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HLA Class I Polymorphism Has a Dual Impact on Ligand Binding and Chaperone Interaction

William H. Hildebrand, Heth R. Turnquist, Kiley R. Prilliman, Heather D. Hickman, Erin L. Schenk, Mary M. McIlhaney, and Joyce C. Solheim

ABSTRACT: This article will describe coordinated analyses of how amino acid substitutions in the HLA class I antigen binding groove modify chaperone interaction and peptide ligand presentation. By parallel testing of ligand presentation and chaperone interaction with a series of natural HLA-B subtypes, this study has discovered that position 116 of the HLA-B15 class I heavy chain is pivotal in both peptide selection and control of interaction between the assembly complex and the class I heavy chain. Correlated with these qualitative differences in peptide selection and chaperone association are quantitative differences in the expression levels of the HLA molecules at the cell surface. These parallel studies, therefore, demonstrate that particular HLA class I polymorphisms can simultaneously influence ligand presentation and interaction with intracellular chaperones. *Human Immunology 63, 248–255 (2002).* © American Society for Histocompatibility and Immunogenetics, 2002. Published by Elsevier Science Inc.

KEYWORDS: antigen presentation; MHC; tapasin; HLA; peptide

MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I

A Polymorphic Receptor

Class I heavy chains are encoded in the major histocompatibility complex (MHC) and their function is to alert cytotoxic T lymphocytes (CTL) to the presence of neoplastic and infected cells. MHC class I molecules are found on the surface of virtually every nucleated cell in the body. Before transport to the cell surface, an MHC class I heavy chain associates with a beta$_2$-microglobulin (β_2m) light chain and an endogenously derived peptide ligand of approximately 9–10 amino acids in length [1]. Assembly of this trimeric heavy chain-β_2m-peptide complex within the endoplasmic reticulum (ER) is assisted by a number of intracellular chaperones, such as calreticulin, tapasin, the transporter associated with antigen processing (TAP), and ERp57 [2–4].

A remarkable characteristic of MHC class I molecules is their polymorphic nature. The amino acid substitutions distinguishing one MHC molecule from one another are, in general, at positions that could modify the binding of peptide ligands [5]. This characteristic supports a hypothesis whereby variations in the MHC class I binding groove allow the various class I molecules in the population to bind and present different portions of viral or other intracellular pathogens for immune targeting. Accordingly, MHC class I variability makes the host an unpredictable, and therefore more elusive, target for such pathogens.

More recent data reveals that substitutions both within and near the class I antigen binding groove can modify interaction of the heavy chain with its intracel-

From the Department of Microbiology and Immunology (W.H.H., H.D.H.), University of Oklahoma Health Sciences Center, Oklahoma City, OK; the Eppley Institute for Research in Cancer and Allied Diseases (H.R.T., M.M.M., J.C.S.), the Department of Pathology and Microbiology (H.R.T., J.C.S.), the Department of Biochemistry and Molecular Biology (J.C.S.), and the Eppley Institute Summer Undergraduate Research Program (E.L.S.), University of Nebraska Medical Center, Omaha, NE; the Division of Immune Regulation (K.R.P.), La Jolla Institute for Allergy and Immunology, San Diego, CA; and the Department of Biochemistry (E.L.S.), Gustavus Adolphus College, St. Peter, MN, USA.

Address reprint requests to: Dr. Joyce C. Solheim, Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, 986805 Nebraska Medical Center, Omaha, NE 68198-6805, USA; E-mail: jsolheim@unmc.edu.

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TABLE 1 Amino acid substitutions distinguishing B*1512, B*1508, B*1501, B*1503, B*1518, and B*1510

	24	45	46	63	67	116	156	166	167
Consensus	*A*	*M*	*A*	*E*	*S*	*S*	*W*	*E*	*W*
B*1512	—	—	—	—	—	—	—	D	G
B*1508	—	—	—	N	F	—	—	—	—
B*1501	—	—	—	—	—	—	—	—	—
B*1503	S	E	E	—	—	—	L	—	—
B*1518	S	E	E	N	C	—	L	—	—
B*1510	S	E	E	N	C	Y	L	—	—

Residues are numbered from the N-terminus of the mature class I heavy chains. Positions of identity with the consensus (italics) drawn from B* 1501 are indicated by dashes (—). Positions above the dark bar are in the α1 domain, and positions above the light-colored bar are in the α2 domain.

lular chaperones. Evidence from several laboratories demonstrates that site-directed mutagenesis of a loop near the binding groove alters the degree of association between the nascent MHC class I molecule and the ER proteins calreticulin, tapasin, and TAP [6–8]. Natural polymorphism in the binding groove of the HLA-B heavy chain has also been reported to alter interactions with TAP. Specifically, a fraction of HLA-B35 subtypes containing aromatic amino acids at position 116 (in the floor of the peptide-binding groove) were found to be associated with TAP, but no such interaction was discovered for HLA-B35 subtypes with serine at position 116 [9]. Analysis of B44 subtypes indicated that position 156 (in an α helix of the peptide-binding groove, with a side-chain pointing in) also contributed to the TAP-association motif [9]. Thus, class I heavy chain substitutions can influence interactions with chaperones that can control the routing of mature MHC class I molecules to the cell surface.

PARALLEL EXAMINATION OF MHC CLASS I INTERACTION WITH CHAPERONES AND PEPTIDES

We hypothesized that natural substitutions in the MHC class I heavy chain affect chaperone interactions and ligand binding in a concurrent fashion. A synergistic approach was used to test this hypothesis. Our initial emphasis was on precisely mapping peptide ligands from closely related HLA-B15 subtypes [10–13]. Peptides were eluted from six B15 subtypes and comparatively mapped by mass spectrometry. Systematic mapping of peptide ligands was facilitated by the production of milligram quantities of soluble HLA molecules using a hollow-fiber cell culture system. The large quantities of endogenously loaded peptides consequently extractable allowed for exceptionally high resolution and consistent analyses. Such precision ligand mapping aided the comparison of peptide maps from HLA subtypes differing by a small number of (or single) naturally occurring substitution(s). Subsequent ligand sequencing then provided direct information as to how single substitutions in the HLA class I heavy chain modified ligand binding tendencies. Validation of this scheme includes the demonstration that peptide ligands eluted from detergent-solubilized class I molecules by other laboratories match both our motifs and individual ligand sequence data.

This study's next focus was on assessing the impact of HLA class I polymorphism upon association of the class I heavy chain with assembly complex proteins [14]. To elucidate how heavy chain substitutions modify chaperone interactions, we analyzed three of the HLA-B15 subtypes for which we had obtained comparative ligand binding data (as described above) for interaction with the chaperones calreticulin, TAP, and tapasin. Our results identify a single amino acid substitution in the binding groove that has an overriding impact on assembly complex association as well as on ligand presentation. This represents the first combined analysis of peptide ligand identification and chaperone association and provides information useful for basic and clinical applications.

Comparison of B15 Motifs

To determine how various polymorphisms in the class I antigen binding groove modify ligand presentation, we examined the peptides presented by the six B15 subtypes listed in Table 1 [10–13]. First, we compared pooled Edman sequencing motifs, using B*1501 as a point of comparison. Two B15 subtypes, B*1512 and B*1503, shared both P2 and P9 preferences with B*1501 (Figure 1). Polymorphisms at positions 166 and 167 (on the α2 domain α-helix) in B*1512 had little effect on the peptide ligand motif, as the B*1501 and B*1512 motifs were virtually identical. Molecule B*1503 also shared P2 and P9 anchors with B*1501, although P2 had diverged from that seen in B*1501 in its preference for a positively charged Lys anchor.

Two other B15 subtypes, B*1508 and B*1518, shared with B*1501 a preference for the aromatic residues Tyr and Phe at the P9 anchor (Figure 1). Although

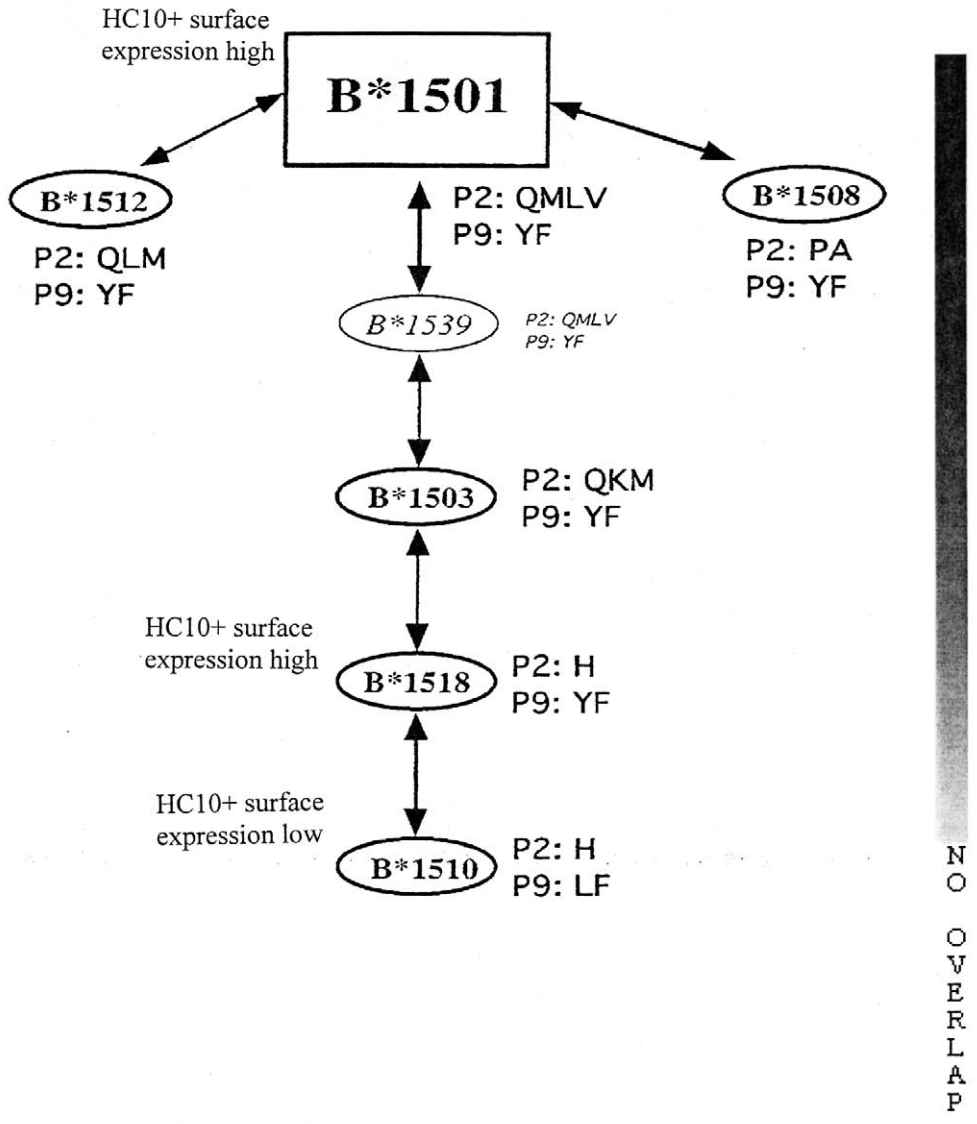

FIGURE 1 Class I HLA-B15 molecules from which peptides were characterized. Edman motif data for each B15 subtype is denoted by dominant and strong P2 and P9 anchors, respectively. The degree of overlap in the individual peptides bound by these subtypes decreases from top to bottom as indicated on the right. Data were obtained from B15 transfectants expressing between 1 and 5 μg sHLA-B15 per milliliter of cell pharm harvest [10–13]. Data for subtype B*1539 are hypothetical. The relative levels of surface expression, as measured by flow cytometry with monoclonal antibody HC10, are indicated for B*1501, B*1518, and B*1510.

both B*1508 and B*1518 shared a hydrophobic P9 with B*1501, they differed noticeably in their P2 preferences. Finally, most distant from B*1501 was the B*1510 motif, sharing only a Phe at P9 with B*1501 (Figure 1). Motif data, therefore, indicated that the B*1508, B*1518, and B*1510 motifs overlapped with the B*1501 motif at the C-terminus of bound ligands but not at the N-terminal anchor position.

Comparison of Individual B15 Ligands

Pooled motif sequencing is not a systematic comparison of individual ligands. In order to determine which, if any, individual ligands bind across polymorphisms in the binding groove, thousands of individual ligands were extracted from the six distinct HLA-B15 allotypes in Table 1. Peptides eluted from each of the six B15 subtypes were individually ion-mapped with a mass spectrometer, and the resulting ion maps were comparatively analyzed. The B*1501 and B*1512 ion maps were virtually indistinguishable, with $> 70\%$ of the ions in the B*1512 map matching ions in the B*1501 map. Ions subsequently subjected to NanoES-MS/MS sequence

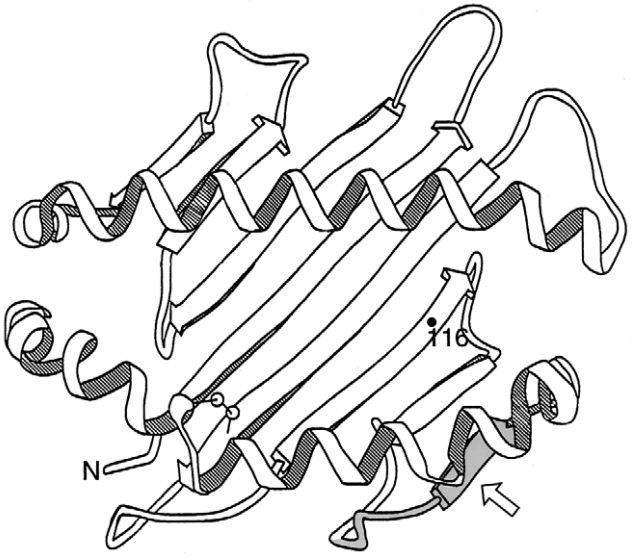

FIGURE 2 Diagrammatic top view of the α1 and α2 domains of an HLA class I molecule. Position 116 is indicated by a dot, and the position of the 128–136 loop is indicated by an arrow.

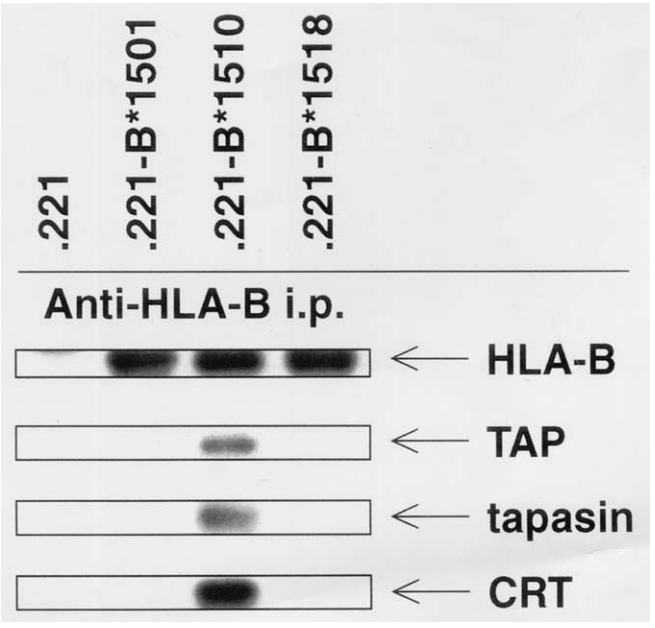

FIGURE 3 HLA-B*1501 and -B*1518 associate inefficiently with calreticulin, tapasin, and TAP. With monoclonal antibody (mAb) HC10, HLA-B heavy chains were immunoprecipitated from lysates of 721.221 cells transfected with B*1501, -B*1518, or -B*1510 cells. The immunoprecipitates were electrophoresed and transferred to membranes that were probed with mAb HC10 (H chain) or with antisera against calreticulin, tapasin, or TAP. The blots were treated with biotin-conjugated secondary antibody and streptavidin-conjugated horseradish peroxidase, developed with ECL reagents, and exposed to film. Figure reprinted from Turnquist *et al.* [14], *European Journal of Immunology* 30:3021, 2000 (reprinted with permission).

analysis confirmed this high level of specific peptide binding identity between B*1512 and B*1501. No other B15 subtype tested demonstrated this level of identity with B*1501.

Following B*1512, subtypes B*1503 and B*1508 manifested the most motif overlap with B*1501 (Figure 1). Comparative ion mapping demonstrated that 14% of the B*1503 ion peaks corresponded with peaks in the B*1501 map. Indeed, individual ligand sequencing identified 12 peptides that bound both B*1501 and B*1503. Ion map comparison between B*1501 and B*1508 revealed that $< 10\%$ of the ions between these allotypes were identical. Subsequent sequence analyses of over 400 individual peptide ligands identified 21 individual peptides that overlap between B*1508 and B*1501. Ion map comparison between B*1501 and B*1518 revealed that $< 1\%$ of the ions between these allotypes were shared. Nonetheless, the one peptide ligand that bound across B*1512, B*1501, B*1508, and B*1503 also bound to B*1518.

Only the B*1510 subtype failed to demonstrate ion map overlaps with any of the other B15 allotypes examined. The molecules B*1518 and B*1510 differ by only a Ser to Tyr substitution at position 116 (Table 1; Figure 2), yet this single substitution was sufficient to eliminate overlaps among the approximately 2000 ions mapped. This lack of overlap was not predicted from the motifs. Thus, one sequence difference dominated the selection of ligands. Specifically, the polymorphism at 116, which distinguishes B*1510 from all of the other B15 subtypes, abrogated all overlap in peptide ligand binding among the B15 subtypes.

Chaperone Interaction With B15 Subtypes

To examine the interaction of B*1501, B*1510, and B*1518 with the assembly complex (calreticulin, tapasin, and TAP), B15 heavy chains were immunoprecipitated with the monoclonal antibody HC10 [15, 16]. These immunoprecipitates were probed on Western blots using antisera reactive with various components of the assembly complex. As illustrated in Figure 3, we found that calreticulin, tapasin, and TAP exhibited little or no coprecipitation with B*1501 and B*1518, although all three ER proteins coprecipitated with B*1510 [14]. The result demonstrating that B*1501 has little or no interaction with TAP is in agreement with the findings of Neisig *et al.* [9], who demonstrated that TAP did not associate with the HLA-B62 serotype (allele B*1501 represents the predominant B62 serotype). In contrast, molecule B*1510, which differs from B*1501 and B*1518 at position 116, is able to interact with the

calreticulin-tapasin-TAP assembly complex. These findings indicate that a loss of tapasin and calreticulin binding is coordinated with the loss of TAP binding for B*1501 and B*1518, and that this coordinated loss is mediated by amino acid residue 116 of the MHC class I heavy chain. We have also performed preliminary analyses on HLA-B*1503, and the results to date report that B*1503 is not tapasin associated (unpublished data). This observation is consistent with our findings with B*1501 and B*1518, which share with B*1503 the serine at position 116 (see Table 1).

Related work has been published by Peh *et al.* [17], who reported that the products of three different HLA-B alleles (B8, B*4402, and B*2705) differed in their dependence on tapasin for surface expression. Notably, the study by Peh *et al.* [17] used tapasin-negative cells to illustrate that B*2705, unlike B*4402, can efficiently form complexes with peptide without associating with tapasin; however, their report did not directly indicate absence of association between B*2705 and tapasin when tapasin was available. To the contrary, when tapasin was transfected into 721.220. B*2705 cells, association between B*2705 and TAP was upregulated, and from this Peh *et al.* [17] concluded that the upregulation was due to bridging of B*2705 to TAP by tapasin.

A previous examination of many HLA types revealed that several HLA-B types interacted inefficiently with TAP but identified no HLA-A types with poor TAP association [9]. We therefore questioned whether there were HLA-A types that, dependent on the nature of position 116, had poor chaperone association. The HLA-A68 subtypes A*6803, A*6807, and A*68012 differ from each other at position 70 and/or 116. In A*6803, residue 70 is a histidine and residue 116 is an aspartic acid; in A*6807 position 70 is a glutamine and 116 is a histidine; and in A*68012 amino acid 70 is a glutamine and 116 is an aspartic acid. Notably, A*6807 and A*68012 differ only at position 116. We have found that TAP is strongly associated with A*6807 but has virtually no association with A*68012 or A*6803, suggesting that position 116 in HLA-A molecules, as in HLA-B molecules, is a primary determinant of chaperone association [18].

QUANTITY OF HEAVY CHAINS AT THE CELL SURFACE

To assess whether the lack of detectable interaction between the assembly complex and B*1501 and B*1518 in the ER affected the surface expression of the mature molecules, we tested the amount of heavy chain on the cell surface by flow cytometry. A small but consistent elevation in the HC10-reactive forms of B*1501 and B*1518, relative to B*1510, was seen ([14] and Figure 1). The lack of strong chaperone interaction by B*1501 and B*1518 within the cells may have resulted in the binding of a pool of slightly suboptimal peptides, which were lost by the HLA-B15 molecule while *en route* to or following arrival at the cell surface. In our HLA-A68 analyses, we found that inefficient association of A68 subtypes with the assembly complex correlated with an increased number of W6/32-reactive A68 molecules at the cell surface [18]. Thus, the overall surface expression of HC10 (open) and W6/32 (folded) forms may be higher for HLA class I molecules that naturally have poor assembly complex association. This scenario would be consistent with the evidence that has been obtained with tapasin-deficient cells suggesting that the assembly complex has a retentive function [19, 20]. Based on these data, together with the observation that HLA-A2 and H-2K^b molecules expressed by tapasin-negative cells are abnormally peptide receptive [17, 21], we believe that HLA types that associate inefficiently with intracellular chaperones may generally demonstrate lower peptide ligand binding affinities.

The expression of some transfected MHC class I molecules has been reported to be low on tapasin-deficient cells [17]. This might seem to contradict our finding that the overall surface expression is higher for HLA class I molecules that we have studied, which have a naturally poor assembly complex association. However, when an MHC class I heavy chain is transfected into a tapasin-deficient cell, the level of surface expression is allele-specific, and the products of some alleles do not exhibit reduction [17]. In fact, when multiple HLA-A2 and HLA-B7 transfectants of .220 (tapasin-deficient cells) or .45 (tapasin-normal cells) were compared, many of the .220 transfectants expressed surface A2 or B7 at higher levels than the .45 transfectants [21].

Furthermore, the distinction should be held in mind that experiments that were performed with tapasin-deficient cells are not exactly equivalent to experiments, such as ours, that were performed with cells coexpressing wild type tapasin and natural HLA molecules that cannot associate with tapasin. Tapasin has the additional function of TAP stabilization, and this function is not dependent on MHC class I binding. The ability of tapasin to stabilize TAP results in a higher level of overall peptide translocation into the endoplasmic reticulum, which could increase MHC class I surface expression [22, 23].

Tapasin Peptide in the B*1510 Groove

A dominant ion peak appeared during the analysis of the B*1510 ion maps, which was absent in the ion maps generated from the other B15 subtypes. Sequence analysis of this strong B*1510 ligand identified a peptide derived from the intracellular chaperone tapasin (tapa-

$sin_{334\text{-}342}$, HHSDGSVSL). Careful analysis of the B*1518 ion maps identified no such corresponding peptide ion. To study the effect of this tapasin sequence on the interaction of the whole tapasin protein with the MHC class I heavy chain, a human tapasin mutant (tapasin Δ334-342) lacking only these nine internal amino acids was constructed. This mutant was transfected into tapasin-deficient 721.220-L^d cells so that the well-characterized anti-L^d monoclonal antibodies 64-3-7 and 30-5-7 could be used for immunoprecipitation. These antibodies react with the open, peptide-free conformation and the folded conformations of L^d, respectively [24]. The ratio of folded to open L^d expressed by human cells has previously been illustrated to be comparable with that of L^d expressed by mouse cells, indicating that L^d assembles similarly in the presence of human or mouse tapasin [25]. By immunoprecipitating L^d and probing a Western blot for coprecipitated tapasin, we found that the deletion of this small segment of tapasin was sufficient to completely abrogate interaction with L^d [26]. This mutant is the second tapasin mutant reported to lack association with the MHC class I heavy chain; the first was a tapasin mutant lacking the N-terminal 50 amino acids [23].

Because deletion of a tapasin segment, even one that is only nine amino acid residues long, might affect the whole tapasin structure and, accordingly, tapasin function, we made point substitutions in the 334-342 region of tapasin by site-directed mutagenesis. By analysis of these mutants, we have recently ascertained that deletion of the entire 334-342 sequence is not necessary to abrogate tapasin/class I interaction. Either of two minor substitutions in tapasin, D337A (a single substitution) or S341R/L342T (substitution of two neighboring amino acids), is sufficient to bring about the same effect (H. Turnquist, S. Vargas, and J. Solheim, manuscript in preparation).

If the tapasin 334-342 peptide has a role in MHC class I assembly similar to that of the invariant chain CLIP peptide, this tapasin-derived peptide might be bound by other MHC class I molecules with a certain (moderate) affinity. To determine whether the tapasin 334-342 peptide can bind in the groove of many different class I molecules, we have tested whether it induces the surface expression of class I on T2 (which expresses a low level of surface, endogenous HLA-A2), T2-L^d, T2-B7-T2-B27, T2-K^d, or T2-D^d. We did not find significant induction of MHC class I surface expression by this peptide. However, Bangia *et al.* [23] have reported that the N-terminus of tapasin is also necessary for tapasin/MHC class I interaction, and there is some evidence that both the MHC class I α2 and α3 domains are involved in tapasin association with class I [27, 28]. Thus it may simply be true that two sites of binding between tapasin and MHC class I must be present for the two proteins to stably associate.

Does Tapasin Interact Directly With the MHC Class I Groove?

We propose that position 116 of the MHC class I heavy chain cooperatively influences peptide binding and chaperone association. Position 116 influences formation of the assembly complex with the MHC class I heavy chain. Our interpretation is that a portion of tapasin is anchored by amino acid 116 into the F pocket of particular heavy chain types during assembly complex formation. The possibility that tapasin might interact with the class I binding groove has been previously proposed [3]. This tapasin-class I assembly complex may represent a relatively stable intermediate that is retained in the ER until the binding groove-associated region of tapasin is either displaced by a high affinity peptide or is cleaved allowing the liberated tapasin334-342 peptide to be transported to the cell surface.

Therefore, the loading of peptides would be directly impacted by the interaction (or lack thereof) of the MHC class I heavy chain with tapasin. For an endogenous peptide to displace tapasin from the binding groove in a stable assembly complex, the endogenous peptide would need to be of a higher affinity than the tapasin segment. For displacement of tapasin to occur, the endogenous peptide would need both strong P2 and P9 anchors. Indeed, we find that P2 and P9 play equivalent roles in anchoring peptides into B*1510, the subtype that associates with tapasin. In contrast, peptides eluted from B15 subtypes that form comparatively less stable assembly complexes (in which tapasin does not associate with the binding groove) tend to have only a single dominant anchor at P9 and are more reactive with HC10 at the cell surface.

An alternative explanation for the influence of position 116 on chaperone association could lie in its proximity to a loop of the α2 domain (positions 128–136; Figure 2) that is involved in assembly complex association [8]. As has been previously pointed out, the F pocket is an unusually mobile region, as demonstrated by the crystallographic models of different MHC class I allele products and in comparisons of particular class I heavy chains binding distinct peptides [29]. In this scenario the presence of different amino acid residues at position 116 could cause regional shifts that affect the α2 domain loop position, and either increase or decrease the stability of assembly complex binding to this loop. However, this explanation is not consistent with the detection of a tapasin peptide in the groove of particular B15 subtypes.

It should be noted that tapasin binds to the open form of MHC class I [30, 31]. This form has an unfolded

groove and is conformationally quite different from the folded, peptide-occupied form, as attested by the finding that multiple antibodies that recognize the folded form do not recognize the open form, and vice versa [32]. Although there are many crystal structures of folded, peptide-occupied MHC class I molecules, the $\alpha 1/\alpha 2$ domains of open, peptide-free MHC class I molecules have not been crystallized. Thus, it is possible, in whatever loose conformation is adopted by the open form of the MHC class I heavy chain, that position 116 and positions 128–136 may be even more proximal than in the folded form, facilitating the interaction of tapasin with both regions.

CONCLUSION

In conclusion, we hypothesize that position 116 impacts ligand presentation directly through interaction with bound peptides and indirectly through assembly complex formation. This hypothesis is based upon the impact that a polymorphism at this binding groove position has upon (1) the anchor residue(s) for bound peptides; (2) the presence of a tapasin-derived ligand in the groove of particular subtypes [13, 33]; and (3) the ability/inability to bind efficiently to the assembly complex. Position 116 clearly impacts class I antigen presentation in distinct, yet overlapping, ways.

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