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Quantitative and Qualitative Influences of Tapasin on the Class I Peptide Repertoire

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Tapasin is critical for efficient loading and surface expression of most HLA class I molecules. The high level surface expression of HLA-B*2705 on tapasin-deficient 721.220 cells allowed the influence of this chaperone on peptide repertoire to be examined. Comparison of peptides bound to HLA-B*2705 expressed on tapasin-deficient and -proficient cells by mass spectrometry revealed an overall reduction in the recovery of B*2705-bound peptides isolated from tapasin-deficient cells despite similar yields of B27 heavy chain and β2-microglobulin. This indicated that a proportion of suboptimal ligands were associated with B27, and they were lost during the purification process. Notwithstanding this failure to recover these suboptimal peptides, there was substantial overlap in the repertoire and biochemical properties of peptides recovered from B27 complexes derived from tapasin-positive and -negative cells. Although many peptides were preferentially or uniquely isolated from B*2705 in tapasin-positive cells, a number of species were preferentially recovered in the absence of tapasin, and some of these peptide ligands have been sequenced. In general, these ligands did not exhibit exceptional binding affinity, and we invoke an argument based on luminal availability and affinity to explain their tapasin independence. The differential display of peptides in tapasin-negative and -positive cells was also apparent in the reactivity of peptide-sensitive alloreactive CTL raised against tapasin-positive and -negative targets, demonstrating the functional relevance of the biochemical observation of changes in peptide repertoire in the tapasin-deficient APC. Overall, the data reveal that tapasin quantitatively and qualitatively influences ligand selection by class I molecules. The Journal of Immunology, 2001, 166: 1016–1027.

The assembly of HLA class I molecules involves a complex chaperone-mediated pathway within the lumen of the endoplasmic reticulum (ER) (1). After targeting to the ER, nascent class I heavy chain (hc) is stabilized by the chaperone calnexin. Once β2-microglobulin (β2m) associates with the hc to form class I heterodimers, calnexin is exchanged for calreticulin. At this stage other chaperones combine to form the class I loading complex, which consists of the class I heterodimer, calreticulin, ERp57, and a specialized chaperone called tapasin (1). Tapasin is a 48-kDa glycoprotein that bridges peptide-receptive class I heterodimers to TAP molecules (2, 3). This interaction is thought to facilitate the loading of antigenic peptides into the binding cleft of class I molecules before their exit from the ER and transport to the cell surface. In addition to a bridging function, tapasin is thought to stabilize the peptide-receptive state of the class I complex (4), influence expression of TAP (4, 5), and increase peptide binding to the TAP heterodimer (6). It has also been demonstrated that tapasin retains empty MHC class I molecules in the ER of insect cells (7). We have also shown that tapasin prevents premature release of class I molecules from the ER of mammalian cells, suggesting that tapasin may also play a role in the retention of suboptimally loaded class I molecules (8). These functions may be accomplished directly by tapasin or indirectly through the concerted actions of other chaperones recruited into the class I loading complex in the presence of tapasin.

Tapasin appears to be highly specific to the class I assembly pathway, while many of the other chaperones involved in class I assembly are more generic and involved in the folding and assembly of most glycoproteins in the ER (9). Therefore, it has been speculated that tapasin may play a role in class I ligand selection and optimization. Several features of tapasin make its putative editorial role attractive; these include the ability to retain empty or suboptimally loaded class I molecules in the ER (7, 8) and the observation that class I molecules that are expressed in tapasin-deficient cells are largely thermolabile and are loaded with a proportion of suboptimal ligands (10–12). Furthermore, recent studies have implied that tapasin interacts with regions of the class I hc that are sensitive to the presence of peptide in the Ag binding cleft (13, 14), reflecting the potential for tapasin to discriminate between peptide-loaded and empty molecules.

Recently, two groups have independently generated tapasin knockout mice (15, 16). The class I molecules expressed in these
mice (H-2Kb and H-2Db) demonstrate pronounced tapasin-dependence for their efficient surface expression. The defect is not absolute, however, with evidence that some pathogen-derived peptides are presented by these class I molecules during infection (16) and that endogenous peptide presentation is sufficient to provide limited positive selection in the CD8+ T cell compartment and to induce tolerance (15). In humans, the tapasin-deficient cell line 721.220 (17) has been used to study the behavior of HLA class I molecules under these conditions. HLA A and HLA B alleles display marked variation in their tapasin dependence for both efficient surface expression and presentation of antigenic determinants to CTL in 721.220 transfectedants (12, 18–20). For example, tapasin is not required for high levels of surface expression of HLA-B*2705 on 721.220 cells or presentation of viral determinants to CTL by these molecules (12). In contrast, functional Ag presentation and surface expression of HLA-B*4402 are highly dependent on tapasin, whereas HLA-B*0801 lies between these alleles in the spectrum of tapasin dependence (12). This variation in surface expression among class I alleles in tapasin-deficient cells most likely reflects their differential abilities to load endogenous peptides in the absence of tapasin. Other factors may also contribute to high levels of surface expression on tapasin-deficient cells. For example, some class I molecules egress to the cell surface in the absence of a functional interaction with tapasin that results either from species incompatibility between the class I molecules and components of the class I loading complex (8) or from mutations in the class I heavy chain itself (11). Thus, the murine class I molecule H-2Kb is expressed at high levels on the surface of the human 721.220 cells despite poor surface expression in tapasin knockout mice (15, 16). This is probably due to 1) greater efficiency of assembly of Kb/human beta2M complexes despite suboptimal ligand selection, and 2) defective retention of Kb molecules by the human class I loading complex (8). Similarly, the HLA A2 mutant T134K is not properly retained in the ER by the class I loading complex and so expresses at high levels at the cell surface (11). However, neither Kb expressed in 721.220 nor the HLA A2 T134K mutant are likely to reflect physiological examples of a tapasin-independent loading pathway, since both function very poorly in Ag presentation and appear to be loaded with suboptimal peptide repertoires (8, 11, 20). By comparison, HLA B*2705 is remarkably efficient at Ag presentation and ligand selection in the absence of tapasin, suggesting that a tapasin-independent pathway of B27-peptide loading might operate at some level even under normal conditions.

Despite achieving normal steady state levels of surface expression, there is evidence that the assembly of HLA B*2705 with peptide is less efficient in the absence of functional tapasin. For example, a delay in the kinetics of B27-restricted viral peptide presentation to CTL was observed in tapasin-deficient 721.220 cells (12). Moreover, surface class I peptide complexes appear less stable in the absence of tapasin, as revealed by more rapid surface decay (10) and an increase in the expression of denatured HLA B*2705 molecules on the surface of 721.220 transfectedants (12). Tapasin also appears to influence the peptide repertoire of HLA B*2705 qualitatively, as highlighted by the ability of certain peptide-sensitive mAbs to differentiate between B*2705 molecules expressed in the presence and the absence of tapasin (10, 12). This differential epitope expression suggests that the peptides presented by HLA B*2705 in the presence and the absence of tapasin might not be identical. Thus, we have chosen to study the repertoire of peptides captured by HLA B*2705 in the presence and the absence of tapasin for the following reasons: 1) a comparison of the peptides presented in the presence and the absence of tapasin provides insights into the function of tapasin; and 2) HLA B27 exhibits redundancy in assembly and Ag presentation pathways (12, 21, 22), and there are grounds to believe that loading of some B27 molecules uses a tapasin-independent pathway in normal cells. Therefore, the principles of repertoire selection under these circumstances are important. 3) The relative tapasin independence of B27 molecules appears unusual among HLA class I alleles, most of which are dependent upon tapasin for high level surface expression and Ag presentation; and 4) any unusual properties or functions of B27 are of great interest because of its strong association with ankylosing spondylitis. Peptides were eluted from B*2705 molecules expressed on the surface of transfected 721.220 (B*27.220) cells and from the same cells reconstituted with human tapasin (B*27.220.hTsn), then were analyzed by a combination of reverse phase HPLC (RP-HPLC) and mass spectrometry. Our findings demonstrate that tapasin not only enhances peptide loading quantitatively, but its expression is associated with qualitative changes in the repertoire of endogenous ligands presented for T cell recognition. We have determined the amino acid sequence of some ligands that are loaded into the cleft of HLA B27 in the presence and the absence of tapasin. The protein source and binding to HLA B*2705 of such peptides reveal that neither cellular abundance nor B27 peptide affinity explain their tapasin independence. The ability of HLA B27 to acquire antigenic peptides via this tapasin-independent pathway is discussed in the context of the association of this allele with inflammatory arthropathies.

Materials and Methods

Cell lines and culture

721.220 is a human lymphoblastoid cell line in which HLA A and B genes have been deleted and a nonfunctional tapasin protein is expressed (17, 23). The transfection of HLA B*2705 and wild-type human tapasin into 721.220 has been described previously (12). The human TAP-deficient cell line T2 transfected with HLA B*2705 (T2.B27) has been described previously (24). All cells were grown in RF-10 (RPMI 1640, Life Technologies, Gaithersburg, MD) supplemented with 2 mM glutamine (Life Technologies), antibiotics, and 10% FBS (Commonwealth Serum Laboratories, Melbourne, Australia). For the peptide elution experiments cells were grown in miniPERM bioreactors (Hereaus, Hanau, Germany) and sequentially conditioned into hybridoma serum-free media (Life Technologies) containing 1% FBS. Flow cytometry and Western blots were used to establish that B*2705 and tapasin were stably expressed in the transfected cell lines during expansion as previously described (25).

Alloreactive CTL and 51Cr release assay

721.220, B27.220, and B27.220.hTsn cells were examined for recognition by a panel of HLA B27-specific alloreactive CTL. These CTL comprised specific alloreactive clones raised against normal B27-positive cell lines that were selected on their ability to recognize B*2705 in a peptide-dependent manner (26, 27). Target cells were grown in RF-10, labeled for 90 min at 37°C with 50 μCi of 51Cr, washed four times, and resuspended in the same medium with 1% FBS. Target cells were then seeded into 96-well plates and coincubated with effector CTL for 4 h at 37°C at given E:T cell ratios. Supernatants of this coculture were harvested and subjected to gamma counting. The percent specific 51Cr release was calculated as ((experimental lysis− spontaneous lysis)/(maximum release− spontaneous lysis)) × 100.

Generation of B27-restricted, peptide-dependent CTL against allogeneic tapasin-deficient APC

The SB and NK series of clones were raised in this study by stimulating PBMC from healthy B27-negative donors with the tapasin-deficient B27.220 cell line as follows. PBMC (2 × 106) from donor SB (HLA A2 A30 B35 B57) or NK (HLA A2 A2 B14 B44) were stimulated in 2 ml of RF-10 with gamma-irradiated (8000 rad) B27.220 stimulator cells at a responder to stimulator ratio of 25:1. After 3 days cells were dispersed and seeded in 0.35% agarose (Seaplaque, FMC BioProducts, Rockland, ME) containing 55% RPMI 1640, 20% FCS, 25% supernatant from MLA-144 cultures, and 30 U/ml rIL-2. Colonies were harvested after an additional 4 days and were amplified in culture with biweekly restimulation with rIL-2, MLA-144 supernatant, and irradiated B27.220 cells.
Preparation of W6/32 affinity matrix

An affinity matrix comprised of 10 mg/ml W6/32 cross-linked to protein A-Sepharose resin was prepared by modifying published methods (28). Briefly, W6/32 was bound (10 mg/ml resin) to protein A-Sepharose, and the resin was washed extensively in borate-buffered PBS. A solution of 40 mM dimethylpyrolium chloride (Sigma, St. Louis, MO) in 0.2 M triethanolamine, pH 8.0, was used to cross-link the Fc region of W6/32 to the protein A matrix, enhancing the spatial distribution and conformational integrity of the immobilized Ab. Cross-linking proceeded for 1 h at room temperature, and the reaction was terminated by addition of ice-cold 40 mM ethanolamine, followed by extensive washing steps.

Metabolic labeling of cells and isolation of HLA B*2705-bound peptides

For metabolic labeling studies, 1 × 10^6 cells were washed in PBS and transferred into arginine-free RPMI (RPMI SelectAmmine kit, Life Technologies) supplemented with 10% dialyzed FBS and 2 mM glutamine. The cells were incubated at 37°C for 1.5 h in arginine-free medium, metabolically labeled with 750 μCi of [3H]arginine (arginine l-([2, 3, 4-3H]mononitrophenyl)hydrochloride, AMRAD, Melbourne, Australia) for 12 h at 37°C. Cells were harvested and lysed in 0.5% Nonidet P-40 (Sigma), 150 mM NaCl, and 10 mM Tris (pH 8.0) supplemented with Complete protease inhibitor cocktail (Roche, Mannheim, Germany). An aliquot of this lysate was retained to normalize for label uptake. HLA B*2705-peptide complexes were immunoprecipitated using W6/32-protein A-Sepharose at a ratio of 50 μl resin/10^6 cells. The resin was washed extensively, and peptides were dissociated from the immobilized B*2705 molecules by treating the resin with 10% acetic acid. The eluate was subsequently passed through a Microcon-3 (Millipore, Bedford, MA) centrifugal filter to separate the peptides from β-m and hcc. The retentate was analyzed by SDS-PAGE and was shown to contain β-m and hcc. The flow-through (≤3 kDa) was then fractionated by RP-HPLC using a SMART system HPLC (Pharmacia Biotech, Upsalla, Sweden). Peptides were separated using a μRPC C18 (2.1 mm, inside diameter) × 10-cm column; Pharmacia Biotech, Upsalla, Sweden) operating initially with a linear gradient from buffer A (0.1% trifluoroacetic acid (TFA)) to 40% B (acetonitrile/0.09% TFA), 1.3%/min at a flow rate of 200 μl/min. Following this gradient a more rapid 40% B to 60% B, 4%/min gradient was employed at a flow rate of 200 μl/min. Individual HPLC fractions (150 μl) were collected, and the incorporated [3H]arginine was measured by a beta liquid scintillation counter (Packard, Meriden, CT).

Purification of HLA B*2705 and peptide analysis

Purification of HLA B*2705 from various cell lines was performed based on the method of den Haan et al. (29). B27.220 or B27.220.hTsn cells were grown in miniPERM bioreactors in hybridoma serum-free medium supplemented with 1% FBS. For accurate comparison these cells were processed in tandem. 5 × 10^6 cells from each line were lysed at 4°C in 0.5% Nonidet P-40, 20 mM Tris, and 150 mM NaCl (pH 7.4) supplemented with Complete protease inhibitor cocktail (Roche). Cells lysates were clarified by two rounds of centrifugation, and the supernatant was filtered and passed over a Tris-blocked Sepharose 4B precolum. The precolured lysates were then applied to columns containing 2 ml of W6/32 affinity matrix, and the columns were washed in buffer containing 30 mM Tris, 150 mM NaCl, and 0.005% Nonidet P-40 (pH 8.0). The columns were subsequently washed extensively with 50 mM Tris and 150 mM NaCl (pH 8.0), a high salt buffer, to remove nonspecifically bound material (50 mM Tris and 500 mM NaCl (pH 8.0)) and finally into 50 mM Tris (pH 8.0). Bound HLA B*2705 peptide complexes were eluted with 10% acetic acid, which also facilitates dissociation of W6/32-bound peptides. The eluate was then chromatographed through a Centricon 3 membrane (Millipore), and the flow-through was concentrated by vacuum centrifugation to a final volume of 300 μl. This material was then subjected to further purification with RP-HPLC using the column and HPLC system described above.

Eluted peptides were resolved from contaminating detergent polymers by employing a rapid gradient from 0 to 60% acetonitrile in 0.1% aqueous TFA (50 mM NaCl/milliliter resin). This material was then subjected to Edman sequencing and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS; as described below). Further purification of the eluted B*2705-restricted peptides was afforded using an optimized gradient with the same HPLC system and column. A linear gradient from buffer A (0.1% TFA) to 40% B (acetonitrile/0.09% TFA; 1.3%/min) at a flow rate of 200 μl/min was used to separate fractions. Peptides (150 μl) were retained and analyzed by MALDI-TOF MS. Identical conditions were used for the purification of B*2705-restricted peptides from both B27.220 and B27.220.hTsn to allow comparison of equivalent fractions from the two cell lines. Importantly, the entire peptide elution experiment was replicated to examine the reproducibility of specific peptide recovery.

Peptide synthesis, sequencing, and MS

Synthetic peptides were purchased from Chiron Technologies (Clayton, Australia) and synthesized with free carbosyl and amino termini using Fmoc-based techniques (30). N-terminal automated Edman sequencing was performed on a Hewlett-Packard G1000A protein sequencer (Hewlett-Packard, Palo Alto, CA) using standard Edman chemistries. MALDI-TOF MS was performed using a Bruker Reflex mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany) operated exclusively in the reflection mode as described previously (31). Aliquots of fractions (1–2 μl) were mixed with an equal volume of α-cyano-4-hydroxycinnamic acid (10 mg/ml in acetonitrile/ethanol, 1/1, v/v), spotted onto a target, and dried for analysis. For comparison of HPLC fractions, identical laser irradiance and repetitions were used to ionize each sample. Care was taken to ensure that uniform matrix/sample crystals were deposited onto the target, ensuring minimal variation in sample ionization between different regions on a given target position and between replicate samples. These measures improve the quantitative nature of the MALDI data. Post-source decay (PSD) experiments were performed under otherwise identical conditions to test the potential and increasing the laser irradiance to optimize the production of fragment ions at each voltage. Assembly of the individual spectra on to a continuous mass scale was performed using FAST software routines within the Bruker XTOF software package. Identification of fragmented ion species was determined by manually assigning C- and N-terminal ion series and comparing parent m/z and fragmentation data in database entries using MS-FIT routines available through the protein prospector program (http://prospector.ucsf.edu). Accurate parent ion mass and fragmentation data allowed assignment of peptide sequence or sequences in several instances. Peptide sequencing by ion trap electrospray ionization MS was performed as previously described (32). Briefly, dried samples were resuspended in 3 μl of methanol/water (1/1) containing 0.1% formic acid. One microfilter of each sample was subjected to peptide sequencing in an LCQ electrospray/ion trap mass spectrometer (Finnigan Thermosquest, San Jose, CA). Collision-induced dissociation and product ion mass spectrometry were individually optimized for each peptide to obtain the optimum fragmentation spectra. Putative peptide sequences were obtained by database comparison of the fragmentation spectra using the PEPSEARCH program (Bioworks package, Finnigan Thermosquest) followed by manual assignment of expected fragments from the highest score sequences. In several instances the authenticity of these sequence assignments was confirmed by comparing the fragmentation spectrum with that of the corresponding retrospectively synthesized peptide.

Competitive HLA B*2705 peptide binding assay

For measurement of relative B27 peptide binding affinities we developed a competition assay in which test peptide was titrated against a fixed amount of labeled reporter peptide and coincubated with T2.B*2705 cells (33). The reporter peptide was synthesized with a biotinylated lysine residue chosen on the basis of predicted solvent accessibility at amino acid position P3, P4, or P5 of the bound peptide ligand in crystal structures (34) or theoretical models (35) of HLA B*2705 complexes. A peptide based on residues 284–292 of the GroEL protein from Chlamydia trachomatis (RR[Ne-bioc]FFMETED) was inserted into the assay based on the B27.220.hTsn data observed. The assay proceeded as follows; 3 × 10^5 T2.B*2705 cells were seeded into each well of a 24-well plate and incubated at 26°C overnight in an atmosphere of 5% CO2. The reporter peptide RRK’AMPEDI was added to each well to a final concentration of 10 μM, and graded concentrations of test peptide (0–50 μM) were added to individual wells, incubated for 1 h, then shifted to a 37°C incubator for an additional 2 h to allow equilibrium to be established at physiological temperature. Cells were harvested on ice and washed three times in wash buffer (1% FBS and 0.02% sodium azide in PBS). The cells were stained with streptavidin-PE (Vector, Burlingame, CA), followed by a biotinylated anti-streptavidin mAb (Vector) and finally with a second round of streptavidin-PE. The cells were then analyzed on a FACSort flow cytometer (Becton Dickinson, San Jose, CA). Competition was visualized as the percent binding of the reporter peptide (stained and corrected for the absence of competitor or in the presence of 50 μM irrelevant peptide (EFGRAFSF)). The inhibitory concentration at which reporter peptide binding was reduced by 50% (IC50) was derived for each test peptide.
Results
Decreased recovery of HLA B*2705-associated peptides in the absence of tapasin

To investigate the influence of tapasin on peptide loading, class I molecules were immunoaffinity purified from $5 \times 10^7$ B27.220 or B27.220.hTsn cells using a matrix consisting of the pan class I mAb W6/32 cross-linked to protein A-Sepharose. Acid-eluted peptides were isolated over a Centricon 3 membrane and crudely separated by RP-HPLC to remove traces of detergent. The retentate on the Centricon 3 filter was analyzed further by SDS-PAGE and revealed equivalent yields of class I hc and $\beta_2m$ from both cell types (Fig. 1A), consistent with the similar levels of surface HLA B*2705 expressed by these cells (12). Importantly, the relative intensities of the HLA B27 hc and $\beta_2m$ bands were similar in both retentates, suggesting that the immunoaffinity-purified material was not substantially composed of altered forms of HLA B27. Furthermore, these gels were electrophoresed under nonreducing conditions, and there was no evidence of B27 hc homodimers ($\sim$90 kDa) (36), consistent with our findings that cell surface hc homodimer is not reactive with W6/32 (C. A. Peh, unpublished observations).

We first determined whether the strong preference for an arginine at position 2 was preserved in B27 ligands captured in the absence of functional tapasin. Aliquots of peptide eluates were pooled sequenced using standard Edman chemistries. Peptides from both tapasin-deficient and reconstituted cell lines displayed prominent arginine peaks in cycle 2 of Edman degradation (Fig. 1B) consistent with the known B*2705 binding motif (37). The contributions of other amino acids to subdominant anchor positions, notably at P1, P3, and P8/9, were also similar for both eluates (data not shown). Thus, the absence of tapasin does not alter the structural constraints that confer the well-recognized binding motif for HLA B*2705. Also evident in Fig. 1B is the reduced yield of peptides containing arginine at P2 derived from tapasin-deficient cells. 721.220 cells lack functional tapasin and have lost HLA A and B loci, but retain low levels of HLA Cw*0102. This allele also has a preferred P2 anchor residue, in this case for alanine (38). Therefore, direct comparison of arginine and alanine yields from cycle 2 of Edman sequencing reflects the relative contributions of peptides derived from HLA B*2705 and Cw*0102 to the peptide pool. In the tapasin-deficient cell line no increase above background was observed for alanine at P2, while a small, but significant, alanine peak was observed in the peptides recovered from the B27.220.hTsn cell line. The other amino acids in this cycle do not significantly deviate from background levels. These data indicate a minor contribution of Cw1 peptides to the eluate from tapasin-reconstituted cells.

We next exploited the preserved P2 arginine motif of B*2705 ligands in tapasin-deficient cells to further assess the quantitative effects of tapasin on the recovery of peptides associated with HLA B*2705. HLA B*2705 transfected cells were metabolically labeled with $[^3]H$arginine, the radiolabeled peptide ligands were isolated and separated by RP-HPLC, and individual fractions were analyzed on a liquid scintillation counter. As shown in Fig. 1C, significantly more labeled peptide was recovered from the B27.220.hTsn cell line compared with B27.220 or the control 721.220 cell line transfected with human tapasin alone (220.hTsn). Peptides recovered from the B*2705 transfecants with and without tapasin exhibited similar broad distributions of labeled species throughout the chromatographic separation, while some hydrophobic species were observed in the immunoprecipitated material from 220.hTsn cells, reflecting low yields of endogenous HLA Cw1-bound peptides. The reduced yield of peptides eluted from B27.220 was reproducible in a number of independent experiments and is probably due to loss of poorly bound B*2705 ligands during the metabolic labeling and purification process. This hypothesis is consistent with decreased yield of arginine in cycle 2 of Edman degradation and the reduced surface stability of the B*2705-peptide complexes expressed by tapasin-deficient cells (10). Therefore, both the current study and previous studies indicate the selection of a proportion of suboptimal ligands by HLA B*2705 in tapasin-deficient cells.

Tapasin quantitatively and qualitatively alters the B*2705-bound peptide repertoire

The biochemical identity of bound peptides contained in the eluates derived from B27.220 and B27.220.hTsn cells was further analyzed by MALDI-TOF MS. Identical ionization conditions were used for each sample to allow accurate comparison of the peptides present in both samples. In addition, care was taken to mix analyte and matrix components identically and to prepare a uniform coverage of sample/matrix fine crystals over the target. This resulted in minimal variation in signal when the analyte was analyzed at different regions on the same target or on replicate target positions. As shown in Fig. 2A, the overall ionization intensity of species within the expected m/z range of 8–12 mer (800–1300 Da) was higher in the peptide eluate from cells expressing wild-type tapasin (spectra of positive polarity). This observation was highly reproducible and is consistent with the reduced recovery of radiolabeled peptides from cells lacking tapasin (Fig. 1C). Comparison of MALDI spectra of B*2705-bound peptides derived from both cell lines revealed a high degree of spectral overlap, with strong coincidence of species with the same m/z values identified in both cell lines. This is particularly evident in Fig. 2B, which represents a portion of the spectra shown in Fig. 2A with an exploded m/z axis. Thus, the majority of bound species isolated from tapasin-positive and -negative cell lines shared m/z values and presumably represent the same peptide. The signal obtained for some species (Fig. 2B), however, differed significantly between tapasin-deficient and reconstituted cells. For example, the signal from the species with m/z of 1098.5 was $\sim$2.5-fold stronger in cells expressing wild-type tapasin. Similarly, species with m/z values of 1090.4, 1115.4, and 1108.4 were all more intense in eluates from tapasin-positive cells. In contrast, species with m/z of 1094.4 and 1095.4 (arrowed) were present at equivalent intensities in eluates from the two cell lines. Although MALDI-TOF MS is not a strictly quantitative tool, these differential effects on signal intensities strongly suggested differential presentation and recovery of B27-peptide ligands in the presence and the absence of tapasin. Overall, the findings indicate that most, but not all, of the peptides loaded into HLA B*2705 in the presence of wild-type tapasin are also loaded in the absence of this chaperone, but generally with lower efficiency. In addition, a spectrum of tapasin dependency was observed for the recovery of individual species. These observations were highly reproducible, with essentially identical results obtained from two independent peptide elution experiments (data not shown).

Quantitative differences in the HLA B*2705-peptide repertoire suggests an editorial function associated with tapasin expression

We were particularly interested in the possibility that some peptides might be captured selectively in the absence of tapasin, but replaced or removed by other peptides in the presence of tapasin. To examine this possibility, eluted peptides were further fractionated using an optimized RP-HPLC separation protocol, and the resultant fractions analyzed were extensively by MALDI-TOF MS. The mass spectra of six representative RP-HPLC fractions

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derived from both B27.220 and B27.220.hTsn eluates (fractions 26, 27, 28, 30, 34, and 36) are shown in Fig. 3. The high degree of overlap in the spectrum of peptides derived from tapasin-deficient and proficient cells was still evident in the mass spectra of individual RP-HPLC fractions, but a greater number of discordant species was now obvious (see arrowed species). Consistent with the mass spectra on the peptide pools, the majority of species were recovered more efficiently from tapasin-positive cells (as depicted in the spectra derived for fraction 30); however, there were several notable exceptions. For example, some species were found only in the presence of tapasin, see \( m/z \) 1058.4 (arrowed) or \( m/z \) 1197.5 in fraction 36, while other species were recovered preferentially from the tapasin-deficient cells, such as the peptide with \( m/z \) 1378.8 (arrowed) from fraction 26. This peptide was isolated in greater abundance (2- to 5-fold) from tapasin-deficient cells in two independent experiments. Likewise, species with \( m/z \) 1169.3 in fraction 27 and 1094.1 and 1797.4 in fraction 28 (arrowed) were all detected at a significantly higher signal in tapasin-deficient cells. Another example is found in fraction 34, where a species of \( m/z = 1019.4 \) (arrowed) was found uniquely in tapasin-deficient eluates, while in the same fraction the species with \( m/z \) 1027.4 was detected with similar intensity in both cell lines. Importantly, in these same fractions some other species were recovered more abundantly in the tapasin-positive eluates, as is obvious for numerous species in fraction 26 and the species of \( m/z = 1031.1 \) (arrowed) in fraction 28.

A potential artifact of these studies is the effect of enhanced expression and peptide binding by HLA Cw1 following introduction of the gene encoding human tapasin into the 721.220 cell line. Data from Edman sequencing and metabolic labeling suggest that Cw1 peptides contribute in only a very minor way to the peptide pool from tapasin-positive 721.220 cells. To formally eliminate the possibility that some of the peptides with differential tapasin dependency in Figs. 2 and 3 were derived from HLA Cw1 we eluted peptides from 5 \( \times \) 10^9 220.hTsn cells in a manner identical with that used for the B27 transfecants. Only a few dominant species were observed in the mass spectrum of these eluted peptides (data not shown), and none had masses corresponding to those species observed in the mass spectrum of the B27 eluted material. Therefore, combined with the Edman sequencing and metabolic labeling studies, these observations indicate that B27-bound peptides overwhelmingly contribute to the peptide eluates examined in this study. Thus, our data indicate that most peptides are loaded into HLA B*2705 less efficiently in the absence of functional tapasin. However, contrary to the majority of peptides, some species recovered from tapasin-deficient cells are missing from the repertoire or are less abundant in tapasin-proficient cells. This reflects a tapasin-dependent, although not necessarily directly tapasin-mediated, editing mechanism and provides the first direct biochemical evidence that tapasin qualitatively affects the composition of species bound to class I molecules.
Biochemical characterization of B*2705 ligands selectively presented by tapasin-positive cells

The MALDI-TOF MS analysis of 40 matched RP-HPLC fractions from tapasin-deficient and tapasin-reconstituted eluates (i.e., 80 fractions in total) revealed qualitative differences in the peptide repertoire for numerous species. Several approaches were taken to further investigate the nature of some of these qualitative changes in HLA B*2705 peptide repertoire. Firstly, a number of previously reported B*2705 ligands (39) were synthesized, and their RP-HPLC retention behavior was modeled. Once the retention time of the synthetic peptides tested. The mass accuracy of synthetic peptides and their putative natural analogues was well within the range seen for peptides that were subsequently sequenced by Edman degradation or mass spectrometry (ΔDa = −1.0 to 0.8). We conclude that several of the matched species are likely to correspond to the modeled peptides; some of these candidates are listed in Table I.

Secondly, individual peptides were identified biochemically using a combination of Edman sequencing and MS of peptides that were deliberately fragmented using collision-induced dissociation or PSD techniques. Two peptide sequences were obtained by PSD analysis. One of the peptides was identified as 38RRYDRKQSGY47, a sequence derived from the 60S human ribosomal protein L44. Analysis of this fraction by Edman degradation and comparison of the PSD spectra and RP-HPLC retention behavior of the retrospectively synthesized RRYDRKQSGY peptide confirmed the identity of this peptide, which was observed to be more abundant in tapasin-positive cells, although still present in the tapasin-deficient cell line. This ligand has previously been reported as a B*2705 ligand (40). A second peptide that has not been reported before as a B*2705 ligand was also identified using a combination of PSD and Edman sequencing. This peptide, 139SRIRKLFNL147, is derived from the 40S ribosomal protein S6 and was also more abundant in the tapasin-expressing cells. Other tapasin-dependent ligands were identified by LCQ electrospray/ion trap mass spectrometry. Sequences delineated included newly identified ligands from the 40S ribosomal protein S11 (76GRILSGVTK79) and a novel peptide from an unknown source (RR[I/L]SRDG[I/L]AI[L]I[K/Q]). The lack of a human or mammalian homologue for this species did not allow assignment of the ambiguous amino acids I/L at P3, I/L at P8, I/L at P10, and K/Q at P11. A bacterial homologue does exist, however (RRLSRDG-LALQ derived from a cycloisomerase involved in benzoate metabolism in Bradyrhizobium japonicum).

The majority of modeled peptides representing previously characterized B*2705 ligands appeared to be dependent on tapasin for efficient presentation (see Table I). Notably, one peptide identified by LCQ electrospray ion trap MS was only observed in eluates derived from cells expressing tapasin, with no detectable recovery of this ligand from tapasin-deficient cells. This species (15GRYS-GKAV33) has not been reported as a B*2705 ligand before and is derived from the 60S ribosomal protein L27.

Biochemical identification of B*2705 ligands present equally in tapasin-positive and tapasin-negative cells

Although most ligands appeared to be recovered more efficiently from tapasin-positive cells, some peptides were present at similar levels in both the eluates from both tapasin-positive and -negative cells. Four peptides whose levels were similar in the eluates from both cell types were sequenced using electrospray ion trap MS. Two of these peptides have been previously characterized as B*2705 ligands: 173GRIDKPLIK181 derived from the 60S ribosomal protein L8 (41), and 36GRLTKHTKFL44 derived from the L36 ribosomal protein (42, 43). A third ligand, 290GRHGVFLEL307 (a previously characterized ligand (39) derived from the proteasome regulator

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subunit S2), was initially recovered more abundantly in the eluate from tapasin-deficient cells. This species represents one of the prominent species (m/z = 1027.4 Da) contained in fraction 34 of the tapasin-deficient eluate, and its relative abundance in the tapasin-deficient and reconstituted eluates is shown in Fig. 3. However, in a second independent experiment, this peptide was recovered in similar amounts from both cell lines, this species was thus classed as a tapasin-independent ligand. The fourth peptide, GRFGTKGLAITF, is a newly identified ligand derived from the ATP-dependent RNA helicase P47 protein. The recovery of this unusually long peptide from both tapasin-positive and -negative cells suggests that tapasin does not generically bias the size of peptides bound to the B*2705 molecule. Similarly, analysis of the overlaid MALDI-TOF spectra of peptides eluted from tapasin-positive and -negative cells did not reveal a significant difference in the capture of longer peptides (>10 aa) by B*2705 molecules in either cell line. The data also imply that tapasin is unlikely to act as a trimming enzyme in the ER.

FIGURE 3. Some peptides are differentially recovered from HLA B*2705 eluates derived from cells with and without expression of functional tapasin. MALDI-TOF mass spectra of fractions derived from the micropreparative separation of peptide eluates (shown in Fig. 2). Six representative fractions from B27.220.tTsn (spectrum of positive polarity) and B27.220 (spectrum of negative polarity) eluates are shown, and preferentially recovered species are highlighted by arrows.
Biochemical characterization of B*2705 ligands selectively presented by tapasin-negative cells

We were particularly interested in identifying the nature of those peptides that were recovered more abundantly in tapasin-deficient cells. Edman sequencing of a fraction derived from tapasin-deficient B27.220 cells identified one such peptide. Interestingly, this peptide was derived from the B*2705 hc itself and has previously been reported as a B*2705 ligand in normal cells (42). As shown in Fig. 3 (fraction 26), the intensity of the 169RRYLENGKETL 

179 peptide (m/z 1378.9) derived from the B*2705 hc was significantly higher in the tapasin-deficient cells (negative spectra) compared with cells expressing wild-type tapasin (positive spectra). This result was highly reproducible, and this species was preferentially recovered from tapasin-deficient eluates in two independent peptide elutions. A number of other species were identified in this study that also were reproducibly recovered in greater abundance (see Table I). Of interest, several of these peptides were derived from the same ribosomal subunit displayed divergent tapasin dependence (e.g., GRHLGFVRL vs GRYSGRKAV). Although some B27-derived oligopeptides of appropriate mass could potentially be liberated from the B27 hc, they did not appear to be appropriate as B27 ligands based on their lack of a canonical arginine 2 anchor motif and the presence of unfavorable amino acids at this position.

Tapasin-independent ligands do not generally display enhanced binding to HLA B*2705

There may be several explanations for the ability of certain peptides to be presented efficiently in the absence of tapasin. These include continued presentation due to high cellular abundance or high affinity for HLA B*2705 relative to tapasin-dependent ligands. The B*2705 hc sequence was scanned for potential peptide fragments corresponding to molecular masses of 1094.1 and 1169.3. Although some B27-derived oligopeptides of appropriate mass could potentially be liberated from the B27 hc, they did not appear to be appropriate as B27 ligands based on their lack of a canonical arginine 2 anchor motif and the presence of unfavorable amino acids at this position.

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Table I. The tapasin dependence of peptides recovered and sequenced in this study

<table>
<thead>
<tr>
<th>Peptide Source</th>
<th>Method of Peptide Identification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Edman MALDE/PSD</td>
<td>LCQ Ion Trap</td>
</tr>
<tr>
<td>Tapasin-independent ligands</td>
<td></td>
</tr>
<tr>
<td>RRYLENKETL HLA B*2705 hc 169–179</td>
<td></td>
</tr>
<tr>
<td>GRHGVLFEI Human 26S proteasome regulatory subunit S2 (P97) 299–307</td>
<td></td>
</tr>
<tr>
<td>GRIDKPLK Human 60S ribosomal protein L8 173–181</td>
<td></td>
</tr>
<tr>
<td>GRLTKHTKF Human analogue of rat ribosomal protein L36 36–44</td>
<td></td>
</tr>
<tr>
<td>GRFPTGKLAIITF Human ATP-dependent RNA helicase P47 379–390</td>
<td></td>
</tr>
<tr>
<td>RRIKEIVKK Human HSP90α 200–208</td>
<td></td>
</tr>
<tr>
<td>Tapasin-dependent ligands</td>
<td></td>
</tr>
<tr>
<td>GRYSGRKAV Human 60S ribosomal protein L27 15–23</td>
<td></td>
</tr>
<tr>
<td>SRIRKLFLN Human 40S ribosomal protein S6 139–147</td>
<td></td>
</tr>
<tr>
<td>RRYDKQSGY Human 60S ribosomal protein RPL44 38–47</td>
<td></td>
</tr>
<tr>
<td>GRILSVVTK Human 40S ribosomal protein S11 70–79</td>
<td></td>
</tr>
<tr>
<td>RR[I/L][RDQ][I/L][A/L][K/Q] Unknown</td>
<td></td>
</tr>
<tr>
<td>MRVTAPRTL HLA B*2705 signal sequence (1–9)</td>
<td></td>
</tr>
<tr>
<td>KRGILITKY Actin 43–51</td>
<td></td>
</tr>
<tr>
<td>RRSKEITVR Human ATP-dependent RNA helicase (77–85)</td>
<td></td>
</tr>
<tr>
<td>KREFGILQR Unknown (possible EF2 peptide)</td>
<td></td>
</tr>
<tr>
<td>ARLFOIRK Human 60S ribosomal protein L13 188–196</td>
<td></td>
</tr>
<tr>
<td>SDRKTMW Human guanine nucleotide-binding protein beta subunit-like protein 35–43</td>
<td></td>
</tr>
<tr>
<td>KRYKSIKY Human fasyl pyrophosphate synthetase 191–199</td>
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</tr>
</tbody>
</table>

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increased HLA B27 expression and turnover in tapasin-deficient cells. Furthermore, it is unlikely that the other peptides preferentially recovered from the tapasin-deficient cells correspond to B27-derived peptides. The B*2705 hc sequence was scanned for potential peptide fragments corresponding to molecular masses of 1094.1 and 1169.3. Although some B27-derived oligopeptides of appropriate mass could potentially be liberated from the B27 hc, they did not appear to be appropriate as B27 ligands based on their lack of a canonical arginine 2 anchor motif and the presence of unfavorable amino acids at this position.
Possible that increased turnover of B27 in tapasin-deficient cells could explain the preferential recovery of the hc 169–179 peptide.

The relative binding efficiency of the peptides isolated from HLA B*2705 was inferred from a competition assay using binding of a biotinylated reporter peptide to temperature-stabilized B*2705 molecules on the surface of transfected human TAP-deficient cell line T2 (22). Representative data for some of the tapasin-independent and -dependent ligands as well as two viral index peptides. A range of IC50 values was observed in all three groups. Representative data for selected tapasin-independent ligands, GRFGTKGLAIF (○), GRIDDPIKLK (▲), RRYLENGKETL (●), and GRHGVFLEL (■), are shown.

Peptide-dependent alloreactive T cell clones discriminate between B*2705 molecules expressed on tapasin-positive and -negative cell lines

To confirm that tapasin qualitatively alters the peptide map presented on the cell surface we examined the functional CTL recognition of HLA B*2705 expressed by tapasin-positive and -negative cell lines. Most T cell allorecognition is thought to depend upon presentation of endogenous self peptides that participate either directly or indirectly in TCR interactions (32, 46, 47). We used two panels of B*2705-specific alloreactive T cell clones to examine the functional presentation of peptide ligands by HLA B*2705. The first of these CTL was raised against normal B27-positive cell lines, and all react with B*2705 targets (26). The second panel of CTL was raised against tapasin-deficient B*2705 targets (i.e., the same clone used for peptide elution studies) during the course of these studies. Both panels of CTL are known to be either peptide-sensitive or peptide-specific, since they recognize the TAP-deficient cell line T2.B*2705 only when sensitized with specific fractions containing peptides eluted from B*2705-bearing cells (27). The natural allo-ligand is known for a number of these CTL. 27S69 recognizes HLA B*2705 in the context of an octamer derived from the 26S proteasome regulatory subunit S2 (GRHGVFLEL). The peptide dependence of these CTL clones was used to probe the peptide repertoire constitutively presented by the B27.220 and B27.220.hTsn cell lines by comparing the patterns of alloreactivity on both cell lines (see Fig. 5, A and B). As expected, none of the clones tested demonstrated lysis of the parental 721.220 cell line. The CTL clones raised against normal B27-positive cells recognized tapasin-proficient cells either more efficiently or at least as well as tapasin-deficient cells. For instance, the CTL clones 37GRK, 18DLH, and 36DLH recognized targets with wild-type tapasin more efficiently (at least a 30% increase in specific 51Cr release) than cells with defective tapasin (error bars represent SDs from the mean of multiple experiments). In contrast, CTL clones 27S69 and 58GRK recognized tapasin-positive and -negative targets equivalently. These patterns of allorecognition were further evident when the lysis of B*2705-positive targets was examined at different E:T cell ratios, as shown in Fig. 5C for selected clones.

Although none of the alloreactive CTL from the first panel showed a clear preference for tapasin-negative B*2705 targets, this is not surprising given that these CTL were elicited in response to a peptide repertoire shaped by the presence of functional tapasin. However, the second panel of CTL (Fig. 5B) either recognized the tapasin-positive and tapasin-negative cells equivalently (SB3, SB5, NK8, SB14, SB15) or recognized the tapasin-deficient cells more efficiently (SB8). These data are consistent with MALDI-TOF MS data that demonstrated higher levels of most, but not all, peptide ligands presented by B*2705 in tapasin-positive vs tapasin-deficient cells.

CTL recognition of B27.220 and B27.220.hTsn was similar for a number of CTL clones, including SB52, 102DRF, 58GRK, and 27S69, which is also consistent with the peptide elution studies showing that some peptides are presented to the same degree in the presence and the absence of tapasin. The peptide ligand recognized by SB52 is presented by both tapasin-positive and -negative cells at similar levels, and, as expected, this CTL recognized B27.220 and B27.220.hTsn with similar efficiency (Fig. 5C). The peptide specificity of this clone is also shown in Fig. 5C using GRHGVFLEL-pulsed T2.B*2705 cells at different E:T cell ratios. These same cells pulsed with other B27-binding peptides or irrelevant peptides were not recognized by this CTL (data not shown). Overall, these data confirm a significant overlap in the repertoire of peptides recognized by CTL when presented by HLA B*2705 expressed in the presence and the absence of wild-type tapasin. Importantly, consistent with the observation that some peptides were
recovered in greater abundance from tapasin-deficient APC, the clone SB8 demonstrated preferential recognition of B27.220 cells, demonstrating the functional consequences of preferential peptide presentation in tapasin-deficient APC.

Discussion

Our study has examined the peptides associated with HLA B*2705 in cells that express wild-type tapasin compared with those that are effectively tapasin negative and express only a defective form of tapasin that lacks the ability to associate with class I molecules. We find that the total amount of recoverable class I-associated peptide is reduced in cells that lack tapasin. This quantitative decrease in peptide determinants is not absolute, since MALDI-TOF MS of the eluted pool of peptides or individual peptide fractions clearly illustrates examples of species that were 1) not detected or differentially decreased in cells that lack wild-type tapasin, 2) present in equivalent amounts in the presence and the absence of tapasin, or 3) preferentially recovered from cells that do not possess wild-type tapasin. A number of peptides with varying tapasin dependence were sequenced in this study. From this dataset there was no clear correlation between tapasin dependence and either cellular abundance or affinity for HLA B*2705. Our observations provide evidence that tapasin functions both to enhance loading of peptides into the cleft of class I molecules and to determine the composition of the peptide repertoire. This effect of tapasin may occur through either a direct mechanism or an indirect editing function mediated through other components recruited by tapasin to the peptide loading complex.

Patterns of alloreactivity using peptide-dependent HLA B*2705-restricted CTL essentially mirrored the biochemical analyses of B*2705 peptide eluates from tapasin-positive and -negative cells. Examples of peptide-dependent allo-CTL that were clearly able to discriminate between tapasin-positive and -negative cells and others that did not probably reflects their dependence on tapasin-dependent or tapasin-independent ligands, respectively. In addition, specific stimulation of allogeneic PBMC from healthy B27-negative donors yielded clones that were not only peptide dependent, but were also peptide specific. One such example is clone SB52, which recognizes the tapasin-positive and -negative cell lines with similar efficiency and was shown in this study to be specific for the GRHGVFLEL peptide. The ability of this clone to kill both targets with equal efficiency directly correlates with the similar levels of GRHGVFLEL peptide recovered from the two cell lines and clearly defines the tapasin independence of this ligand. Furthermore, the clone SB8 demonstrated greater lysis of tapasin-deficient APC, confirming that some peptides are presented more abundantly on the surface of tapasin-deficient cells. The peptide ligand recognized by clone SB8 was not defined in this study. SB8 did not recognize T2.B*2705 pulsed with known B27 ligands or any of the peptides defined in this study. SB8 did not recognize T2.B*2705 pulsed with known B27 ligands or any of the peptides defined in this study, reflecting the peptide-dependent and peptide-specific manner by which this clone recognizes HLA B*2705.

Tapasin has a number of distinct functions that offer some clues to the mechanism of its role in repertoire modulation. These functions include colocalization of class I and TAP molecules, increasing the level of TAP expression, and possibly affecting peptide translocation from the cytosol into the lumen of the ER (5, 6). In addition, as highlighted from studies with soluble tapasin (4), this chaperone may stabilize class I complexes and influence peptide loading independently of TAP association. Our data suggest that an alternative pathway of peptide acquisition by HLA B*2705 operates when tapasin is limiting or when TAP association is abrogated, as with the T134K HLA A2 mutant (11) and RT1.1^{+} molecules expressed on the TAP^{−} background (48). However, TAP-translocated peptides remain the primary source of the peptides selected by HLA B*2705 under these alternative conditions (49). Under normal circumstances it is generally assumed that peptide-receptive MHC class I heterodimers acquire antigenic peptide
when bridged to the TAP (1, 50). However, it is possible that some B*2705 molecules might initially acquire peptides via an alternative tapasin-independent pathway and then subsequently be localized to TAP via tapasin binding. Physical association with TAP may allow a degree of peptide exchange with less abundant or shorter half-life peptides as well as peptides of better fit. This idea is consistent with some peptides having an absolute requirement for tapasin for class I loading through a process that may involve direct interactions among tapasin, the class I complex, the TAP molecule itself, and perhaps the peptide. Such an arrangement could allow a direct role for tapasin in the editing process. There is also ample scope for tapasin to exert an indirect influence on this putative editing process; tapasin modulates TAP function by enhancing the translocation of peptides and therefore might alter the competitive advantage of certain peptides. The presence of tapasin is also required for efficient recruitment of other components of the class I loading complex, which may exert individual or concerted effects on peptide editing (1).

How tapasin exerts its influence on the peptide repertoire is still unclear, though obviously tapasin is important in the assembly of nascent class I heterodimers with peptide and in retaining these complexes in the ER until a suitable peptide ligand is bound. Other opportunities for fine-tuning the bound ligand may also exist. Recent studies indicate that class I molecules associate stably with the TAP for periods of up to several minutes (51) and are still associated with tapasin before dissociating from the TAP complex (52). These studies also revealed that once dissociated from the TAP complex, peptide-loaded molecules did not egress to the cell surface immediately, suggesting that a window of opportunity for additional editing of bound peptides may exist after TAP-mediated loading. A putative tapasin binding site on class I MHC molecules includes amino acid residues 128–136, which form a loop in the α2 domain, and conceivably interaction with this region of the class I molecule could influence peptide binding by modulating cleft conformation (13, 14). Combined with our data we propose an integral role for tapasin in both peptide loading of class I molecules and ligand optimization. From our small dataset it does not appear that tapasin-independent ligands possess enhanced binding affinity for HLA B*2705, nor does the cellular source of the ligands indicate that cytoplasmic abundance necessarily correlates with tapasin dependency. We propose that the ER luminal availability of the peptide plays an important role in dictating class I loading in tapasin-deficient cells. Luminal availability is conceivably a product of TAP-dependent translocation of peptides into the ER, the resistance of the peptides to ER protease, and their requirement for processing or trimming following translocation from the cytoplasm into the ER lumen. Further analysis of the peptides defined here and in future studies will explore whether further rules regarding the selection of peptide ligands by tapasin exist.

Recent studies with tapasin knockout mice suggest a generalized defect in Ag presentation in tapasin-deficient cells (15, 16). However, these studies observed a low level of Ag presentation in knockout mice sufficient for some positive selection of CD8+ T cells and tolerance induction. This is entirely consistent with our biochemical findings described here that some peptides continue to be presented for T cell recognition in the absence of tapasin. We suggest that the extent of Ag presentation will be highly dependent on the class I allele and ligand availability. It is clear from several studies using the 721.220 cell line that human class I alleles are differentially affected by tapasin deficiency and that HLA B*2705 is perhaps the most successful allele in overcoming such defects. A prediction of our study is that in normal APC some degree of tapasin-independent loading of HLA B*2705 may operate and that a proportion of B27 molecules may be more receptive to exogenous ligand due to the acquisition of suboptimal peptides (10, 12). Indeed, such peptide receptivity has been observed for B27 molecules under normal conditions (22). The physiological significance of these phenomena remains unclear, but it is interesting to speculate that this property of HLA B*2705 may contribute to this allele’s very strong association with ankylosing spondylitis and other inflammatory arthropathies.

The potential for viruses or intracellular pathogens to target tapasin as a mechanism for evading CTL recognition has been highlighted in a study that examined the role of adenovirus protein E19 in preventing class I presentation (53). HLA alleles that encode tapasin-independent molecules may have evolved in response to such selective pressures and permit the host CTL compartment to respond to pathogens that specifically target this aspect of class I assembly. It is interesting to note that the HLA B27 group of alleles is evolutionarily successful, being distributed in nearly all ethnic groups, and is associated with slow progression of HIV infection (54, 55). Our data would suggest that if tapasin function was abrogated there is potential for the up-regulated presentation of poorly tolerized self-peptides. If this occurs in an inflammatory context this may also elicit autoimmune responses, since these peptides would not normally be presented at high density to the immune system by tapasin-proficient cells. It is interesting to note that the B27 he peptide 169–179 that is preferentially presented by tapasin-deficient cells has previously been espoused as a potential arthritogenic peptide (42). The exact role of this peptide in disease is unclear, since this peptide is prominent in peptide pools derived from some of the B27 subtypes reported not to be associated with ankylosing spondylitis (56) (our unpublished observations). Moreover, widespread reactivity toward self-peptides (vasoactive intestinal peptide 1 receptor 400–408 and LMP2a236–244) has been observed in patients with ankylosing spondylitis (57), highlighting the importance of the repertoire of self-peptides in ankylosing spondylitis and presumably other B27-associated inflammatory arthropathies. Given the qualitative and quantitative influence of tapasin on the self-peptide repertoire it will be of great interest to determine whether other ligands that are preferentially presented in the absence of tapasin are involved in autoimmunity.

Acknowledgments

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