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Tapasin Facilitation of Natural HLA-A and -B Allomorphs Is Strongly Influenced by Peptide Length, Depends on Stability, and Separates Closely Related Allomorphs

Linda Geironson,* Camilla Thuring,* Mikkel Harndahl,† Michael Rasmussen,† Søren Buus,‡ Gustav Røder,‡ and Kajsa M. Paulsson*  

Despite an abundance of peptides inside a cell, only a small fraction is ultimately presented by HLA-I on the cell surface. The presented peptides have HLA-I allomorph-specific motifs and are restricted in length. So far, detailed length studies have been limited to few allomorphs. Peptide–HLA-I (pHLA-I) complexes of different allomorphs are qualitatively and quantitatively influenced by tapasin to different degrees, but again, its effect has only been investigated for a small number of HLA-I allomorphs. Although both peptide length and tapasin dependence are known to be important for HLA-I peptide presentation, the relationship between them has never been studied. In this study, we used random peptide libraries from 7- to 13-mers and studied binding in the presence and absence of a recombinant truncated form of tapasin. The data show that HLA-I allomorphs are differentially affected by tapasin, different lengths of peptides generated different amounts of pHLA-I complexes, and HLA-A allomorphs are generally less restricted than HLA-B allomorphs to peptides of the classical length of 8–10 aa. We also demonstrate that tapasin facilitation varies for different peptide lengths, and that the correlation between high degree of tapasin facilitation and low stability is valid for different random peptide mixes of specific lengths. In conclusion, these data show that tapasin has specificity for the combination of peptide length and HLA-I allomorph, and suggest that tapasin promotes formation of pHLA-I complexes with high on and off rates, an important intermediary step in the HLA-I maturation process.  

preferences of the HLA-I peptide binding groove with regard to which peptides are eventually presented on the surface of APCs. From a given viral proteome, only very few peptides have immunodominant status and are responsible for the majority of produced CD8+ T cells (32). A broad bottom-up approach with as many potential immunodominant epitopes as possible is hence desirable for selection of peptide epitopes for peptide-based vaccines. HLA-I binding of longer peptides (11–25 aa) has indeed been shown, and presentation efficiency was found to be equal or even greater as compared with shorter peptides (33, 34). It has been proposed that crucial factors controlling what lengths of peptides are presented include Ag-processing variables, such as proteasomal cleavage products and TAP-peptide transport preferences (33).

Although tapasin has been shown to strongly influence the presented peptide repertoire, its effect on HLA-I binding to peptides of different lengths has not been studied. As neither the HLA-I allomorph preferences nor the effect of tapasin, for different peptide lengths, are firmly established, in this study, we used random peptide libraries of 7- to 13-mer-long peptides (from here on referred to as X7–X13 libraries) to study peptide–HLA-I (pHLA-I) complex formation and facilitation by tapasin. Tapasin shapes the presented peptide repertoire in terms of quality and quantity, and keeps a pool of peptide-receptive HLA-I molecules, for which peptides have been suggested to associate and dissociate faster, in the ER allowing peptide optimization and subsequent rapid surface presentation in the case of, for example, viral infection (5).

Partly with base in the suggestion that tapasin preferably interacts with pHLA-I complexes of lower stability, we also studied the stability of pHLA-I complexes as a function of peptide length.

Materials and Methods

Recombinant protein production

HLA-I H chains and β2-microglobulin (β2m) were generated as previously described (35, 36). In brief, HLA-I H chains encoding the soluble part of the protein were fused at the C termini with a histidine affinity tag and a biotinylation-signal peptide. The protein was expressed in Escherichia coli inclusion bodies, extracted, and purified. The degree of biotinylation (usually >95%) was determined with gel-shift assays. The HLA-A*02:01-T134K protein preparations in our hands repeatedly suffer from a lower quantified in a W6/32-based AlphaScreen assay, which recognizes folded complex formation to reach a steady-state. pHLA-I complexes were incubated at 18˚C for 48 h to allow the pHLA-I complex formation to reach a steady-state. pHLA-I complexes were incubated at 18˚C overnight, equilibrated to reader temperature for 1 h, and subsequently read in a plate reader (EnVision; Perkin Elmer). The plates were incubated at 18˚C overnight, equilibrated to reader temperature for 1 h, and subsequently read in a plate reader (EnVision; Perkin Elmer). The conversion of AlphaScreen signal to concentrations of folded pHLA-I complex was done using a prefolded pHLA-I standard of known concentration.

Stability assay

A scintillation proximity-based assay was used to measure pHLA-I complex stability (38). In brief, a mix of 50 mM biotinylated HLA-I H chain,[152I]-labeled β2m (final sp. act. of 125 copm/μl), and 0.1 mg/ml peptide library (X7–X13) was incubated in a streptavidin-coated FlashPlate (SMP103; Perkin Elmer) at 18˚C for 24 h. Dissociation of the pHLA-I complexes was initiated by adding an excess (1 μM) of unlabeled β2m followed by continuous reading in a liquid scintillation counter (TopCount NXT; Perkin Elmer) set to 37˚C. The dissociation curves were analyzed using GraphPad Prism 5.0 software. Nonlinear regression was carried out with the least-squares fitting method. An extra sum-of-squares F-test was performed and a more complex curve fit model was selected to compare models when the null-hypothesis could be rejected (i.e., F < 0.05). In all cases, dissociation data were fit to biphasic dissociation equations (p < 0.0001) with the plateau set to zero (i.e., two-phase decay was always prefered over one-phase decay with p < 0.0001), whereas more complex dissociation models cannot be excluded.

Data analysis

Tapasin facilitation was calculated as (the highest [pHLA-I] in the presence of Tpn1–87)/(the highest [pHLA-I] in the absence of Tpn1–87). The values for the average HLA-I allomorph were calculated after normalization of

FIGURE 1. Peptides of varying lengths differentially bind to HLA-A*02:01 and HLA-A*02:01-T134K. Synthetic random peptide libraries (X) of 7–13 aa (X = 19 L-amino acids, cysteine excluded) were analyzed for pHLA-I complex formation in an LOCI assay. The highest amount of [pHLA-I] formed with each peptide length is shown in arbitrary units (AU) after normalization against the highest amount of [pHLA-I] formed with X9 (highest complex formation). (A) Complex formations of HLA-A*02:01 with X7–X13 libraries. (B) HLA-A*02:01-T134K complex formations with X7–X13 libraries. Results shown are representatives from duplicate setups from several similar experiments.
the highest amount of [pHLA] for each length against the highest value of [pHLA-I] of any length. Average tapasin facilitation per peptide length was calculated based on values for each allomorph after normalization using the following ratio: \( \frac{(\text{the highest [pHLA-I] + Tpn}_{1-87} \times n)}{(\text{the highest [pHLA-I] + Tpn}_{1-87} \times X_{\text{highest value for any length}})} \)\( \frac{(\text{the highest [pHLA-I] + Tpn}_{1-87} \times X_{\text{highest value for any length}})}{\text{number of studied natural allomorphs}}. \) The values representing all studied HLA-I allomorphs were based on the average of the normalized values calculated for each separate allomorph. All data were analyzed in GraphPad Prism 5.0.

Results

Peptides of various lengths bind to HLA-A*02:01

The preference for certain amino acids at specific peptide positions varies for different HLA-I allomorphs, even for allomorphs differing in only one amino acid (17). Peptide-binding motifs have traditionally been studied with a focus on sequence rather than length, based on the central dogma that HLA-I molecules bind peptides of 8–10 aa in length (31). HLA-I presentation of short peptides is rare; however, a number of different studies have shown that HLA-I binds and presents peptides considerably longer than 8–10 aa (39–42). In particular, the binding and presentation of longer peptides up to 14 mers has been reported for A*02:01, including several CTL epitopes of 11 aa in length, challenging the exclusiveness of peptide lengths of 8–10 aa (43–45). To further our understanding of how peptides of different lengths bind A*02:01, we set out to study the influence of peptide length on binding to wild type A*02:01 and mutant A*02:01-T134K using peptide libraries with random sequences of 7–13 aa in length, that is, \( X_n \), where \( n = 7, 8, 9, 10, 11, 12, \) or 13. A high number of peptide-A*02:01 complexes were formed in the presence of peptides with \( \geq 9 \) aa, 8-mer peptides formed intermediate numbers, and 7-mers low numbers of peptide-A*02:01 complexes (Fig. 1A). Complex formation of peptides with A*02:01-T134K was also dependent on peptide length (Fig. 1B).

HLA-A and -B molecules prefer peptides of different lengths

To study the length preferences of an extended group of HLA-I allomorphs, we used peptides of various lengths, \( X_7–X_{13} \) and recombinant versions of 16 allomorphs. We observed that the HLA-B allomorphs had a stronger preference for binding 8- to 10-mers (i.e., the classic lengths presented by HLA-I), whereas the HLA-A allomorphs were less length sensitive and bound slightly longer peptides best (i.e., 10- or 11-mers formed the most complexes under our experimental conditions; Fig. 2). The finding that HLA-B molecules bind well to 8-, 9-, and 10-mers is not surprising, and a high occurrence of presented peptides of these lengths has been reported in the SYFPEITHI database of natural HLA-I ligands. The exact differences in binding grooves that allow shorter peptides to bind better and/or exclude longer peptides from binding HLA-B and explain why HLA-A allomorphs tolerate or even prefer longer peptides remain to be explored.

HLA-A and -B allomorphs are facilitated to different degrees by tapasin

Despite recent advances in defining HLA-I sites and regions that are important for interaction with tapasin, the exact molecular

![FIGURE 2. Peptide length influences the degree of tapasin facilitation of HLA-A and HLA-B molecules with partly allomorph-specific preferences. HLA-A and HLA-B allomorphs were incubated with \( X_7–X_{13} \) libraries of random peptides in the presence or absence of Tpn\(_{1-87}\). The formation of pHLA-I complexes was analyzed with an LOCI assay. The allomorphs were sorted into the three groups based on the degree of tapasin facilitation. The bars show the highest [pHLA-I] values for each peptide length in the presence (white) or absence of Tpn\(_{1-87}\) (gray) for each studied HLA-I allomorph. Data shown are the averages of duplicate samples from one of two independent setups.](http://www.jimmunol.org/)
features in different HLA-I allomorphs that dictate tapasin dependence variation are unknown (46). Previous studies of tapasin dependence have been limited to labor-intensive cellular models that could study only a few HLA-I allomorphs at a time, and variation in the amount of HLA-I expressed after transfection made it difficult to compare HLA-I allomorphs (15, 20). We used a recombinant pHLA-I folding assay that allowed us to simultaneously study several HLA-I allomorphs in the presence and absence of the first 87 N-terminal amino acids of tapasin (Tpn1–87) under identical conditions. The assay is both sensitive and specific, and is suitable for analysis of a large number of samples. We have previously observed that Tpn1–87 facilitates folding of A*02:01, B*08:01, B*44:02, and B*27:05 to different degrees, but not A*02:01-T134K (4). This is perfecty consistent with other studies of full-length tapasin and these HLA-I allomorphs in cellular models (15, 20) and of A*02:01-T134K (21, 22). Based on the prevailing idea that 8- to 10-mers are abundant natural ligands and of optimal length for HLA-I binding (31), we studied tapasin facilitation of 16 HLA-I molecules during binding of X8–X10 peptide libraries.

The allomorphs were divided into three groups based on their tapasin facilitation: high (>2.3), intermediate (2.3–1.3), and low (<1.3; Fig. 3). The high tapasin-facilitated group included B*44:02, B*51:01, B*27:03, and B*08:01. Intermediate facilitation was observed for B*15:01, A*01:01, B*27:05, A*24:02, A*30:01, A*11:01, B*40:01, B*18:01, and A*02:01. The low facilitation group was composed of B*35:01, A*02:10, and the mutant A*02:01-T134K. These data suggest that the span of tapasin dependence is very broad, with both HLA-A and HLA-B allomorphs distributed all over the spectra. Interestingly, all HLA-I allomorphs with very high dependence on tapasin belonged to the HLA-B group.

**Tapasin-facilitated X7–X13 peptide binding is allomorph dependent**

We next set out to study tapasin facilitation using peptides shorter and longer than the traditionally considered optimal length of 8–10 aa, including X7, X11, X12, and X13 libraries (Fig. 2). When HLA-I allomorphs were organized according to tapasin facilitation based on the averaged results of all Xn libraries studied, the order of HLA-I allomorphs within the different groups changed, but no allomorph shifted its position to an extent that allowed inclusion into a new group (i.e., no allomorph shifted from high to low or intermediate to low or vice versa; Supplemental Fig. 1). In both the presence and absence of Tpn1–87, the less tapasin-dependent HLA-I allomorphs form more pHLA-I complexes compared with the highly tapasin-dependent allomorphs (Fig. 4).

**Tapasin facilitation is lowest for binding of 10- and 11-mer peptides for most allomorphs**

As expected, 7- and 13-mers form the least number of pHLA-I complexes for most allomorphs, whereas 9- to 11-mers form the most pHLA-I complexes in both the presence and absence of Tpn1–87 (Fig. 2). Allomorphs in the low tapasin-facilitated group and some of the intermediate-facilitated allomorphs, that is, A*02:01, B*27:05, B*18:01, and A*24:02, are not as adherent to the prevailing dogma that optimal peptides for binding are 8–10 aa in length; rather, they formed a high number of complexes even with peptides >11 aa. The most tapasin-facilitated allomorphs (B*44:02, B*51:01, B*27:03, and B*08:01) have the strongest preference for specific peptide lengths and form most complexes with 8- and 9-mers, and also 10-mers for B*27:03. The amount of B*44:02 formed without Tpn1–87 is so low that any conclusions regarding differences between X7- and X13-induced levels of maximum folded complex should be considered with extreme caution. The highly tapasin-facilitated allomorphs form several times less pHLA-I complex in the absence of optimal peptide lengths (Figs. 2, 4), indicating a stricter length requirement. For all studied allomorphs, the binding of 10- and 11-mers was consistently least facilitated by tapasin (Fig. 5).

**High tapasin facilitation correlates with lower stability**

Cell-surface Ag presentation requires pHLA-I complexes that are stable enough to be exposed to scanning CD8+ T cells for sufficient time to allow signal transmission via TCRs specific for the presented pHLA-I complex. Hence a key requirement for pHLA-I complex functionality is stability, which in vitro settings involves several factors, including maturation/folding time, temperature, detergent, and chaperone presence. We previously analyzed the effect of Tpn1–87 on HLA-I stability after folding for 24–48 h at 18˚C without any effect on the dissociation of A*02:01 with different peptides (4, 47). These studies showed that high stability and low tapasin facilitation correlated for pHLA-I complexes formed with the selected studied peptides. Because tapasin facilitation has been shown to correlate with stability for a limited number of specified peptides, we wanted to investigate, independently of peptide sequence, whether two different HLA-I molecules distinctively separated by a degree of tapasin facilitation also had different stabilities. Because A*02:01 and its mutant version...
A*02:01-T134K are well separated in terms of tapasin facilitation, we used X 8–X10 libraries to study the stability of these two molecules by closely monitoring the dissociation of pHLA-I complexes over 24 h at 37˚C. Consistent with the theory that more stable complexes are less facilitated by tapasin, the more tapasin-facilitated A*02:01 was indeed less stable than the mutant A*02:01-T134K, which is the least tapasin-dependent HLA-I molecule studied to date (Fig. 6A). The higher stability of HLA-I allomorphs that are less facilitated by tapasin can be considered as partly a function of intrinsic features that are less affected by peptide binding and tapasin facilitation. Consistent with previous work on A*02:01, the pHLA-I complexes formed with peptides from either of the X8 libraries dissociated with a suggested biphasic or even triphasic pattern, with a major proportion of pHLA-I complexes formed with peptides from either library following a fast dissociation curve (Fig. 6A) (47).

Peptide length strongly influences not only the tapasin facilitation but also the stability of pHLA-I complexes (Figs. 6, 7). To investigate whether stability of pHLA-I complexes is influenced by peptide length, we analyzed the dissociation of pHLA-I complexes formed with X7–X13 libraries. The 7- and 8-mers formed peptide-A*02:01 complexes of the poorest quality, whereas the most stable complexes were formed with the 13-mer library peptides, which clearly had the highest proportion of remaining pHLA-I complexes after 24 h (Fig. 6B). The dissociation of complexes formed with 9- to 12-mer peptides all had similar dissociation patterns placing these complexes in a group of intermediary stability compared with the 7- to 8-mers and 13-mers. The finding that the spread in stability of A*02:01-T134K with X7–X13 is narrower supports the hypothesis that A*02:01-T134K stability is less dictated by bound peptides. We show in this article that tapasin facilitation is at least partly a function of stability of pHLA-I complexes and relates not only to differences in specific peptide sequences or HLA-I allomorphs, but is also applicable on and differentially affects peptide libraries of different lengths.

Discussion
Based on the recent finding that it is possible to use Tpn1–87 to determine tapasin dependence and technological advances (i.e., robotic sample preparation systems and sensitive readouts with high signal-to-noise ratios) that make it feasible to simultaneously investigate a large set of HLA-I allomorphs, we set out to study the impact of peptide length on HLA-I binding, the impact of tapasin on the binding of peptides of different lengths, to determine the degree of tapasin facilitation and how peptide length and tapasin facilitation relate to the stability of pHLA-I com-
plexes. Among 16 HLA-I allomorphs, we found that the studied HLA-B allomorphs adhere more strictly than HLA-A allomorphs to the classic rule of binding 8- to 10-mers. Two of nine HLA-B molecules preferred 8-mers, whereas all studied HLA-A allomorphs had 10-mers as preferred ligands. Interestingly, in the presence of Tpn1–87, 5 of 9 HLA-B molecules formed the most complexes with 8-mers, whereas 5 of 7 HLA-A allomorphs had 10-mers as preferred ligands and tolerated binding to even longer peptides (Fig. 2).

The amino acid positions in the HLA-I structure that influence tapasin interaction are known to some extent, but the exact molecular features responsible for tapasin-dependent or -independent phenotype are still not characterized (46). It has been hypothesized that the ionic/hydrophobic environment at the bottom of the F...
of allomorphs with a higher propensity for binding of longer peptides was also less tapasin dependent. The ability of HLA-I molecules to form complexes with longer peptides suggests an efficient utilization of ER luminal resources, in that it allows binding to a large proportion of TAP-transported peptides, which are usually 8–16 aa in length (50). The dominance of 8- to 10-mer peptides presented at the cell surface may be explained partly by the presence of ER peptidases, such as ERAP1 and ERAP2 (51, 52).

Recently, not only stability but also tapasin facilitation has been suggested as a good indicator of immunogenicity (4). However, the question of differential stability in tapasin-dependent HLA allomorphs compared with less tapasin-dependent allomorphs has not been clearly answered. We studied in this article the stability of two HLA-I molecules with different tapasin facilitation after folding and binding to X7–X13 peptide libraries. The more tapasin-facilitated A*02:01 was clearly less stable than its mutated, less tapasin-facilitated counterpart A*02:01-T134K (Fig. 6A). Accordingly, A*02:01 binding to 7- and 8-mers showed the lowest stability and the highest tapasin facilitation (Figs. 2, 6B). To our surprise, despite low tapasin facilitation and a low initial count of 13-mer-A*02:01 complexes, these were the most stable over time (Fig. 6B and schematically shown in Fig. 6C). This could reflect an assembly/binding process that requires a high level of energy, with corresponding energy-consuming dissociation. The high tapasin facilitation of 7-mers in particular, and to some extent 8-mers, together with their poor stability, suggests that tapasin facilitation generates easily opened peptide-exchangeable complexes. On the other hand, tapasin facilitation was low for 13-mers, which may reflect another type of binding kinetics for

FIGURE 7. The relative average proportions of optimal and suboptimal peptides in X7–X13 random peptide libraries. The proportion of peptides binding to HLA-I was calculated as an average for all studied HLA-I allomorphs based on the highest [pHLA-I] values for each separate allomorph. (A) The highest [pHLA-I] for each length +/- Tpn1–87 was respectively normalized to the highest value of [pHLA-I] in the presence of Tpn1–87 for any length. Values for each length are shown as an average for all 15 studied natural allomorphs. (B) The average HLA-I Tpn1–87 facilitation for each length was calculated as the normalized ratio of (((pHLA-I) Tpn1–87)/(pHLA-I)Xmax) to (((pHLA-I) Tpn1–87highest)/(pHLA-I)Xhighest))/15 allomorphs.
binding of longer peptides to A*02:01. A possible model is that once a 13-mer has bound, it produces a more stable complex and possibly a more locked peptide-binding groove, where the action of tapasin is important for the exchange of suboptimal preoccurring peptides but not for actual locking after a longer peptide has bound. The low tapasin facilitation of more stable pHILA-I complexes suggest that these molecules are either not in a conformation that allows tapasin to interact or have a conformation for which association has no effect.

Fig. 7 shows the average highest amount of pHILA-I complexes based on normalized binding values in the absence and presence of Tpν1-87 of the HALA-I allomorphs we studied (for the values of the average HALA-I allomorphs representing the groups of high, intermediate, and low facilitated HALA-I allomorphs; see Supplemental Fig. 2). The peptide pool bound to the “average HALA-I allomorph” during maturation in the absence of Tpν1-87 is suggested to constitute optimal peptides. This is explained by the lower overall amount of pHILA-I complexes and the lack of the additional pHILA-I complexes chaperoned by Tpν1-87. In the presence of Tpν1-87, both suboptimal and optimal peptides are suggested to bind, with the main increase in the amount of pHILA-I complexes likely to arise from bound suboptimal peptides, which are the likely substrate for which Tpν1-87 increases pHILA-I Bmax (4). However, they must not be confused with the end products, that is, optimal peptides such as natural ligands from the SYFPEITHI database presented at the cell surface after tapasin editing. It is also important to consider the distinction between suboptimal peptides and poor or nonbinders. Suboptimal peptides are believed to be important during intermediate HALA-I maturation stages and are retained in the ER by tapasin until the optimal peptide has been loaded (9). The facilitation of binding of 10- and 11-mers was lower than for 8- and 9-mers (Figs. 5, 7B), and in the absence of Tpν1-87, the formation of pHILA-I complexes was higher than for 7- to 9- and 12- and 13-mers (Figs. 2, 7A). This suggests that there are both a smaller proportion of suboptimal peptides and a smaller proportion of optimal peptides in the X12 and X13 libraries than in the X10 and X11 libraries (Fig. 7).


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Disclosures

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Supplementary figure 1. Tpn1-87 facilitates peptide binding of 15 natural HLA-I molecules to a higher degree than tapasin-inert HLA-A*02:01-T134K and separates them over a broad spectrum. Using 16 different recombinant HLA-I molecules and Tpn1-87 in assays with X7–X13 libraries, complex formation was studied using a LOCI assay (see figure legend 1 and material and methods). The average Tpn1-87 facilitation of each allomorph bound to X7–X13 is shown as a line, while the specific tapasin facilitation for each peptide length is shown in the plot by the number of the peptide length, i.e., 7–13. The data shown is the average of duplicate samples from one of two independent setups.
Supplementary figure 2. The proportions of optimal and suboptimal peptides in X7–X13 random peptide libraries that bound to HLA-I vary for allomorphs with high, intermediate and low tapasin facilitation. The proportion of peptides binding to HLA-I was calculated as an average for HLA-I allomorphs with low, intermediate and high tapasin facilitation respectively, using the highest [pHLA-I] values for each separate allomorph. A) The highest [pHLA-I] for each length +/- Tpn1-87 was respectively normalized to the highest value of [pHLA-I] in the presence of Tpn1-87 for any length. Values for each length are shown as an average for HLA-I in each tapasin facilitation group, i.e., high, intermediate and low. B) The average HLA-I tapasin facilitation for each length and each tapasin facilitation group is shown. The data shown is the average of duplicate samples from one of two independent setups.