The major histocompatibility complex class I molecules consist of three subunits, the 45-kDa heavy chain, the 12-kDa \( \beta_2 \)-microglobulin (\( \beta_2 \text{m} \)), and an \(~8–9\)-residue antigenic peptide. Without \( \beta_2 \text{m} \), the major histocompatibility complex class I molecules cannot assemble, thereby abolishing their transport to the cell membrane and the subsequent recognition by antigen-specific T cells. Here we report a case of defective antigen presentation caused by the expression of a \( \beta_2 \text{m} \) with a Cys-to-Trp substitution at position 25 (\( \beta_2 \text{m}^{25\text{W}} \)). This substitution causes misfolding and degradation of \( \beta_2 \text{m}^{25\text{W}} \) but does not result in complete lack of human leukocyte antigen (HLA) class I molecule expression on the surface of melanoma VMM5B cells. Despite HLA class I expression, VMM5B cells are not recognized by HLA class I-restricted, melanoma antigen-specific cytotoxic T lymphocytes even following loading with exogenous peptides or transduction with melanoma antigen-expressing viruses. Lysis of VMM5B cells is restored only following reconstitution with exogenous or endogenous wild-type \( \beta_2 \text{m} \) protein. Together, our results indicate impairment of antigenic peptide presentation because of a dysfunctional \( \beta_2 \text{m} \) and provide a mechanism for the lack of close association between HLA class I expression and susceptibility of tumor cells to cytotoxic T lymphocyte-mediated lysis in malignant diseases.

The human leukocyte antigen (HLA)\(^\text{a} \) class I molecules, encoded by the genes located in the major histocompatibility complex, are composed of three subunits including a 45-kDa HLA class I heavy chain (HC), a 12-kDa \( \beta_2 \)-microglobulin (\( \beta_2 \text{m} \)), and an \(~8–9\)-residue peptide (1). Expression of these molecules on the cell surface requires the stepwise assembly of HCs, \( \beta_2 \text{m} \), and peptides in the endoplasmic reticulum (ER) followed by the transport of the trimeric molecule to the plasma membrane. These processes are dependent on a functional antigen processing machinery (APM), which includes the proteasome subunits, the peptide transporters TAP1 and TAP2, and a number of ER-resident chaperons such as calnexin, calreticulin, ERP57, and tapasin (2, 3). \( \beta_2 \text{m} \) plays an integral part in the assembly and transport of HLA class I molecules because it stabilizes the HC-\( \beta_2 \text{m} \) heterodimer through non-covalent protein-protein interactions, thereby allowing binding of endogenous antigenic peptides with the help of TAP and tapasin (4). As a result, the assembled HC-\( \beta_2 \text{m} \)-peptide trimeric complexes can travel to the cell surface, where they are recognized by HLA class I-restricted, antigen-specific cytotoxic T lymphocytes (CTLs).

The lack of HLA class I molecule expression on the cell surface usually reflects defects in \( \beta_2 \text{m} \) protein synthesis caused by mutations in the \( \beta_2 \text{m} \) gene, as has been found mostly in malignant cells thus far (5). This abnormality renders tumor cells resistant to tumor antigen-specific CTLs and may have a negative impact on the elimination of tumor cells by host CTLs. The defects underlying \( \beta_2 \text{m} \) loss have thus far been found to be structural in nature, involving lack of translation because of small deletions or point mutations in most cases and lack of transcription because of large deletions in a few cases (5). Because of a lack of \( \beta_2 \text{m} \) expression, the resulting HLA class I loss cannot be corrected by interferon (IFN-\( \gamma \)), a cytokine that up-regulates the expression of most of the molecules participating in the assembly and transport of HLA class I molecules. On the other hand, a low level of HLA class I expression on cells usually reflects nonstructural defects in the APM components because this abnormality can be corrected by IFN-\( \gamma \) (6).

In the present study, we have elucidated the mechanism underlying HLA class I down-regulation identified in a melanoma cell line and in the metastasis from which the cell line was derived (7). This HLA class I down-regulation phenotype cannot be corrected by IFN-\( \gamma \) and was unexpectedly found to be caused by an abnormal full-length \( \beta_2 \text{m} \) protein that can neither form stable complexes with HCs nor assist in loading peptides onto the HLA class I peptide binding groove.

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The VMM5A and VMM5B melanoma cell lines were established from two sequential metastatic melanoma deposits surgically removed from patient VMM5 at two time points (7). Other melanoma cell lines included DM6 cells (a gift from Dr. T. L. Darrow, Duke University, Durham, NC), which express HLA-A2 and multiple melanocytic differentiation antigens including gp100, melanoma antigen recognized by T cells (MART-1) and tyrosinase, and FO-1 cells, which do not express HLA class I molecules because of \( \beta_2 \text{m} \) loss (8). LG-2 is a human B lymphoblastoid cell line. All of these cell lines were maintained

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\(^{8}\) This work was supported in part by Public Health Services Grants R01 CA67108 and P30 CA160056 (to S. F.) and R01 CA57653 (to C. L. S.) awarded by NCI, National Institutes of Health, Department of Health and Human Services. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\(^{1}\) Recipient of the Susan G. Komen Breast Cancer Foundation dissertation research award.

\(^{2}\) An Elaine Shepard cancer investigator supported by the Cancer Research Institute.

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\(^{4}\) The abbreviations used are: HLA, human leukocyte antigen; APM, antigen processing machinery; \( \beta_2 \text{m} \), \( \beta_2 \)-microglobulin; CTL, cytotoxic T lymphocyte; ER, endoplasmic reticulum; HC, HLA class I heavy chain; IFN-\( \gamma \), interferon-\( \gamma \); LMP, low molecular mass polypeptide; LOH, loss of heterozygosity; mAb, monoclonal antibody; MFI, mean fluorescence intensity; TA, tumor antigens; TAP, transporter associated with antigen processing; GPDH, glyceraldehyde-3-phosphate dehydrogenase; TyrVac, tyrosinase-expressing vaccinia strain.
in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum in a 5% CO2 atmosphere at 37 °C. A tyrosinase-specific CTL line was derived from patient VMM119 who had been vaccinated with a mixture of four peptides including tyrosinase369–377D (YMDGTMSQV) (9).

### Monoclonal and Polyclonal Antibodies

The following mAbs were developed and characterized as described: the mAb W6/32, which recognizes a monomorphic determinant expressed on HLA-A, -B, and -C heavy chains (10); the mAb LG III-147.4.1, which recognizes a determinant expressed on HLA-A heavy chains except A23, A24, A25, and A32 (11); the mAb B1.23.2, which recognizes a determinant restricted to HLA-B and -C antigens (12); the mAb HC-10, which recognizes a determinant expressed on all HLA-B and -C heavy chains and on HLA-A10, -A28, -A29, -A30, -A31, -A32, and -A33 heavy chains (13–15); the mAb 637 m-specific mAbs L368 (16) and KJ-2, which recognize HLA class I heavy chain-free 2m; the delta (Y)-specific mAb SY-5; the MB1 (X)-specific mAb SJJ-3; the Z-specific mAb NB-1; the LMP2-specific mAb SY-1; the LMP7-specific mAb HB-2; the LMP10-specific mAb TO-7 (17); the TAP1-specific mAb NOB-1 (18); the TAP2-specific mAb NOB-2 (18); the calnexin-specific mAb TO-5 (19); the calreticulin-specific mAb TO-11 (19); the ERp57-specific mAb TO-2 (19); and the tapasin-specific mAb TO-3 (19). The human actin-specific mAb (sc-8432) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-idiotypic mAb MK2–23 (20) and mouse IgG2a (BD Biosciences) were used as isotype controls. R-phycoerythrin-conjugated goat anti-mouse F(ab')2 fragments and goat anti-mouse IgG antibodies were purchased from Dako (Carpinteria, CA) and GE Healthcare, respectively.

### Peptides, IFN-γ, Wild-type Human 2m, and Pharmacological Inhibitors

The HLA class I-associated peptides HER2/neu369–377D, KIFGSLAFL, and tyrosinase369–377D were synthesized with a free amide N terminus and free acid C terminus by standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) chemistry using a model AMS422 peptide synthesizer (Gilson Co. Inc., Middleton, WI). Peptides were purified to >98% purity by reverse-phase high pressure liquid chromatography on a C-8 column (Vydac, Hesperia, CA) at the University of Virginia biomolecular core facility. Purity and identity were confirmed using a triple quadrupole mass spectrometer (Finnigan, San Jose, CA). Recombinant human IFN-γ was purchased from Roche Applied Science. The wild-type human 2m was purchased from Sigma. The proteasome inhibitor MG132 and the trans-Golgi-to-secretory vesicles

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β2-Microglobulin Dysfunction and Antigen Presentation Defects

Log fluorescence intensity (FL2-H)

FIGURE 2. β2m expression in VMM5B cells. A, control- and IFN-γ-treated VMM5A and VMM5B cell lysates were stained with HLA class I heavy chain-specific HC-10 and β2m-specific L368 mAbs and rabbit polyclonal antibodies by Western blotting. Actin expression was analyzed as an indication of equal loading. B, control- (thin line) and IFN-γ-treated (thick line) VMM5A and VMM5B cells were stained intracellularly with the β2m-specific mAb L368 and analyzed by flow cytometry. The numbers above each open profile are -fold MFI. Representative results of one of three experiments are shown. The difference in -fold MFI between control- and IFN-γ-treated cells is statistically significant (p < 0.05).

Recombinant Vaccinia Virus—Virus encoding full-length human tyrosinase (TyrVac) was constructed as described previously (25). Purified recombinant vaccinia virus stock was titered and tested for proper expression of tyrosinase using specific HLA-A2-restricted CTL (data not shown).

Cytotoxicity Assay in Vitro—Standard 51Cr release assays were performed to determine CTL recognition of the HLA-A2-restricted
epitope from the melanocyte differentiation protein tyrosinase (tyrosinase 369–377D). Target cells were prepared by either loading with tyrosinase 369–377D peptide (40 μg/ml) for 1 hour at room temperature or by infecting with TyrVac (10 plaque-forming units/cell, 10^6 target cells) in 1 ml of Hanks’ balanced salt solution supplemented with 0.1% bovine serum albumin, 1.6 mM MgSO4, and 1.8 mM CaCl2 for 1 h and then 4 ml of RPMI 1640 medium supplemented with 10% fetal bovine serum for 8 h to allow for expression followed by labeling with 100 μCi of Na51CrO4 for 1 h for a standard 4 h 51Cr release assay. Percent cytotoxicity was calculated by the formula: % specific lysis = \((\text{experimental release} - \text{spontaneous release})/\text{total release} - \text{experimental release})\) × 100.

RESULTS

Marked HLA Class I Down-regulation on VMM5B Melanoma Cells—Fluorescence-activated cell sorting analysis of cells stained with mAb W6/32 showed that HLA class I molecules were barely detectable on melanoma cells VMM5B as compared with autologous VMM5A melanoma cells (Fig. 1A). The low level of staining of VMM5B cells, as well as FO-1 cells, by mAb W6/32.

To corroborate the specificity of the staining of VMM5B cells by mAb W6/32, cells were treated with low pH (pH 3.0) and then stained with mAb W6/32, a method used to disintegrate the trimeric HLA class I complex on the cell surface (21). As shown in Fig. 1B, following treatment with pH 3.0, VMM5B cells were not stained by mAb W6/32. These results imply the dissociation of peptides and β2m from the HCs because the antigenic determinant recognized by mAb W6/32 requires the association of HCs with β2m for its expression (10). This possibility is supported by the restoration of the staining with mAb W6/32 following loading of low pH-treated VMM5B cells with wild-type β2m along with a HLA-A2-binding peptide (HER-2/neu369–397, KIFGSLAFL, τ = 481.2 min). This result reflects the reassembly of the trimeric HLA class I complex on the cell surface. It is noteworthy that the level of the restored HLA class I expression is similar to that on untreated cells, remaining at a low level, unlike the full restoration of HLA class I expression on low pH-treated autologous VMM5A cells following loading with exogenous peptide and β2m (data not shown). These results suggest that the amount of HCs transported to the plasma membrane is the limiting factor in VMM5B cells. This finding is different from the efficient transport to the cell surface of open-form (peptide-free or low affinity peptide-bound) HCs, which can subsequently be refolded to their native, bioactive conformation following loading with a high affinity peptide, as has been observed in the TAP-deficient T2 cells (3).
To determine whether the low level of HLA class I molecules on the membrane of VMM5B cells is caused by a reduced level of all HCs and/or by a defect in β₂m, HC and β₂m expression in VMM5A and VMM5B cell lysates were analyzed by Western blotting. As shown in Fig. 2A, HCs were detected in both cell lysates and were markedly up-regulated by IFN-γ. In contrast, β₂m was barely detectable with β₂m-specific rabbit polyclonal antibodies in a VMM5B cell lysate following IFN-γ treatment. β₂m was not detected in a VMM5B cell lysate with a panel of β₂m-specific mouse mAb (Fig. 2A and data not shown). The latter results may reflect the insufficient sensitivity of the Western blotting technique because β₂m was detected in VMM5B cells by intracellular staining with the β₂m-specific mAb L368 (Fig. 2B). These results in conjunction with the lack of up-regulation of HLA class I molecules by IFN-γ suggest that the abnormal HLA class I phenotype of VMM5B cells is caused by a defect in β₂m.

Cys-to-Trp substitution in β₂m Caused by a Point Mutation in the β₂m Gene in VMM5B Cells—To investigate whether structural mutation(s) underlie the low β₂m level and its functional abnormalities, we amplified the full-length β₂m cDNA fragments (Fig. 3A) and performed nucleotide sequencing of the open reading frame of the β₂m gene in VMM5B cells. As shown in Fig. 3B and C, a cytosine-to-guanine transversion mutation at position 135 in exon 2 was detected. This mutation changes codon 25 from Cys to Trp (β₂mC25W) (Fig. 4A), abolishing the disulfide bond between residue 25 (Cys-25) and 80 (Cys-80) of the full-length β₂m protein (B). It is noteworthy that this mutation was not acquired during in vitro culture of the VMM5B cell line because the identical mutated nucleotide was detected in the genomic DNA extracted from the melanoma metastasis from which the cell line had been derived (Fig. 5A). Moreover, the wild-type sequence of the β₂m gene in VMM5B cells was not detected suggesting that one β₂m gene copy was lost. This pos-
sibility is supported by the LOH identified at two chromosome 15
short tandem repeat sites (D15S126 and D15S209) (23) flanking the
\( \beta_2m \) gene in genomic DNA extracted from VMM5B cells and the
corresponding melanoma metastasis (Fig. 5, B and C).

**Instability of \( \beta_2m^{C25W} \) in VMM5B Cells**—To test whether loss of a
disulfide bond in the mutant \( \beta_2m \) identified in VMM5B cells caused
conformational instability of the full-length protein, we examined
the effect of Cys-to-Trp substitution at residue 25 on the stability of
\( \beta_2m \) structure using molecular modeling. The wild-type
\( \beta_2m \) is a \( \beta \)-sandwich structure composed of two anti-parallel \( \beta \)-pleated
sheets connected by a disulfide bond between Cys-25 and Cys-80
(Fig. 6A). Each of the \( \beta \)-pleated sheets contains three \( \beta \) strands. In
the wild-type Cys-25–Cys-80 hydrophobic core, the linked sulfur
atoms exhibit favorable van der Waals contacts with respect to the
surrounding atoms 4.43, 4.81, and 3.88 Å for Cys25SG-Val27CG1,
Cys25SG-Gln8CG, and Cys80SG-Val82CG2, respectively (Fig. 6B).
However, when Cys-25 is replaced with Trp-25, the bulky indole ring
of Trp side chain displays drastic steric clashes with the side chains of
the neighboring residues. These clashes occur with all 14 possible
Trp-25 side chain rotameric conformations analyzed. Fig. 6C shows one
representative conformation in which the ring carbon and nitrogen members
are within the unfavorable van der Waals distances with the neighboring
atoms 2.63, 1.62, 2.74, and 1.95 Å for Trp25CZ3-Tyr66CD1, Trp25CZ2-
Val27CG1, Trp25NE1-Val82CG2, and Trp25CD1-Cys80SG, respectively.
Because all of these distances are below 3.0 Å, the minimum distance
between two nonbonded carbon atoms, the free energy is drastically
increased, leading to a thermodynamically unstable state of the mutant \( \beta_2m \)
(\( \beta_2m^{C25W} \)).
Degradation of $\beta_2m^{C25W}$ and Lack of Stable HC-$\beta_2m^{C25W}$ Association in VMM5B Cells—Next we tested whether $\beta_2m^{C25W}$ was degraded by the proteasome in VMM5B cells, especially because it was present at a very low level intracellularly, approximately at a level 29-fold and 41-fold lower than that in VMM5A cells under basal conditions and following incubation with IFN-$\gamma$ (300 units/ml), respectively (Fig. 2B). To this end, VMM5B cells were sequentially incubated with IFN-$\gamma$ (300 units/ml) for 24 h at 37 °C and with the proteasome inhibitor MG132 (5 $\mu$M) for 12 h at 37 °C. The intracellular levels of the steady-state free $\beta_2m^{C25W}$ free HCs, and HC-$\beta_2m^{C25W}$ complexes in IFN-$\gamma$/MG132-treated and in IFN-$\gamma$-treated VMM5B cells were compared utilizing fluorescence-activated cell sorting analysis of cells intracellularly stained with mAbs. As shown in Fig. 7, A and B, both the $\beta_2m^{C25W}$ and HCs were increased ~2-fold following incubation of cells with IFN-$\gamma$, but the level of HC-$\beta_2m^{C25W}$ complexes remained unchanged. In the presence of IFN-$\gamma$ and MG132, the level of $\beta_2m^{C25W}$ increased markedly (5-fold above that in untreated cells) along with an ~3-fold increase in HCs, but the level of HC-$\beta_2m^{C25W}$ complexes still remained unchanged.

On the other hand, increased secretion of $\beta_2m^{C25W}$ did not appear to play a role in its low intracellular accumulation because the level of $\beta_2m^{C25W}$, HCs, and HC-$\beta_2m^{C25W}$ complex expression was not increased by the addition, 4 h before harvest, of monensin, an inhibitor of trans-Golgi-to-secretory vesicles traffic, to MG132-treated cells cultured in the presence of IFN-$\gamma$. Therefore, $\beta_2m^{C25W}$ was removed through proteasome-mediated degradation. Even when it accumulates, the mutant $\beta_2m$ cannot form stable complexes with HCs. This conclusion is corroborated by the lack of immunoprecipitation of HC-$\beta_2m^{C25W}$ complexes with mAb W6/32 from MG132 and IFN-$\gamma$-treated VMM5B cell lysates (Fig. 7C). Attempts to obtain biochemical evidence for the degradation of $\beta_2m^{C25W}$ in pulse-chase experiments failed because the available methods were not sufficiently sensitive to detect the mutant $\beta_2m$ in immunoprecipitation (Fig. 7C and data not shown).

Restoration of HLA Class I Expression and Peptide Presentation on VMM5B Cells following Exogenous and Endogenous Reconstitution of Wild-type $\beta_2m$ Expression—Despite HLA class I expression, VMM5B cells were resistant to lysis by HLA-A2-restricted, tyrosinase-derived peptide tyrosinase$^{369–377D}$ (YMDDTMSQV, $t_1/2 = 212.6$ min)-specific CTLs, even following pulsing target cells with the tyrosinase$^{369–377D}$ peptide (Fig. 8A and D). To determine whether reconstitution of wild-type $\beta_2m$ expression is sufficient to restore HLA class I expression and its ability to present peptides to CTLs, we transfected VMM5B cells with a wild-type $\beta_2m$ cDNA. As shown in Fig. 8B, expression of HLA class I molecules was restored on stable clones of $\beta_2m$-transfected VMM5B cells (VMM5B.$\beta_2G$) compared with mock-transfected VMM5B cells (VMM5B.neo). Similarly, the gene products of HLA-A and HLA-B, HLA-C loci were restored, although the latter molecules were expressed at a level ~10-fold lower than the former ones (Fig. 8B).

To test whether the lack of peptide presentation is caused by the mutated $\beta_2m$ ($\beta_2m^{C25W}$) associated with HC, VMM5B.neo cells were treated with low pH to dissociate the HC-$\beta_2m^{C25W}$ complexes on the cell surface before being loaded with the wild-type $\beta_2m$ together with the tyrosinase$^{369–377D}$ peptide and tested for susceptibility to lysis by tyrosinase$^{369–377D}$-specific CTLs. VMM5B.$\beta_2G$ cells were treated in a similar fashion and analyzed in parallel as a control. Although acid-stripped VMM5B.$\beta_2G$ cells became sensitive to CTL lysis following loading with either the tyrosinase$^{369–377D}$ peptide by itself or both, the wild-type $\beta_2m$ and the tyrosinase$^{369–377D}$ peptide (Fig. 8C, left panel), VMM5B.neo cells were sensitive to CTL lysis only following loading with both the wild-type $\beta_2m$ and the tyrosinase$^{369–377D}$ peptide (Fig. 8C, right panel). These results indicate that peptide presentation is restored on VMM5B cells only when both the wild-type $\beta_2m$ and the tyrosinase$^{369–377D}$ peptide are present. Therefore, the presence of $\beta_2m^{C25W}$ does not allow exogenous peptide loading onto HLA class I molecules.

To test whether $\beta_2m^{C25W}$ affects endogenous peptide loading, VMM5B.neo cells were transduced with TyrVac, a tyrosinase-expressing vaccinia strain, and analyzed for susceptibility to lysis by HLA-A2-restricted, tyrosinase$^{369–377D}$ peptide-specific CTLs. As shown in Fig. 8D, TyrVac-infected VMM5B.neo cells were resistant to CTL lysis, whereas TyrVac-infected VMM5B.$\beta_2G$ cells were sensitive. In these experiments, the tyrosinase-expressing DM6 cells were used as a positive control, whereas the untransduced VMM5B, VMM5B.neo, and VMM5B.$\beta_2G$ cells were used as specificity controls. Therefore, in VMM5B cells, the endogenously processed tyrosinase$^{369–377D}$ peptide cannot be presented by $\beta_2m^{C25W}$ associated with HLA-A2 heavy chains unless the wild-type HLA-A2-$\beta_2m$ dimer is available in sufficient amount following reconstitution with wild-type $\beta_2m$ expression. These
FIGURE 7. Lack of stable HC-β2m association despite accumulation of β2m following inhibition of proteasome function in VMM5B cells. A, VMM5B cells were treated with the indicated combination of IFN-γ, MG132, and monensin or mock-treated before being intracellularly stained with β2m-specific mAb KJ-2, HC-specific mAbs HC-A2 and HC-10, and HLA class I-specific mAb W6/32 and analyzed by flow cytometry. The numbers above each histogram profile indicate the fold MFI increase above the background. B, the fold differences in the levels of free β2m, free HC-A, -B, and -C and HC-β2m complexes among the indicated treated groups are shown. C, lysates of VMM5B cells treated with IFN-γ and/or MG132 were immunoprecipitated with mAb W6/32 and analyzed by SDS-PAGE. A VMM5A cell lysate was used as a positive control. The bottom panel shows equal loading of proteins in each lane as monitored by Western blot analysis of an equal portion of each lysate preparation with calnexin-specific mAb TO-5. CNX, calnexin.
FIGURE 8. Restoration of HLA class I expression and function following reconstitution of wild-type β2m expression on VMM5B cells. A, VMM5B cells (VMM5B.neo) were analyzed for susceptibility to lysis by HLA-A2-tyrosinase369–377-specific CTL in cytotoxicity assays following loading with the tyrosinase369–377 peptide (YMDGTMQV). VMM5A, VMM5B.β2.7G, and DM6 cells were used as positive controls. B, β2m-transfected VMM5B cells (VMM5B.β2.7G) and their mock-transfected counterpart VMM5B.neo cells were stained with HLA class I-specific mAb W6/32 (thick line), HLA-A2-specific mAb KS-1 (thin line), and HLA-B and -C-specific mAb B1.23.1 (dotted line) and analyzed by flow cytometry. C, acid-treated VMM5B.β2.7G (left panel) or VMM5B.neo cells (right panel) loaded with the tyrosinase369–377 peptide or with the tyrosinase369–377 peptide and wild-type β2m were analyzed for susceptibility to lysis by HLA-A2-tyrosinase369–377-specific CTLs in cytotoxicity assays. Acid-treated VMM5B.β2.7G and VMM5B.neo cells incubated in medium were used as controls. D, VMM5B.neo and VMM5B.β2.7G cells infected with a TyrVac were analyzed for susceptibility to lysis by HLA-A2-tyrosinase369–377-specific CTLs in cytotoxicity assays. Control-infected cells and DM6 cells served as a specificity control and a positive control, respectively. E:T, effector-to-target ratio.
data indicate that although expressed on the cell membrane, HLA class I heavy chains, associated with β₂mC25W, cannot present peptides.

To exclude the possibility that defects in antigen presentation by VMM5B cells were caused by APM abnormalities, the level of APM component expression in VMM5A and VMM5B cells was compared. No marked difference was detected, except for lower LMP7, LMP10, calnexin, and tapasin expression in VMM5B cells than in VMM5A cells, as measured by Western blotting (Fig. 9) and by flow cytometry (for results, see Ref. 7). However, LMP7, LMP10, calnexin, and tapasin down-regulation did not appear to have an impact on antigen presentation because peptide presentation was restored in VMM5B cells transfected with a wild-type β₂m cDNA (VMM5B.β2.7G) (Fig. 8D). This observation in conjunction with the failure of exogenous peptide loading onto VMM5B cells, as compared with the wild-type β₂m-transfected VMM5B.β2.7G cells, indicates that defective HLA class I-associated antigen presentation in VMM5B cells is caused by a dysfunctional β₂m.

**Restoration of Low Level of HLA Class I Expression on β₂m-deficient Cells Transfected with a cDNA Construct Encoding the Mutant β₂mC25W**—To exclude the possibility that the observed low level of HLA class I expression on VMM5B cells is a cell line-specific phenomenon, we transfected a β₂mC25W-expressing cDNA construct into FO-1 cells, a well characterized β₂m-deficient cell line harboring a large deletion in the β₂m gene (8). Fourteen stable clones were established and analyzed for HLA class I expression on the cell surface by flow cytometry with mAb W6/32. Ten of them were stained weakly by mAb W6/32 as compared with the mock-transfected (FO-1.neo) and wild-type β₂m-transfected (FO-1.β2) controls (Fig. 10 and data not shown). These results indicate that the mutant β₂mC25W alone is able to restore low level of HLA class I expression on the cell surface regardless of the differences in the genetic background.

**DISCUSSION**

In this report, a naturally occurring, full-length mutant β₂m (β₂mC25W) in the melanoma cell line VMM5B and the metastasis from which the cell line originated was found to cause HLA class I down-regulation rather than loss because of its impaired association with HCs. Notably, the HC-β₂mC25W dimer does not appear to constitute a conventional peptide-receptive conformation because neither exogenous nor endogenous peptides can be presented on it, as indicated by the lack of induction of lysis by the cognate CTLs. These findings represent the first example of a β₂m structural abnormality that does not cause total HLA class I loss but causes defects in antigen presentation associated with their down-regulation. Importantly, the ability of the mutant β₂mC25W to restore a low level of HLA class I expression is not restricted to VMM5B cells because the same phenotype was observed in the allogeneic β₂m-deficient cell line FO-1 transfected with a β₂mC25W-expressing construct.

Several examples of single amino acid substitutions in proteins involved in HLA class I-associated antigen presentation, such as TAP1,
have shown that such mutations do not affect the level of protein expression (26). However, this is not the case for $\beta_{m}\text{C25W}$ presented here. The steady-state level of $\beta_{m}\text{C25W}$ in VMM5B cells is ~29-fold lower than that of its wild-type counterpart in VMM5A cells. Such a low level of accumulation could be explained by the increased degradation of $\beta_{m}\text{C25W}$ because of the structural instability caused by the Cys-to-Trp substitution at position 25. It is likely that a drastic alteration in the mode of folding of the nascent $\beta_{m}$ polypeptide chain occurs, resulting in either a folded protein with an altered three-dimensional conformation or an unfolded protein with a thermodynamically unstable structure. The latter possibility is supported by the results of our structural analysis because: (i) the strong steric hindrance by the Trp side chain is likely to interfere with the hydrophobic interaction between the two $\beta$-pleated sheets, and (ii) the disappearance of the disulfide link between Cys-25 and Cys-80 may not keep $\beta_{m}$ in an energetically favored conformation. The accumulation of $\beta_{m}\text{C25W}$ in VMM5B cells incubated with a proteasome inhibitor further suggests that $\beta_{m}\text{C25W}$ is unstable and ready for proteasome-mediated degradation. On the other hand, the predicted unfolding effect of the C25W substitution may not occur when Cys is replaced with a small, hydrophobic amino acid, such as alanine. In this situation, no apparent steric hindrance would take place, and the two $\beta$-pleated sheets may remain closely contacted, resulting in the proper folding of the $\beta_{m}$ protein. The importance of disulfide bonds in the structure and function of a protein has been indicated in several examples; one is represented by the replacement of Cys at position 634 of the RET (rearranged during transfection) proto-oncogene with a small, hydrophobic amino acid, such as alanine. This possibility is supported by the lack of refolding on the cell surface of the mouse major histocompatibility complex molecule H-2Dd HC asssociated with an artificially mutated $\beta_{m}\text{C25W}$ complexes was not increased significantly in IFN-γ- and MG132-treated VMM5B cells despite the marked elevation of indoleamine 2,3-dioxygenase (27).

It is noteworthy that not all the tumor cells in the metastasis, from which the VMM5B cell line had been derived, carry the described antigen presentation defect because the freshly isolated tumor cells showed a certain degree of susceptibility to tyrosinase-specific CTLs, as reported in our previous study (7). In this regard, the VMM5B cell line is likely to represent a subpopulation of tumor cells in the metastasis that has escaped control by CTLs in the course of the disease. Also worthy of mentioning is that the patient did not receive any type of immunotherapy in the course of the disease. Therefore, the elicited CTL response was spontaneous in nature as opposed to the response introduced by T cell-based immunotherapy. This is also reflected by the location of the identified $\beta_{m}$ gene mutation (TGC to TGG in exon 2), outside the mutational hot spot in exon 1, which in melanoma has been found mostly in patients exposed to T cell-based immunotherapy-related immune selective pressure (29). Whether this type of mutation will represent a common one among malignant cells exposed to spontaneous CTL immune selective pressure remains to be determined. From a practical viewpoint, the resistance of VMM5B cells to lysis by CTLs despite HLA class I expression on the cell surface represents another example of lack of correlation between HLA class I expression and CTL-mediated lysis in malignant cells (30). Understanding the mechanisms underlying this phenomenon as reported here may contribute to defining the molecular basis of tumor escape and optimization of the criteria to select patients to be treated with immunotherapy.

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β₂-Microglobulin Dysfunction and Antigen Presentation Defects