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Distinct Assembly Profiles of HLA-B Molecules

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MHC class I polymorphisms are known to influence outcomes in a number of infectious diseases, cancers, and inflammatory diseases. Human MHC class I H chains are encoded by the HLA-A, HLA-B, and HLA-C genes. These genes are highly polymorphic, with the HLA-B locus being the most variable. Each HLA class I protein binds to a distinct set of peptide Ags, which are presented to CD8+ T cells. HLA-disease associations have been shown in some cases to link to the peptide-binding characteristics of individual HLA class I molecules. In this study, we show that polymorphisms at the HLA-B locus profoundly influence the assembly characteristics of HLA-B molecules and the stabilities of their peptide-deficient forms. In particular, dependence on the assembly factor tapasin is highly variable, with frequent occurrence of strongly tapasin-dependent or independent allotypes. Several polymorphic HLA-B residues located near the C-terminal end of the peptide are key determinants of tapasin-independent assembly. In vitro refolded forms of tapasin-independent allotypes assemble more readily with peptides compared to tapasin-dependent allotypes that belong to the same supertype, and, during refolding, reduced aggregation of tapasin-independent allotypes is observed. Paradoxically, in HIV-infected individuals, greater tapasin-independent HLA-B assembly confers more rapid progression to death, consistent with previous findings that some HLA-B allotypes shown to be tapasin independent are associated with rapid progression to multiple AIDS outcomes. Together, these findings demonstrate significant variations in the assembly of HLA-B molecules and indicate influences of HLA-B–folding patterns upon infectious disease outcomes.


Major histocompatibility complex class I molecules bind and present antigenic peptides to CD8+ T cells and thus mediate immune responses against intracellular pathogens and cancers (reviewed in Ref. 1). MHC class I molecules are also important regulators of the activities of NK cells (reviewed in Ref. 2). MHC class I molecules comprise a H chain, a L chain called β2-microglobulin (β2-m), and a peptide, which are assembled in the endoplasmic reticulum (ER) of cells. The H chain is highly polymorphic. There are hundreds of variants of the HLA-A, HLA-B, and HLA-C genes, which encode human MHC class I H chains. The polymorphisms influence the specificities of peptide binding in the assembled MHC class I proteins to allow for the presentation of a distinct and diverse pool of antigenic peptides by each HLA class I molecule.

HLA class I molecules are known to exert profound influences on disease progression in a number of infectious diseases and cancers (reviewed in Refs. 3–6). Among all genetic factors known to influence outcome to HIV infection, the strongest associations link to HLA class I genes. The peptide-binding characteristics of individual MHC class I proteins are shown to be a major factor that determines immune control of HIV (7, 8), but other characteristics of the HLA molecules, such as those relating to variation in the assembly and stability of individual HLA class I molecules, may also have an influence on disease outcomes. By virtue of their highly polymorphic nature, the MHC class I molecules present unique challenges to the cellular protein-folding machinery. Thousands of variants (across the population) must be correctly assembled for immunity to be effective at the individual level. Folding and assembly of MHC class I molecules are critically dependent on rare and transient peptides within the ER lumen. HLA class I molecules that are suboptimally assembled are either retained in the ER or rendered unstable at the cell surface (9). Thus, the assembly and stability characteristics of individual HLA class I allotypes in addition to their peptide-binding specificities may also exert influences on disease outcomes.

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Abbreviations used in this article: ER, endoplasmic reticulum; HR, hazard ratio; LIC, ligation-independent cloning; β2m, β2-microglobulin; MFI, mean fluorescence intensity; PLC, peptide-loading complex; TAP, transporter associated with Ag processing; TEV, tobacco etch virus.
The assembly of MHC class I molecules occurs in the ER lumen, with the help of a multiprotein peptide-loading complex (PLC) (reviewed in Ref. 10). The transporter associated with Ag processing (TAP) is responsible for translocation of peptides from the cytosol into the lumen of ER and also serves as a scaffold for the PLC assembly (reviewed in Ref. 11). Tapasin, a key component of the PLC (12, 13), bridges a physical interaction between MHC class I and TAP to localize MHC class I in the vicinity of an incoming pool of TAP-translocated peptides. Tapasin also recruits the oxidoreductase ERp57 (14, 15) and the associated ER chaperone calreticulin (16–21) to facilitate MHC class I–peptide assembly in the ER. Although there may be multiple levels of quality control exerted on suboptimally assembled MHC class I proteins, tapasin has a propensity to interact with peptide-deficient forms of MHC class I molecules (19, 22). Tapasin stabilizes the peptide-free conformation of MHC class I molecules, quantitatively increasing peptide associations with MHC class I molecules (19, 23, 24). Tapasin also optimizes the MHC class I peptide repertoire in favor of high-affinity sequences (19, 24–26). Some HLA-B molecules are known to vary in their dependencies on tapasin for assembly and Ag presentation. For example, HLA-B*4402 and HLA-B*4405, which differ by a single residue at position 116 of the peptide-binding groove, are highly tapasin dependent or independent, respectively, for their assembly (25, 27, 28), which in turn affects intracellular trafficking (28–30). Tapasin-dependent or tapasin-independent HLA class I assembly is expected to influence the stability of antigenic peptide associations with MHC class I molecules. Additionally, tapasin-dependent or tapasin-independent HLA class I assembly could reflect intrinsic structural features of empty forms of MHC class I proteins. Because tapasin is a key determinant of the assembly and cell surface stability of several MHC class I proteins, in this study, we examined tapasin dependencies of several of the most frequent HLA-B allotypes observed in North American populations. To further examine potential relationships between intrinsic assembly and stability of empty HLA class I proteins and tapasin dependencies of their intracellular assembly, we also compared refolding and assembly efficiencies of soluble forms of the most highly tapasin-dependent or independent HLA-B allotypes. These studies revealed important differences between HLA-B molecules at the level of assembly and stability. Finally, we examined the influences of tapasin-dependent or independent assembly on progression to various AIDS outcomes.

Materials and Methods

Construction of ligation-independent cloning (LIC) pMSCVneo

The plasmid pMSCVneo (Clontech) was used as the parent vector for construction of a ligation-independent cloning (LIC) variant. This was accomplished by inserting a unique sequence that could be digested with restriction enzyme Pmel. LIC overhangs could then be generated by treatment of the linear plasmid vector with T4 DNA polymerase exonuclease activity. The inserted sequence was constructed by annealing two oligos to generate a fragment with overhangs complementary to an EcoRI restriction site. The oligo sequences used were 5′-AATTAGAGAGTTTCACTGCTGTGATGCA-3′ or 5′-GGAAATTTAACAGTCCATTGCGATCCG-′3 for the forward sequences and 5′-GAATTGTGAAGTTTCTTAAGCTTGAGGACACATC-′3 for the stop sequence. Some HLA-B sequences were previously described (30). HLA-B*3701 was from C. Traversari (MoImed S.P.A), HLA-B*1501 and HLA-B*1518 were from W. Hildebrand (Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center,) and HLA-B*3801 was from C. Gardiner (NK Cell Research Group, School of Biochemistry and Immunology, Trinity College, Dublin, Ireland). Irrespective of their source, all HLA-B sequences were cloned into retroviral vector MSCVneo using LIC. The oligos used for screening MSCVneo constructs were forward, 5′-CACCTTAAACCTGCTGCTCC-′3′ and reverse, 5′-AAATAAGGCCAGGAGCCAGCCAGG-′3′.

LIC was also used to transfer all HLA-B constructs into the pMCSG7 vector (31) for bacterial expression. DNA constructs encoding soluble HLA-B molecules containing N-terminal hexahistidine tags and tobacco etch virus (TEV) protease cleavage sites (and lacking signal sequences and transmembrane domains) were amplified by PCR using 5′-TACTTTCCATTCAATCTGTCAGCCACCTCCATGA-′3′ for the start sequence and 5′-TTATCCACATCTAAATGCTGCAGCCCTCAA-′3′ for the stop sequence. HLA-B-MSCVneo vectors were used as templates, and PCR products were cloned into pMCSG7 vector (31).

For LIC, overhangs of 15 bp were generated in the insert DNA by processing 0.2 pmol PCR products with 0.5 U LIC-qualified T4 DNA polymerase (Novagen) in the presence of 4 mM dCTP (for ligation with pMSCS7) or 4 mM dGTP (for ligation with pMSC-neo) and 5 mM DTT, 20 mM Tris–HCl reaction buffer, 1 mM MgCl2, 1.6–2.0 μl total, were set up with 1.6–2.0 μg linear DNA and 3.75 U LIC-qualified T4 polymerase (Novagen) in the presence of 4 mM dGTP (for pMSCS7) or 4 mM dCTP (for pMSC-neo) and 5 mM DTT. Reactions were mixed on ice and incubated, as described above, for inserts. Insert and vector DNA were annealed in 96-well plates by combining 1 μl processed vector and 2 μl processed insert per well, followed by incubation in a PCR machine at 22°C for 15 min with addition of 1 μl 25 mM EDTA after 10 min of incubation. Annealed DNA was used to transform competent XL1-Blue cells. All HLA-B constructs in the pMSCV or pMCSG7 vectors were sequenced by the University of Michigan DNA Sequencing Core.

Cell lines

A human melanoma cell line M553 (obtained from N. Bangia, Roswell Park Cancer Institute) (32) and CEM cells were grown in RPMI 1640 (Life Technologies) supplemented with 2 mM glutamine. BOSC cells (obtained from K. Collins, University of Michigan) were grown in DMEM (Life Technologies). All growth media were supplemented with 10% (v/v) FBS (Life Technologies), 100 μg/ml gentamicin, and 100 U/ml penicillin (Life Technologies).

Viruses and cell infections

Retroviruses were generated, as previously described, using BOSC cells and used to infect M553 cells (30). Cells were infected with retroviruses encoding the HLA-B molecules, selected by treatment with 1 μg/ml
G418 (Life Technologies), and maintained in 0.5 mg/ml G418. Exogeneous MHC class I expression was verified in M553 cells by flow cytometric analyses using the W6/32 Ab (33) and by immunoblotting analyses of cell lysates using the HC10 (34) or 171.4 Abs (35). M553 cells expressing HLA-B molecules were infected with the tapasin retrovirus and selected by treatment with 1 μg/ml puromycin (Sigma-Aldrich), and cells were maintained in 0.5 μg/ml puromycin. Tapasin expression in M553 cells was verified by immunoblotting analysis of cell lysates using rabbit anti-tapasin antisera (generated against an N-terminal peptide of tapasin; obtained from T. Hansen, Washington University). For immunoblotting analysis, the cells were lysed in Triton X-100 lysis buffer (1% Triton X-100 in PBS containing EDTA-free protease inhibitors [Roche] [pH 7.4]) for 1 h on ice. The lysates were centrifuged at 4°C for 30 min to remove cell debris, and protein concentration in lysates was determined by a bicinchoninic acid protein assay (Pierce, Thermo Scientific). Equal microgram amounts of cell lysates were separated on 10% SDS-PAGE and transferred to Immobilon membranes (Millipore) for immunoblotting. Membranes were blocked in 5% milk in TBS for 1 h at room temperature, followed by an overnight incubation with primary Ab in TBS–TWEEN 20 at 4°C. Western blotting analysis was done using HC10 or 171.4 Ab to detect MHC class I H chain and using rabbit anti-tapasin antisera to detect tapasin. Membranes were washed for 2 h in TBS–TWEEN 20, incubated for 60 min with secondary Ab, and washed again for 2 h. The secondary Abs were GAM-HRP, GAR-HRP (Jackson ImmunoResearch Laboratories), or GAM-IRDye 800CW (LI-COR Biosciences). The Western blots were developed for chemiluminescence using the GE Healthcare ECL Plus kit or scanned for IRDye fluorescence using Odyssey System (LI-COR Biosciences).

Flow cytometric analysis to assess MHC class I cell surface expression

A total of 1 × 10^5–1 × 10^6 cells was washed with FACS buffer (PBS [pH 7.4] containing 1% FBS) and then incubated with W6/32 Ab at 1:250 dilutions for 30–60 min on ice. Following this incubation, the cells were washed three times with FACS buffer and incubated with PE-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) at 1:250 dilutions for 30–60 min on ice. Following incubations, the cells were washed three times with FACS buffer and analyzed using a FACSCanto cytometer. The FACS data were analyzed with WinMDI software (J. Trotter, Scripps Institute, Flow Cytometry Core Facility) or FlowJo software (Tree Star).

MHC class I refolding and gel filtration analysis

The baculovirally expressed HLA-B constructs lacked signal sequences but contained an N-terminal MIHHEHHHSSGVDLGETNYFQSNF fusion sequence, including a histidine tag for nickel affinity chromatography and a TEV protease site for removal of the majority of the N-terminal tag (leaving a tripeptide sequence [SN]A) prior to the start of the mature HLA-B sequences. All soluble HLA-B constructs were truncated prior to the transmembrane domain after 276. The HLA-B-pMCSG7 vectors were transformed into BL21 cells for protein preparation. Inclusion body preparations of selected HLA-B H chains and β2m were undertaken, as previously described (36). The inclusion bodies were first solubilized in 6 M guanidine hydrochloride and diluted in 6 M guanidine hydrochloride to 200 or 25 μM stocks for β2m and H chains, respectively. Refolding reactions were initiated with 0.45 ml refolding buffer (100 mM Tris [pH 8.0], 400 mM l-arginine, 2 mM EDTA, 0.5 mM oxidized glutathione, 5 mM reduced glutathione) combined in some experiments with 5–10 μg TEV protease. Heterodimers were typically refolded by sequential dilution to 200 or 25 μM stocks for β2m and H chains, respectively. Refolding buffer with adequate stirring (using microspin bars), to final concentrations of 4 μM β2m and 2 μM H chains. The refolding reactions were conducted at room temperature for 1 h or at 4°C overnight, in the absence or presence of TEV, respectively, as indicated. Following refolding, insoluble proteins were removed by centrifugation in a microfuge at 13,000 rpm and 4°C for 30 min. Refolded soluble proteins were analyzed by gel filtration chromatography on a fast protein liquid chromatography system using Superdex 75 10/300 GL column (GE Healthcare). TEV-digested samples were incubated with nickel beads to remove uncleaved proteins, and the unbound fraction was used for gel filtration analyses. Indicated gel filtration fractions (peaks 1 and 2) were pooled and tested for peptide binding. For the HLA-B7 supertype, pooled peak 1 and peak 2 fractions (10–20 μM) were directly used in binding assays. For the HLA-B44 supertype, peak 1 and peak 2 fractions were typically concentrated to 200–400 nM prior to use in binding assays, but similar results were obtained if protein was used at 10–20 μM in binding assays, without a prior concentration step. Protein fractions were incubated with 10-fold molar excess of purified β2m and peptide at 37°C for 2 h or at room temperature for 24 h. Following incubations, the samples were separated by 15% native-PAGE, and gels were scanned using a Typhoon scanner (GE Healthcare) for fluorimaging analyses.

Statistical analysis

For Figs. 1, 2, 4, and 5, statistical analyses were performed using the GraphPad Prism 6 (GraphPad Software). The p values were calculated using the paired or unpaired Student t test or one-way ANOVA with Tukey’s multiple comparison test. A p value of 0.05 was considered significant. Pearson correlation and linear regression analyses were also done using GraphPad Prism 6.

AIDS progression studies

The 496 HIV–1–infected subjects for whom the dates of seroconversion were known were derived from three cohorts: the Multicenter AIDS Cohort Study (37), the Multicenter Hemophilia Cohort Study (38), and the D.C. Gay Cohort Study (39). SAS (version 9.2, SAS Institute) procedure PROC PHREG was used for Cox proportional hazards regression analyses. Four AIDS-related outcomes were considered in the regression models, as follows: a CD4+ T cell count of <200 cells/ml, progression to AIDS according to the 1993 definition of the Centers for Disease Control, progression to AIDS according to the more stringent 1987 Centers for Disease Control definition, and death from an AIDS-related cause. Statistical significance refers to two-sided p values <0.05. PROC PHREG with STEPWISE selection was performed, including mean fluorescence intensity values as a continuous variable and presence versus absence of all individual HLA-B alleles with ≥5% frequency in the Cox model. The significance level for selecting a variable to stay in the model was p < 0.05.

Results

Tapasin dependencies of HLA-B assembly

Using immortalized B cell lines from HLA-typed donors, cDNAs were generated for several of the most frequent HLA-B allotypes observed in North American populations. The sequences were cloned into retroviral vectors and verified, and the different HLA-B alleles were expressed in a tapasin-deficient human melanoma cell line (M553) (32). Cell surface expression of HLA-B allotypes was analyzed by flow cytometry (Fig. 1A). HLA-B allotypes showed large variations in cell surface expression in M553 cells (Fig. 1A). Cell surface expression of HLA-B*3501, B*4001, B*1801, B*4405, B*3503, and B*1501 was high and ~7– to 15-fold higher than the cell surface expression of the endogenous MHC class I of M553 cells (Fig. 1A), which are HLA-A*28, HLA-B*5001, and HLA-B*5701 (32). Cell surface expression of B*3801, B*5701, B*1302, B*5101, B*0801, B*4901, B*5021, B*4403, B*5802, and B*4402 was very low, and on average either undetectable or <2-fold above endogenous MHC class I cell surface expression (Fig. 1A). Other HLA-B allotypes, such as HLA-B*1510, B*5703, B*4201, B*5301, B*0702, B*3701, B*5801, B*2705, B*1518, B*1503, and B*4501, showed intermediate phenotypes, which were 2.5- to 8-fold higher than endogenous MHC class I cell surface expression (Fig. 1A). The levels of HLA-B allotype expression shown in Fig. 1 are averaged from five independently infected sets of cell lines. Thus, the observed effects are not due to differences in individual retroviral vector preparations.

In general, there was poor correlation between extracellular MHC class I expression assessed by flow cytometry (Fig. 1A) and total cellular expression assessed by quantitative immunoblotting analyses for HLA class I H chains (Fig. 1B). The latter analyses indicated that the intracellular expression of the majority of HLA-B alleles was within 2-fold of each other and of HLA-B*1801 (one of the higher expressing HLA-B allotypes) (Fig. 1C). Serial dilutions of HLA-B*1801–expressing cell lysates (Fig. 1C, last three lanes on the right) verified that the protein loads used were within the linear ranges of the quantitative immunoblotting assays. Some tapasin-dependent HLA-B allotypes consistently displayed lower total cellular expression compared with tapasin-independent allotypes (for example, HLA-B*4403 and HLA-B*4402 compared with...
Variable expression of HLA-B molecules in tapasin-deficient cells. The y-axes show cell surface expression ratios (MFI ratios) of MHC class I staining in M553 cells (a tapasin-deficient melanoma cell line) infected with retroviral constructs encoding the indicated HLA-B allotypes relative to M553 cells that were infected with a virus lacking MHC class I (vector). MHC class I surface expression was analyzed by flow cytometry using the W6/32 Ab. Statistical analyses were done using a one-way ANOVA test, followed by a Tukey’s multiple comparisons procedure for all pairwise differences of means. Significant differences are indicated (with an asterisk) on the graph (p < 0.05). Data represent averaged MFI ratios derived from 10–15 independent flow cytometric analyses from five independent infections (infections 1–5) of M553 cells. In infection 5, a Pearson analysis was used to examine correlation between cell surface MFI ratios assessed by flow cytometry and total cellular expression ratios assessed by immunoblotting of cell lysates. A significant correlation was not observed. (C) Top panel, Cell lysates from infection 5 of M553 cells were tested by quantitative fluorescence-based immunoblotting with the H chain–specific 171.4 Ab. The bar graphs show total cellular expression ratios (relative to cells expressing HLA-B*1801) of the indicated HLA-B molecules. Averaged values from three independent sets of immunoblotting analyses of infection 5 are shown. Lower panel, Representative quantitative immunoblots with the 171.4 Ab of indicated cell lysates from infection 5. A quantity amounting to 10 μl total cell lysate was loaded in each lane, unless otherwise indicated. The last three lanes show HLA-B*1801 H chain expression in M553-HLA-B*1801 lysates (incremental loads of 2.5–10 μl).

HLA-B*4405 [representative data are shown in Fig. 1C]), suggesting the possibility of enhanced ER-associated degradation of some HLA-B allotypes.

We next examined cell surface expression of MHC class I molecules in each of the HLA-B–expressing M553 cells following further infections with a tapasin-encoding virus (using a separate retroviral vector with a different drug selection vehicle) (Fig. 2A). Similar levels of tapasin were expressed in most cell lines as assessed by immunoblotting analyses (data not shown). Expression of tapasin strongly induced cell surface expression of endogenous MHC class I of M553 cells (>20-fold; Fig. 2B). The mean fluorescence intensity (MFI) ratios (+tapasin/−tapasin) were compared for the different HLA-B–expressing cell lines, after subtracting background signals from the corresponding M553 cells lacking exogenous HLA-B expression (Fig. 2A). There was a strong inverse correlation between low cell surface expression under tapasin-deficient conditions and the extent of tapasin-mediated induction (Fig. 2C).
normalizations of the relative protein concentrations and the addition of excess \( \beta_{2m} \). These binding studies revealed that the refolded forms of allotypes with high tapasin independence (HLA-B*1801 and HLA-B*4405) bound more efficiently to the peptide than those with low tapasin independence (HLA-B*4402 and HLA-B*4403) (Fig. 3C). Within the B7 supertype, the HLA-B*3501 and HLA-B*3503 allotypes share similarities with each other and with HLA-B*5101 (which is strongly tapasin dependent for its assembly) in peptide binding, with common P2 and some common P9 anchor residues (46–49). The HIV-1 Pol-derived peptide IPLTEEAEL elicits cytotoxic T cell responses in the context of both HLA-B*3501 and HLA-B*5101 (50). We used a modified version of IPLTEEAEL (IPLK\textsuperscript{PITC}EEAEEL) to compare peptide binding by the refolded HLA-B35 and HLA-B*5101 allotypes. Again, the highly tapasin-independent HLA-B35 allotypes bound to the peptide more efficiently, compared with HLA-B*5101 (Fig. 3D).

For the tested HLA-B allotypes, both the peak 1 and 2 fractions were competent for binding fluorescent peptides in the presence of excess \( \beta_{2m} \), with slightly reduced assembly competence for peak 1 compared with peak 2, as assessed by native-PAGE gels and fluorimaging analyses (Fig. 3C, 3D). In comparison, the protein fraction recovered in the void volume was poorly assembly competent (data not shown). Comparisons of the refolding patterns of the most tapasin-dependent and tapasin-independent allotypes revealed a hierarchy of refolding efficiencies (Fig. 4A, 4B). Although not absolute, there was a trend toward increased recovery of assembly-competent (peak 1 + peak 2) fractions for the peptide-deficient forms of tapasin-independent allotypes compared with their tapasin-dependent counterparts (Fig. 4C, 4D). Compared with overnight refolding at 4°C, refolding differences between tapasin-dependent and tapasin-independent allotypes were more pronounced following room temperature refolding for 1 h, which generally increased the level of aggregation (Fig. 4C, 4D; TEV was not included in the 1-h refolding reactions, as an overnight reaction is recommended for completion of TEV digestions). Together, the findings of Figs. 3 and 4 are consistent with the model of decreased conformational heterogeneity of peptide-deficient forms of several tapasin-independent allotypes compared with their tapasin-dependent counterparts, which results in greater assembly competence and reduced aggregation of the peptide-deficient forms of tapasin-independent allotypes.

Determinants of tapasin-independent assembly

The Bw4/6 Ab epitope is contained within residues 77–83 of the HLA-B H chains, and is a key determinant of NK cell-inhibitory receptor engagement by HLA-B molecules (51). Expression studies in M553 cells indicated that the most strongly tapasin-independent allotypes were of the HLA-Bw6 serotype (with the exception of HLA-B*4405), whereas many allotypes that were poorly expressed in M553 cells were of the HLA-Bw4 serotype (with the exception of HLA-B*0801) (Fig. 1A). HLA-Bw4 versus HLA-Bw6 groups are significantly different for tapasin-independent allotypes (Fig. 5A). Additionally, in the refolding analyses, the yields of the folding competent fractions are significantly different for Bw6 epitopes compared with Bw4 epitopes (Fig. 5B).
although a hierarchy of assembly efficiencies is also observed within each group (Fig. 4). Together, these findings suggested that the region corresponding to the Bw4/6 epitopes, a known determinant of NK recognition (51), is also a key determinant of the assembly and

![Image](http://www.jimmunol.org/)

**FIGURE 3.** Higher assembly competence of soluble refolded tapasin-independent HLA-B allotypes. A soluble form of the indicated HLA class I H chains (2 μM) was refolded with β2m (4 μM) overnight at 4°C in the presence of TEV. Following refolding, the soluble fractions were analyzed by gel filtration chromatography using a Superdex 75 10/300 GL column. Representative gel filtration analyses of (A) B1801, B4402, B4403, and B4405 (B44 supertype) and (B) B3501, B3503, and B5101 (B7 supertype) are shown. The vertical lines show peak position ranges of the gel filtration column void volume (left), peak 1 (middle), and peak 2 (right). (C and D) Peak 1 and peak 2 fractions from (A) and (B) were assessed for their peptide-binding competence. Peak 1 and peak 2 fractions were normalized for protein concentration (to 200 nM [peak 1] or 400 nM [peak 2] for B44 supertypes, and 20 nM [peaks 1 and 2] for B7 supertypes) and incubated with a 10-fold excess of refolded β2m and FITC-labeled peptide (peptide EEFGKFITCAFSF for B44 supertypes and peptide IPLKFITCEEAEL for B7 supertypes). Peptide-bound heterodimers were separated by native gel electrophoresis and visualized by fluorescence scanning. Both peak 1 and peak 2 fractions are competent for peptide binding. Representative data of four independent measurements are shown.

**FIGURE 4.** Variable levels of aggregation of soluble empty HLA-B molecules following refolding. Soluble forms of the indicated MHC class I H chains were refolded with β2m. (A) overnight at 4°C with TEV or (B) at room temperature (22°C) for 1 h without TEV. Following refolding, soluble proteins were analyzed by gel filtration chromatography. Percentages of fractions corresponding to the void volume and peak 1 + peak 2 (peptide-binding competent fractions) of different allotypes are shown (left y-axis), and total protein yields (right y-axis) are also shown. Data represent averaged values from three independent refolding reactions with two different inclusion body preparations for all allotypes except HLA-B*0801 and HLA-B*1302, which represent averaged values of two independent refolding reactions with a single inclusion body preparation. (C and D) Percentages of peak 1 + peak 2 fractions, derived from data in (A) and (B), were compared for the tapasin-independent and tapasin-dependent allotypes. Differences between two groups are significant when refolding is conducted under the more stringent conditions (1 h at 22°C). Statistical analyses were performed using an unpaired t test. **p < 0.01.
stability characteristics of HLA-B molecules. To further examine individual residue contributions to tapasin-independent assembly, HLA-B allotypes were grouped based on dimorphic or polymorphic residue distributions at each position of their extracellular domain sequences, and averaged MFI ratios derived in Fig. 1A were compared between groups. A total of 47 dimorphic or polymorphic HLA-B residues was compared (corresponding to positions 9, 11, 12, 24, 30, 32, 41, 45, 46, 62, 63, 65, 66, 67, 69, 70, 71, 74, 77, 80, 81, 82, 83, 94, 95, 97, 99, 103, 113, 114, 116, 131, 143, 145, 147, 152, 156, 158, 163, 167, 171, 177, 178, 180, 194, 199, and 282 of the H chain sequences). Statistically significant averaged MFI differences were observed for polymorphisms corresponding to positions 77 (S versus N), 80 (N versus I), 81 (L versus A), 82 (R versus L), and 83 (G versus R) (Fig. 5C–H), residues that define the Bw4/Bw6 epitope differences.

Despite significant influences of residues within the HLA-Bw4/Bw6 region upon tapasin-independent assembly (Fig. 5C–H), these residues alone are insufficient to fully determine tapasin independence. Within the HLA-Bw4 group, N77A81L82R83 is present within the most tapasin-dependent HLA-Bw4 allotypes, but also in the strongly tapasin-independent HLA-B*4405 and several strongly tapasin-dependent HLA-Bw4 molecules (Fig. 6A, 6B). Within the Bw6 group, S77N80L81R82G83 is present in the most tapasin-independent HLA-Bw6 allotypes as well as in HLA-B*0801, which is strongly tapasin dependent (Fig. 6A). Similar to the HLA-Bw4 group, amino acids within/near the F-pocket region may constitute the determinants of assembly differences between HLA-B*0801 and tapasin-independent HLA-Bw6 molecules, including residues 9, 74, 97, 114, and 156 (Fig. 6A, 6C). Although residue variations at positions 9, 74, 94, 95, 97, 113, 114, 116, and 156 do not result in significant differences in tapasin independencies between the tested HLA-B allotypes (Fig. 5H–P), these residues are expected to influence the structure and conformation of the peptide-binding groove in the vicinity of the F-pocket, and thus also determine the nature of interactions with the peptide C terminus.

Influences of tapasin-independent assembly on progression to various AIDS outcomes

We examined whether tapasin-dependent or independent assembly influences progression to AIDS and survival in HIV infections. Individuals were assigned tapasin-independence scores based on their HLA-B genotypes. The MFI ratio in M553 cells (Fig. 1A) was
used to compute mean tapasin-independent expression of the two relevant HLA-B allotypes in each individual. Cox models were used to examine the influences of mean tapasin-independent expression as a continuous variable upon progression to three AIDS outcomes in European-American subjects infected with HIV. Significant effects of MFI values on progression to different AIDS outcomes were observed (death, $n = 496$, $p = 0.001$, hazard ratio [HR] = 1.11; AIDS 87, $n = 496$, $p = 0.02$, HR = 1.06; CD4, $n = 458$, $p = 0.02$, HR = 1.06), in which the HR represents an increase in 1 MFI unit. Thus, the difference between homozygotes for the most extreme tapasin-independent and dependent alleles, B*3501 and B*4402, is 11.94 U with HR = 2.0. When all HLA class I alleles with frequencies $\geq 3\%$ were included in the model, the effect of MFI values upon progression to death remained significant ($n = 496$, $p = 0.001$, HR = 1.12). These data suggest that greater tapasin-independent assembly confers more rapid progression and that the effect of tapasin-independent assembly is primarily a late effect in AIDS progression.

**Discussion**

Low and high tapasin independence were prevalent among 37% (10 allotypes) and 22% (6 allotypes), respectively (Fig. 1A), of the 27 HLA-B allotypes considered in this study. Based on the extent of assembly (Fig. 3) or the extent of aggregation of soluble refolded proteins (Fig. 4), tapasin-independent allotypes were found to be more assembly competent and more stable in their empty forms compared with tapasin-dependent allotypes. Higher intrinsic assembly competence of tapasin-independent allotypes is consistent with findings from other recent in vitro refolding/assembly studies (41), and with previous findings of more rapid intracellular trafficking of tapasin-independent allotypes compared with their tapasin-dependent counterparts belonging to the same supertype (for example, HLA-B*4405 compared with HLA-B*4402 and HLA-B*4403, and HLA-B*3501 compared with HLA-B*3501 (28–30, 53, 54)). These findings support the view that peptide-binding grooves of tapasin-independent HLA-B allotypes are in a more stable peptide-receptive conformation compared with tapasin-dependent HLA-B molecules. Molecular dynamics simulation studies have suggested significant differences in the dynamics of the F-pocket regions of HLA-B*4402 and HLA-B*4405, with greater differences between the peptide-bound and peptide-free forms of HLA-B*4402 compared with those of HLA-B*4405 (55). Thus, differences in the structure and flexibility of MHC class I molecules in the absence of bound peptide may play a key role in determining the requirement for tapasin during peptide loading.

The studies described in this work point to several residues in the vicinity of the C-terminal end of the peptide, including those comprising Bw4/Bw6 epitope differences, as key determinants of tapasin-independent assembly (Figs. 5, 6). Consistent with these findings, tapasin-mediated peptide selection is suggested to disrupt hydrogen bonds near the peptide C terminus (24), and peptides bound to MHC class I molecules of tapasin-deficient cells are elongated and contain a broader set of C-terminal residues compared with those derived from wild-type cells (56). Near the peptide C terminus, polymorphic Bw4/Bw6 determinants 77 and 80 and invariant residue 84 interact with the peptide main chain (for example, Refs. 28, 52, 57). Residue 80 forms hydrogen bonds with the peptide C-terminal carboxyl group. S77 could allow for bulky/hydrophobic P9 side chains to be more readily accommodated within the F-pocket compared with N77, and polymorphic residue 81 (L versus A) could alter the nature of interactions with the peptide P9 residue. Furthermore, residues 82 and 83 could determine the overall conformational stability of interactions mediated by residues 77, 81, and 84 with the peptide main chain and side chain. The combination of interactions with the peptide C terminus could intrinsically be more favorable for tapasin-independent allotypes. Additionally, the significant Bw4/Bw6 differences in tapasin independence suggest that tapasin is particularly critical for guiding the expression or peptide repertoires of HLA-B molecules that are recognized by NK receptors.
Our findings show that HLA-B molecules vary in their requirements for tapasin for their assembly (Fig. 1A), and additionally that refolded empty HLA-B molecules have distinct assembly competencies and patterns of stability of their empty forms (Figs. 3, 4). Such differences can influence the stabilities of antigenic peptide associations with HLA-B molecules, as well as interactions with ER quality control factors, and thus the abilities of different HLA-B molecules to mediate immune responses during infections. Our findings indicate that the presence of tapasin-independent allotypes links to a greater HR for death following HIV infections. Consistent with these findings, allotypes such as HLA-B*5701 and HLA-B*2705 that are associated with slow progression to AIDS (reviewed in Refs. 3, 4) display low or intermediate tapasin independence (Fig. 1A). Some studies also suggest protective effects of HLA-Bw4 homozygosity in HIV infections (58), and low tapasin independence is strongly prevalent within the HLA-Bw4 serotype (Figs. 1, 5). Conversely, some allotypes such as HLA-B*3503 and HLA-B*3501 that are associated with more rapid AIDS progression (4) are highly tapasin independent for their assembly. Tapasin is suggested to function as a peptide editor, facilitating MHC class I occupancy with high-affinity peptide (19, 24–26). MHC class I molecules loaded with a slow-dissociating peptide are expected to be more stable at the cell surface and thus may be able to present Ags to the CD8+ T cells over longer durations. In contrast, the tapasin-independent allotypes may be loaded with fast-dissociating suboptimal peptides. Suboptimal epitope selection could result in more transient and suboptimal Ag presentation to the CD8+ T cells. Although more detailed mechanistic studies are needed to understand the cellular and molecular basis for the differences in disease progression, these findings suggest that assembly characteristics of HLA-B molecules can and do influence survival outcomes following infections. Whereas the presence of tapasin-independent HLA-B molecules enhances progression to death in HIV infections, it is possible that tapasin-independent assembly confers advantages in other infectious contexts, particularly those that interfere with MHC class I assembly in the ER. Further studies are needed to better understand the influences of HLA-B assembly/ stability characteristics upon disease outcomes in other disease contexts and upon global CD8+ T cell responses during infections.

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References


