mechanism to escape from immune surveillance.

In vitro studies indicate that HLA class I antigen loss may have differential effects on susceptibility of melanoma cells to lysis by cytotoxic effector cells.  $\beta_2m$  gene defects are an extremely effective mechanism for melanoma cells to escape from killing by HLA class I-restricted, MAA-specific CTL (34). On the other hand, complete HLA class I antigen loss may result in increased susceptibility of melanoma cells to lysis by natural killer (NK) cells (35). Restoration of HLA class I antigen expression by transfection with a wild-type  $\beta_2m$  gene reduces susceptibility of melanoma cells to NK cell-mediated lysis (35). Since the melanoma cell lines analyzed in this study and in previous ones (8, 9, 33) have been established from lesions of patients with advanced disease, it appears that these cells have acquired mechanisms to escape from in vivo NK cell recognition and killing and/or that the NK cell effector system is defective in these patients.

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