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Stability of peptide–HLA-I complexes and tapasin folding facilitation – tools to define immunogenic peptides

Linda Geironson [a], Gustav Røder [b], Kajsa Paulsson [a,b,*]

[a] Immunology Section, BMC-D14, Lund University, Experimental Medical Sciences, SE-221 84 Lund, Sweden
[b] Department of Experimental Immunology, Institute of International Health, Immunology and Microbiology, University of Copenhagen, Panum Institute 18.3, Blegdamsvej 3B, DK-2200 Copenhagen, Denmark

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ABSTRACT

Only a small fraction of the peptides generated inside the cell end up being presented by HLA-I on the cell surface. High stability of peptide–HLA-I complexes and a low HLA-I tapasin-facilitation have been proposed to predict immunogenicity. We here set out to investigate if these parameters correlated and defined immunogenic peptides. Both peptide–HLA-B*08:01 and peptide–HLA-A*02:01 complexes showed small differences in tapasin-facilitation and larger differences in stability. This suggests that the stability of immunogenic peptide–HLA-I complexes vary above an HLA-I allomorph dependent lower limit (e.g. >2 h for HLA–A*02:01), immunogenicity predicted by tapasin-facilitation may be defined by an equally allomorph unique upper value (e.g. tapasin-facilitation <1.5 for HLA–A*02:01), and variation above the stability-threshold does not directly reflect a variation in tapasin-facilitation.

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1. Introduction

Human leukocyte antigen class I (HLA-I) molecules present peptides to CD8+ T-cells, and are crucial in the clearance of viral pathogens and of cells in the process of malignant transformation. The peptide–HLA-I (pHLA-I) complex is formed in a highly regulated process, in which several different ER proteins work together to assure the formation of stable pHLA-I complexes. Once the HLA-I heavy chain (HC) has associated with β_2-microglobulin (β_2m), the HLA-I molecule is bridged by tapasin to the transporter associated with antigen processing (TAP), thereby forming the peptide-loading complex (PLC), which also consist of calreticulin (Crt) and ERp57 [1]. Inside the PLC, HLA-I is kept in a peptide-receptive state until an optimal peptide is bound. Peptide-receptive and suboptimally peptide-loaded HLA-I molecules are suggested to be both chaperoned and retained in the ER by tapasin until a peptide inducing a stable pHLA-I complex have bound. Binding of optimal peptides induces release of pHLA-I from tapasin and egress to the cell surface.

In the PLC, tapasin is believed to play a key role in the late stage maturation of HLA-I molecules, allowing cell surface expression of stable pHLA-I complexes. Since its discovery, tapasin has been attributed several functions – to increase the available local peptide concentration by bridging HLA-I molecules to TAP [2–4], to stabilize TAP expression and improve its capacity to bind peptide prior to the translocation step [5–8], to keep HLA-I molecules peptide-receptive in the PLC [9–11], to recycle empty or suboptimally loaded HLA-I molecules back to the ER [4,12–14], and, to act as a peptide-editor, allowing exchange of suboptimal peptides for optimal peptides [1,15,16]. However, the exact molecular mechanisms of the tapasin-mediated HLA-I quality control remain unknown. The optimization process is suggested to work towards pHLA-I molecules of higher stability but the boundaries in half-lives defining immunogenic pHLA-I complexes are poorly defined.

From the law of mass action, the equilibrium dissociation constant (K_D, a measure of affinity) of a bimolecular receptor–ligand interaction is equal to the ratio of the rate of dissociation (k_d, the dissociation rate coefficient) to the rate of association (k_a, the association rate coefficient):

$$K_D = k_d/k_a \quad (1)$$

Under conditions of limited HLA-I (receptor) concentration and high peptide (ligand) concentration EC_{50} is a reasonable approximation of K_D (i.e. affinity). The off-rate/dissociation-rate coefficient is here a measure of stability. Since the HLA-I HC – β_2m dimer is highly unstable in the absence of peptide and it has been demonstrated that dissociation of ^{125}I-labeled β_2m precisely

* Corresponding author at: Immunology Section, BMC-D14, Lund University, Experimental Medical Sciences, SE-221 84 Lund, Sweden.
E-mail address: kajsa_m.paulsson@med.lu.se (K. Paulsson).

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corresponds to dissociation of peptide, we here used dissociation of β_2m as a measure of peptide dissociation [17]. Given the law of mass action, affinity and stability are related but do not always follow each other, e.g. high affinity does not always correspond to high stability. Although a general perception of affinity is a good measurement of potential immunogenicity, different studies have indeed suggested stability as a more reliable parameter [18,19]. In this study we selected HLA–A*02:01 and HLA–B*08:01 binding peptides from the SYFPEITHI database (i.e. natural ligands largely defined as being immunogenic), determined folding and stability of these pHLA-I complexes, and studied complex formation and dissociation under the influence of Tpn_{1-87} (amino acids 1–87 of the mature tapasin protein [20]). Although all peptides were of high affinity the stability varied and also to some extent the ratio of the increase in B_{max} (B_{max} = amount pHLA-I complex formed) in presence and absence of Tpn_{1-87}, termed tapasin-facilitation. The hierarchy between pHLA-I complexes based on changes in tapasin-facilitation did not correlate with the, slightly larger, changes in stability. Moreover, the effects of Tpn_{1-87} were more pronounced on HLA–B*08:01 than HLA–A*02:01, and absent on the tapasin-independent allomorph HLA-A*02:01-T134K, in line with the previously established hierarchy of tapasin-dependency for these HLA-I molecules [21]. Although stability is a good tool to predict immunogenicity, a change in stability not necessarily equals a change in immunogenicity. Moreover, the lower limit of stabilities defining immunogenic pHLA-I complexes differs for different HLA-I allomorphs and makes predictions of immunogenicity more complicated. Similarly, predictions based on a low-degree of tapasin-facilitation should be done first after determining the upper limit of tapasin-facilitation for immunogenic peptides for the specific allomorphs studied. Although a previous study of large sets of HLA–A*02:01 non-SYFPPEITHI and SYFPEITHI peptides indicated that the tapasin-facilitation and the stability to a very high degree of specificity discriminate SYFPEITHI peptides from non-SYFPEITHI peptides [18], and although the peptides studied here for both HLA–A*02:01 and HLA-B*08:01 are all defined by both a reasonable high stability (over 2 and 1.5 h half-life respectively) and a low tapasin-facilitation (less than 1.5 and 1.8 respectively), studies have shown that stability does not always correlate with immunogenicity [22]. Hence, we propose that for best sensitivity, and to a lesser extent specificity, both stability and tapasin-facilitation thresholds should be determined and used as complementary tools in the development of candidates for peptide-based vaccines.

2. Materials and methods

2.1. Peptides

All peptides were purchased from Schafer-N (Denmark). Peptides intended to be used in setups with ^{125}I-labeling the sequence of the original SYFPEITHI peptides were modified. $Ataxin_{324-333}$ (QVFPGLLERV) was modified with substitutions of F to Y, and R to K, resulting in the QVY peptide, QVYPGLLEKV. Ribosomal protein $L10a_{8-16}$ (TLYEAVREV) was modified with substitution of E to K, resulting in the TLY peptide, TLYEAVRKV. Cadherin 17 $precursor_{2-10}$ (ILQAHLHSL) was modified with substitutions of Q to Y, and of S to K, resulting in the ILY peptide, ILYAHLHKL. FLRGRAYGL (EBV EBNA3A), QAKWRLQTL (EBV EBNA3) and ELRSRYWAI (Influenza A) were synthesized as original SYFPEITHI sequences. Briefly, all peptides were synthesized (Applied Biosystems, model 431A), using conventional F-moc (*N*-(9-fluorenyl) methoxycarbonyl) chemistry. The peptides were subsequently purified by RP-HPLC and dissolved in PBS.

2.2. Peptide–HLA-I folding assay

Peptide–HLA-I (pHLA-I) folding was monitored in a luminescence oxygen chanelling immuno (LOCI) assay (commercialized as amplified luminescence proximity based homogenous assay (AlphaScreen)) as previously described [20,23]. Briefly, 2 nM biotinylated recombinant HLA-I HCs were diluted in a buffer containing different concentrations of peptide, recombinant human 30 nM β_2m and presence or absence of 20 nM Tpn_{1-87}. The reaction mixtures were incubated at 18 °C for 48 h to allow pHLA-I complex formation to reach steady-state. pHLA-I complexes were quantified in a W6/32-based AlphaScreen assay, which recognizes folded pHLA-I complexes. Detection of folded pHLA-I complexes was done by adding 15 μl folding reaction to 15 μl PBS containing 10 μg/ml of AlphaScreen Donor beads (PerkinElmer, 6760002; conjugated with streptavidin) and Acceptor beads (PerkinElmer, 6762001; in-house conjugated with the W6/32 antibody). The plates were incubated at 18 °C over-night, and then 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 pre-folded pHLA–A*02:01 standard of known concentration.

2.3. Peptide–HLA-I stability assay

To measure stabilities of pHLA-I complexes a biochemical proximity assay was used [24]. Briefly, 50 nM biotinylated HLA-I HC, ^{125}I-labeled β_2m (final specific activity of 125 cpm/μl), and 1 μM of an appropriate strong binding peptide was incubated in the presence or absence of 500 nM Tpn_{1-87}. The reactions were incubated in a streptavidin coated FlashPlate (PerkinElmer, SMP103) at 18 °C for 24 h. Dissociation of the pHLA-I complexes was initiated by adding excess (1 μM) of unlabeled β_2m followed by continuous reading in a liquid scintillation counter (TopCount NXT, Perkin Elmer) thermostated to 37 °C. The half-lives were calculated from the dissociation curves using Prism 5 (GraphPad). Non-linear regression was made with least squares fit. To compare models, an extra sum of square *F*-test was done, and more complex curve fit model was selected when the null-hypothesis could be rejected (i.e. $P < 0.05$). Dissociation data were in all cases fit to biphasic dissociation equations (P-value < 0.0001) with the plateau set to zero.

3. Results

3.1. SYFPEITHI peptides of high affinity bind to both HLA–A*02:01 and HLA–B*08:01, but Tpn_{1-87} facilitates complex formation only with HLA–B*08:01 to a significant extent

To study the role of tapasin in HLA-I maturation we have previously produced a recombinant protein consisting of the first 87 amino acids of tapasin, Tpn_{1-87} [20]. When Tpn_{1-87} was present during de novo folding of recombinant HLA–A*02:01 we observed that an increased amount of pHLA–A*02:01 complexes was generated. These observations, together with recent results for different HLA-I molecules (i.e. HLA–B*44:02, HLA–B*08:01, HLA–A*02:01, HLA–A*02:01(T134K), and HLA–B*27:05), suggest that Tpn_{1-87} facilitates folding of pHLA-I complexes to a degree depending on both the peptide and HLA-I HC identity [18]. Importantly, Tpn_{1-87} was shown to discriminate HLA–A*02:01 immunogenic peptides (i.e. peptides reported in the SYFPEITHI database as natural MHC-I ligands) from non-immunogenic HLA–A*02:01 binding peptides of equal affinity. Moreover, stability analysis of pHLA–A*02:01 complexes suggested that also the stability of pHLA-I complexes is a good parameter to identify immunogenic peptides among

HLA-I binders, as has been suggested before [19,25]. Here, to investigate the two parameters, that both can be used to define immunogenic peptides for HLA–A*02:01, we focused on three HLA–A*02:01 binding peptides, QVYPGLLEKV, TLYEAVRKV and ILYAHLHKL. The three peptides were derived, by substitution of non-anchor positions, from peptides present in the SYFPEITHI database, and thus the chosen peptides would probably be peptides conferring high-stability and/or non-facilitated (below a defined threshold of 1.5) by Tpn_{1-87}. To also study a more tapasin-dependent HLA-I molecule we selected HLA–B*08:01 and three high-affinity SYFPEITHI peptides for this allomorph. Predictions based on an artificial neural network based tool (NetMHC 3.2) [26,27] suggested that the synthesized peptides bind well to the HLA–B*08:01 and HLA-A*02:01 respectively, and that the substitutions in the peptides not significantly alter their predicted affinities for HLA–A*02:01 (Table 1). We next experimentally determined the affinities, of the peptides to HLA–A*02:01 using an AlphaScreen assay – an HLA-I binding assay in which dose-titrated amounts of peptide is added to biotinylated HLA-I HC, β_2m and presence or absence of Tpn_{1-87} allowing folding of pHLA-I complexes [23]. Under conditions of limited receptor concentration ($[HLA] \leqslant K_D$), the concentration of ligand leading to half-saturation (the EC_{50}) is a reasonable approximation of the equilibrium dissociation constant, K_D. After folding, streptavidin-coated AlphaScreen donor beads, capturing the folded pHLA-I complexes, and acceptor beads conjugated with the MHC-I conformation specific antibody W6/32 are added. Successfully folded pHLA-I complexes bound to donor beads also bind W6/32 coated acceptor beads, creating a proximity that allows diffusion of reactive oxygen species from the donor to the acceptor bead, generating a luminescent signal to be released and read in a plate reader. From the binding curves, EC_{50} values were calculated confirming that all the peptides were high affinity binders in the very upper end of the spectrum for binders to HLA–A*02:01 (Table 2 and Fig. 1A). When Tpn_{1-87} was present during the folding we observed a small increase in B_{max} (i.e. 20–25%) for folded pHLA–A*02:01 complexes (Fig. 1A–C), which should be compared to the increase of B_{max} of non-SYFPEITHI peptides that typically are facilitated over 50–100% (see Supplementary Fig. 1) [18]. We have previously defined the ratio between the maximum amount of folded complexes, B_{max}, in the presence and absence of Tpn_{1-87} as the *Tpn_{1-87} facilitated folding* [18]. Here, the tapasin facilitation was low (1.2–1.25) for all three pHLA–A*02:01 complexes, consistent with the previous observations that binding of SYFPEITHI peptides to HLA–A*02:01 is only slightly or not at all facilitated by Tpn_{1-87} [18]. As a control the mutant HLA–A*02:01-T134K molecule, that is not able to interact with tapasin [28], was used and shown not to be facilitated regardless of what kind of peptide that was supplied (Fig. 1B) [18,20]. Indeed, the tapasin-facilitation of peptide binding was similar for both the wild type HLA–A*02:01 and the mutant HLA–A*02:01-T134K, further emphasizing the lack of tapasin-facilitation for the here studied A*02:01 binding peptides.

Table 1
In silico predicted affinities of HLA–A*02:01 and HLA–B*08:01 binding peptides.

Original SYFPEITHI Peptide	Predicted binding affinity (nM)	Modified peptide	Predicted binding affinity (nM)
IL**Q**AHLH**S**L	16	IL**Y**AHLH**K**L	12
QV**F**PGLLE**R**V	317	QV**Y**PGLLE**K**V	394
TLYEAVR**E**V	14	TLYEAVR**K**V	28
FLRGRAYGL	24		
QAKWRLQTL	35		
ELRSRYWAI	13		

Table 2
Affinities determined from dose response curves of peptides binding to HLA–A*02:01 and HLA–B*08:01 as analyzed after folding with AlphaScreen assay.

Peptide	EC_{50} (nM)
ILYAHLHKL	<1
QVYPGLLEKV	<1
TLYEAVRKV	<1
FLRGRAYGL	16
QAKWRLQTL	69
ELRSRYWAI	12

The affinities of the peptides binding to the more tapasin-dependent allomorph HLA–B*08:01 confirmed that also for this allomorph the selected peptides were very good binders, although with slightly lower affinities than the HLA-A*02:01 peptides (i.e. a low affinity = a high EC_{50} value) (Table 2). The tapasin dependent phenotype of HLA–B*08:01 was confirmed based on a stronger facilitation effect exerted by Tpn_{1-87}. Indeed, two of the three SYFPEITHI peptides were facilitated above 1.5 (the threshold determined, from large sets of peptides, to discriminate SYFPEITHI from non-SYFPEITHI binding to HLA–A*02:01), suggesting that different thresholds might be applicable for identification of natural ligands for different HLA-I allomorphs (Fig 1D).

*3.2. pHLA–A*02:01 and pHLA–B*08:01 complexes differ in stability depending on the bound peptide*

Both HLA-I HC and peptide are important determinants of tapasin-facilitation, but the exact factors that define pHLA-I tapasin-facilitation are not known. Here, we set out to investigate the relationship between tapasin-facilitation and stability of pHLA–A*02:01 and pHLA–B*08:01. We measured the stability of the pHLA–A*02:01 and pHLA–B*08:01 complexes using a biochemical scintillation proximity assay [24]. In brief, ^{125}I-labeled β_2m and HC was folded in the presence of peptide. After reaching steady state, excess of unlabeled β_2m was added to block re-association of dissociated ^{125}I-labeled β_2 and the samples incubated at +37 °C, and the dissociation was followed continuously. Two of the HLA–A*02:01 binding peptides, QVYPGLLEKV and TLYEAVRKV, resulted in pHLA–A*02:01 complexes with similar stability (6.5 and 7.5 h respectively), while the ILYAHLHKL peptide produced complexes with a half-life of 13.3 h (Fig. 2A). The difference between 6.5 and 7.5 h for QVYPGLLEKV and TLYEAVRKV respectively and 13.3 h for ILYAHLHKL suggested a significantly more stable pHLA–A*02:01 complex for ILYAHLHKL. Moreover, to determine whether Tpn_{1-87} would stabilize or de-stabilize the pHLA–A*02:01 complexes at physiological temperature we also measured the half-lives in the presence of Tpn_{1-87} (Fig. 2B). Consistent with a previous study of other pHLA–A*02:01 complexes using the same conditions we found that Tpn_{1-87} does not affect the dissociation of pHLA–A*02:01 complexes [18].

Binding of each of the HLA–B*08:01 SYFPEITHI peptides resulted in HLA–B*08:01 complexes of less stability than the pHLA–A*02:01 complexes (Fig. 3A). The stability varied for the pHLA–B*08:01 complexes but not in accordance to tapasin-facilitation (Figs. 3D and 1D). A small but reproducible decrease in stabilization in the presence of Tpn_{1-87} was seen for all the here studied pHLA–B*08:01 complexes. All the pHLA-I dissociation data was fitted to biphasic dissociation curves (P-value < 0.0001) indicating the presence of more than one pHLA-I species at time point 0. This is in agreement with models of both MHC-I and HLA-II maturation that include transition state of HLA complexes on their way to final maturation by optimization/exchange of their suboptimal peptide [29–31].

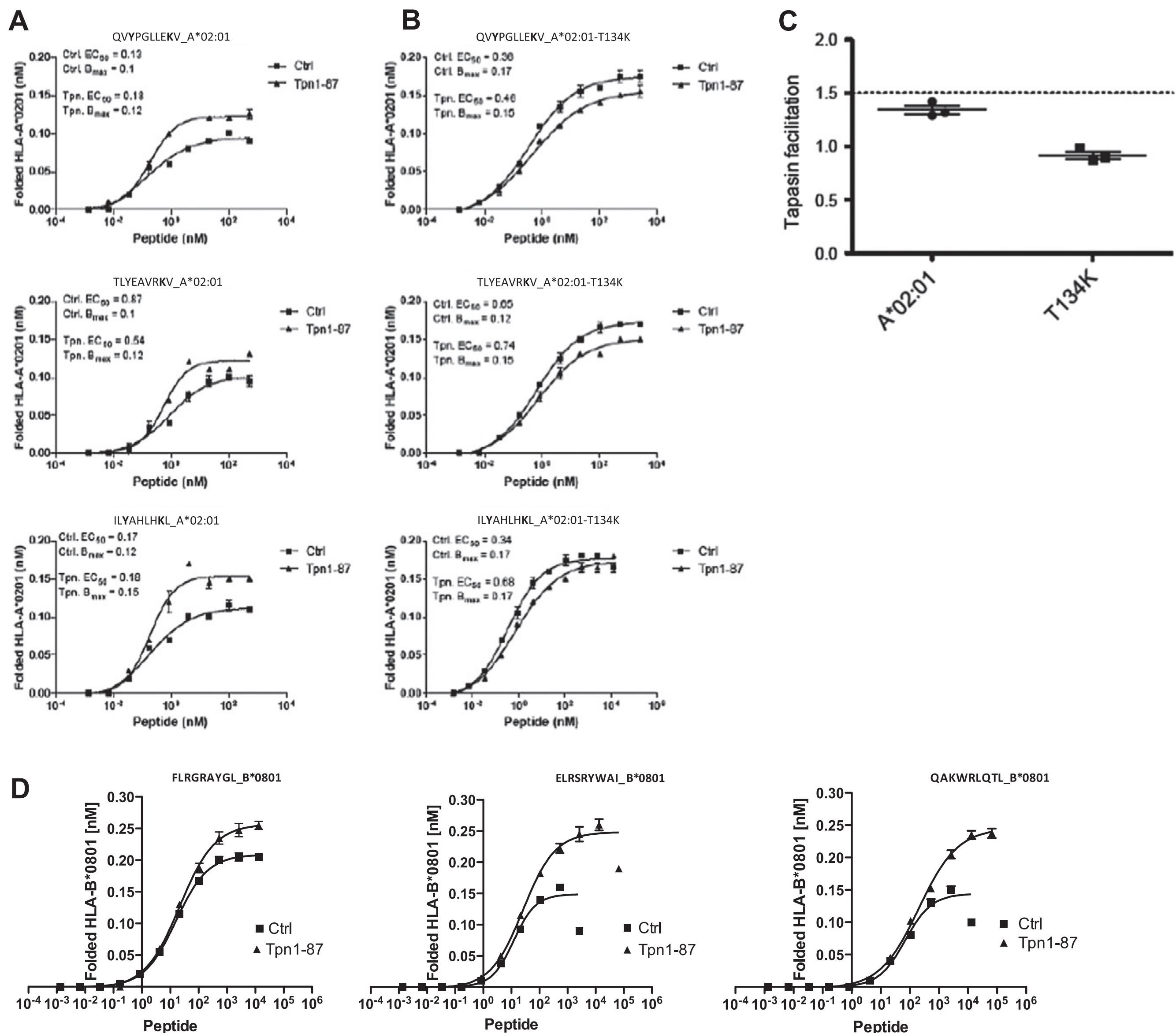

Fig. 1. Tpn_{1-87} slightly facilitates folding of peptide–HLA–A*02:01 complexes and to a greater extent folding of peptide–HLA–B*08:01 complexes. Measurement of peptide-binding affinities (i.e. EC_{50}), maximal folding and effect of Tpn_{1-87}. (A) Recombinant HLA-A*02:01 heavy chain, or (B) recombinant HLA–A*02:01-T134K heavy chain, or (D) recombinant HLA–B*08:01 heavy chain, and β_2m was mixed with different concentrations of the indicated peptides. The molecules were allowed to fold into peptide–HLA-I complexes in the absence (Ctrl) or presence (Tpn_{1-87}) of excess Tpn_{1-87}. The concentrations of folded peptide–HLA-complexes were measured using a biochemical luminescence oxygen channeling immunoassay (LOCI), in which the MHC-I β_2m-heavy chain conformation specific monoclonal antibody, W6/32, allows detection of folded pHLA–A complexes. The maximum concentration of folded peptide–HLA-I complexes obtainable, B_{max}, was calculated from the plateau at high peptide concentrations. The peptide concentration required to reach half the value of B_{max} is termed EC_{50}, and is used as an approximation of the peptide binding affinity to HLA-I. (C) A statistical comparison of tapasin-facilitation of wild type HLA–A*02:01 and the mutant HLA–A*02:01-T134K. All experiments were done in duplicates, and standard deviations are shown in each point. Experiment shown is representative out of three similar experiments.

4. Discussion

HLA-I matures inside the ER, and several ER proteins control the maturation process. The ultimate purpose is to optimize the pHLA-I complexes to generate efficient pHLA-I display at the cell surface. Inside the PLC, the HLA-I is kept in a peptide-receptive conformation possibly enabling exchange of an already bound suboptimal peptide with an optimal one, or alternatively trimming of a suboptimal peptide already bound resulting in a more optimal version. Tapasin is one of the ER proteins known to have a crucial role in HLA-I maturation. Tapasin-deficient cells have reduced HLA-I cell surface expression and the resulting pHLA-I complexes are less stable compared to complexes from wild type cells [2,4]. Interestingly, it has been found that different HLA-I molecules depend differently on tapasin for efficient cell surface expression, so that HLA–B*27:05 is hardly affected by the absence of tapasin, whereas HLA–B*44:02 cannot be expressed efficiently without tapasin [21]. HLA–A*02:01 is an intermediary in terms of tapasin-dependency and is in addition one of the most widely distributed HLA-I allomorphs. The degree of tapasin-facilitation correlates with the tapasin-dependency determined in cellular models with and without full length tapasin; and since it has been shown that both stability and tapasin-facilitation are good parameters to discriminate immunogenic from non-immunogenic (i.e. SYFPEITHI from non-SYFPEITHI) HLA–A*02:01 binding peptides a possible hypothesis is that the more stable the pHLA-I complex, the less Tpn_{1-87} facilitation and vice versa [18].

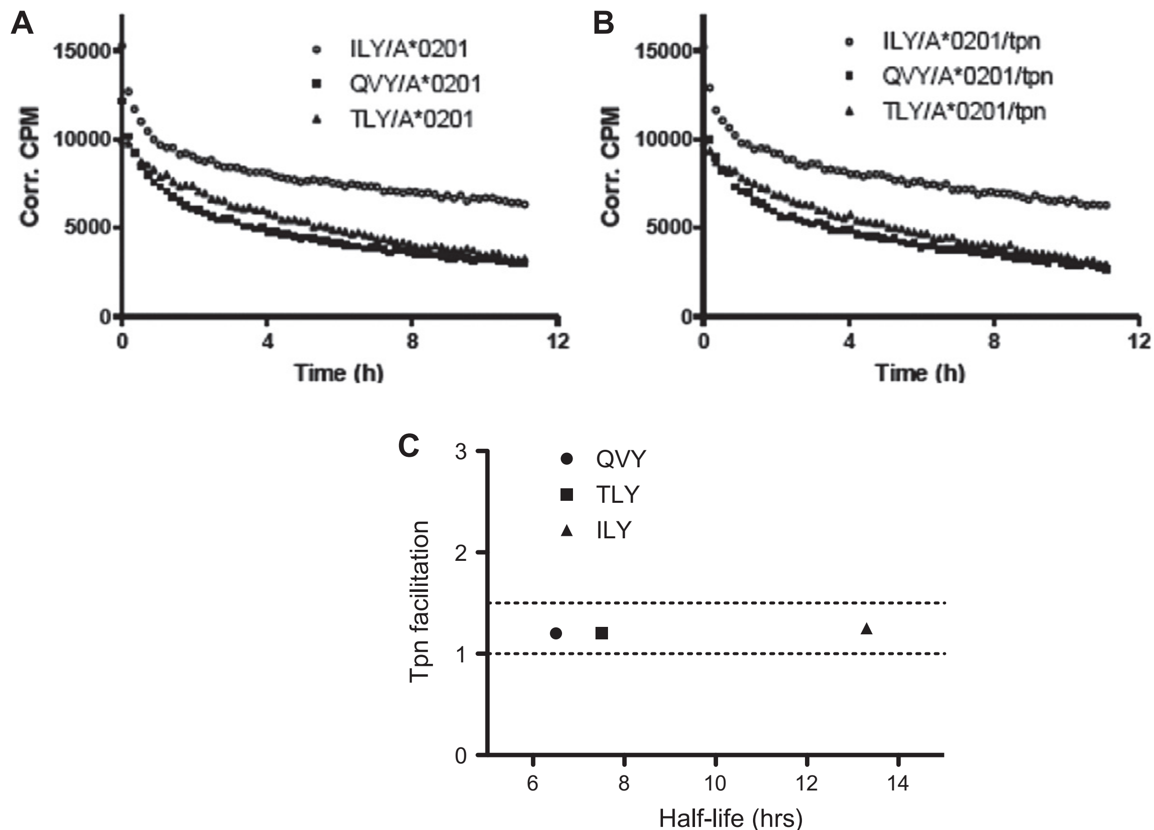

Fig. 2. Different stabilities, but no effect of Tpn_{1-87} on the stability of three different peptide–HLA–A*02:01 complexes. Peptide–HLA–A*02:01 complexes were folded with ^{125}I-labeled β_2m. An excess of unlabeled β_2m was added and the dissociation of the complexes was initiated by incubating at +37 °C. Bound radiolabeled β_2m was read at the time points indicated. (A) Dissociation of ILY, QVY and TLY–HLA–A*02:01 complexes. (B) Dissociation of ILY, QVY and TLY–HLA–A*02:01 complexes were monitored in the presence or absence of Tpn_{1-87}. All experiments were done in duplicates, data were fitted in GraphPad (Prism) and best fit was selected with *P*-value < 0.0001. The half-lives were calculated as the time required to half the amount of bound labeled β_2m. Experiment shown is representative out of three independent experiments. (C) The degree of tapasin-facilitation plotted against the half-life of the peptide–HLA–A*02:01 complexes. The dotted line at tapasin-facilitation of 1.5 indicates the threshold for low/absent tapasin-facilitation for A*02:01, defining the upper limit indicating immunogenic peptides.

Here, we have studied three peptides each for HLA–A*02:01 and HLA–B*08:01 that have been registered in the SYFPEITHI database. The peptides were predicted to be high-affinity binders, and we here confirmed these predictions in a biochemical peptide–HLA-I binding assay, and found that QVYPGLLEKV, TLYEAVRKV, ILYAHLHKL peptides were high affinity binders to HLA–A*02:01 and FLRGRAYGL, QAKWRLQTL, ELRSRYWAI to HLA-B*08:01 (Fig. 1 and Table 2). We also found that all three HLA-A*02:01 peptides conferred stable pHLA–A*02:01 complexes, although the pHLA–A*02:01 complex with the ILYAHLHKL peptide was significantly more stable than the other pHLA–A*02:01 complexes despite equal binding affinity as TLYEAVRKV and QVYPGLLEKV to HLA–A*02:01 (Figs. 1 and 2). The folding of all pHLA–A*02:01 complexes were equally unaffected by Tpn_{1-87} (i.e. tapasin-facilitation to very low degrees (1.2–1.25)) (Fig 2C). These results are consistent with observations of other pHLA–A*02:01 complexes [18], showing that the folding of pHLA–A*02:01 complexes with half-lives above 2 h are not readily facilitated by Tpn_{1-87}.We also suggest that the stability for tapasin facilitated pHLA–A*02:01-complexes is in general rather low (i.e. less than 2 h) [18], but there are also exceptions in the direction of even less stable pHLA–A*02:01 complexes that still not are Tpn_{1-87} facilitated (unpublished data, Geironson et al.), hence the two criteria (i.e. low tapasin-facilitation and high stability) are to a great extent defining the same peptide pool but caution should be taken since there might be exceptions identified only by one or the other of the selection tools (Fig. 4). In conclusion for HLA–A*02:01, we here show, by using different peptides of high affinity, that the stability of potential immunogenic pHLA–*02:01 complexes varies to a significant extent but above a threshold 2 h, and that neither binding nor dissociation is affected by Tpn_{1-87} for the here three studied high-affinity peptides.

Also the stability for the pHLA–B*08:01 complexes varied significantly (i.e. 1.7–4.8 h). The pHLA–B*08:01 with the lowest tapasin-facilitation was the intermediary pHLA-I complex in terms of stability. It is worth again noting that tapasin-facilitation differs for different HLA-I allomorphs and that a threshold (i.e. 1.5) has so far been defined for HLA–A*02:01 only, separating potentially immunogenic versus non-immunogenic peptides for this allomorph [18]. For binding of peptides to HLA–A*02:01-T134K no tapasin-facilitation is seen, neither with immunogenic nor with non-immunogenic peptides, and for HLA–B*08:01 a trend for separation based on tapasin-facilitation has been shown although the threshold should most likely be set higher for this allomorph. All the pHLA–B*08:01 had a lower stability than the pHLA–A*02:01, which is in line with other data suggesting HLA-B allomorphs to be less stable than HLA-A allmorphs (Harndahl et al., manuscript in preparation). Moreover, a reproducible trend suggesting a negative influence by Tpn_{1-87} on pHLA–B*08:01 stability in a dissociation assay at 37 °C was seen. A destabilizing effect of Tpn_{1-87} was not seen for pHLA–A*02:01, which could reflect the less tapasin-dependent nature of this allmorph. For the highly tapasin-dependent allomorph HLA–B*44:02 an even stronger destabilizing effect is seen (unpublished data, Geironson et al.).

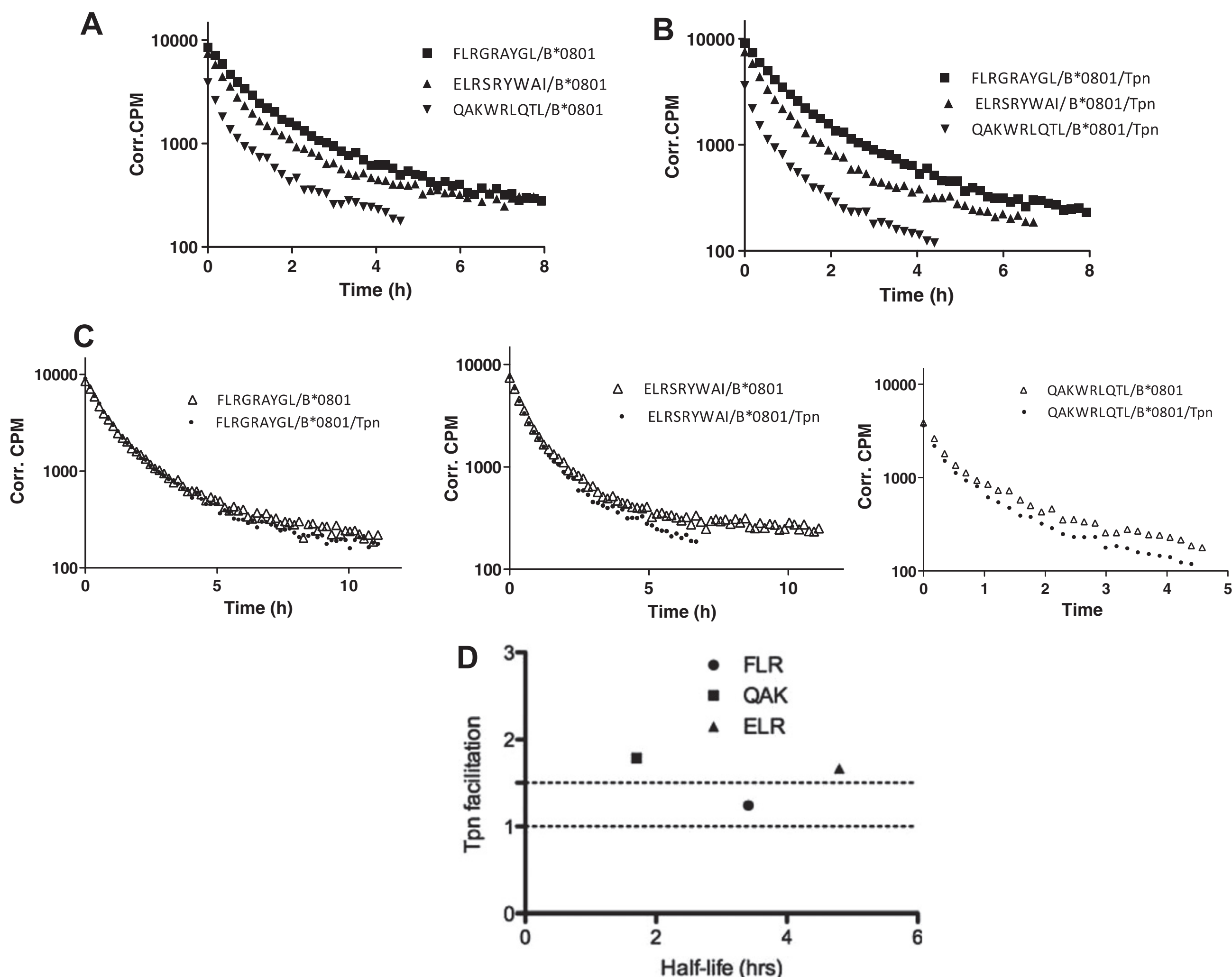

Fig. 3. Different stabilities and a destabilizing tendency of Tpn_{1-87} on three different peptide–HLA–B*08:01 complexes. Peptide–HLA–B*08:01 complexes were folded with ^{125}I labeled β_2m. Excess unlabeled β_2m was added and the dissociation of the complexes was initiated by incubating at +37 °C. Bound radiolabeled β_2m was read at the time points indicated. (A) Dissociation of ILY, QVY and TLY-HLA–A*02:01 complexes. (B) After folding of FLR, QAK and ELR-HLA–A*08:01 complexes dissociation was monitored in the presence or absence of Tpn_{1-87}. All experiments were done in duplicates, data were fitted to dissociation models and best fit was selected with *P*-value < 0.0001, and the half-lives were calculated as the time required to half the amount of bound labeled β_2m. Experiment shown is representative out of two independent experiments. (C) Dissociation curves of peptide–HLA–B*08:01 complexes in the presence and absence of Tpn_{1-87}. (D) Degree of tapasin-facilitation plotted against the half-life of the peptide–HLA-A*02:01 complexes. The dotted line at tapasin-facilitation of 1.5 indicates the threshold for low/abscent tapasin-facilitation for A*02:01.

The maturation of pMHC-I complexes proceeds through several equilibria. The proportions of HLA-I in different phases have been suggested to be dictated by HLA-I allomorph tapasin-dependence (Fig. 5) [32]. The nature of dissociation of pMHC-I complexes is influenced by several factors including peptide cargo, the presence of tapasin, temperature and HLA-I allomorph [29]. A soluble tapasin fos-jun zippered to HLA–B*08:01 has been shown, in a peptide specific manner at 20 °C, to shift biphasic dissociation with a high half-life to monophasic dissociation with lower half-life. Our results are achieved from a stability assay at 37 °C and show only a trend for Tpn_{1-87} influence on dissociation, which is likely due both to the temperature difference and the absence of binding forces strong enough to allow the truncated Tpn_{1-87} to bind pHLA-I complexes at these conditions. The here used stability assay conditions are not ideal for the study of the very weak interactions between Tpn_{1-87} and HLA-I molecules, or the effect of this, but are instead good to provide information of HLA-I half-lives at physiological temperature.

To design peptide-based vaccines it is crucial to be able to select peptides with high immunogenic potential. In a study using vaccinia virus (VACV) and HLA–A*02:01 as a model system it was shown that only 15 epitopes out of >115,000 possible VACV-derived 9- and 10-mer peptides were recognized after VACV infection in HLA–A*02:01 transgenic mice [22]. There are several known as well as unknown factors that contribute to this immunodominance among peptides. The expression of viral proteins, the processing and presentation, and the TCR repertoire as well as immune-regulatory mechanisms are all factors to have in mind when designing peptide-based vaccines. To date the cleavage preferences for the proteasome are known [33], as well as the preferences for human TAP to transport into the ER in terms of peptide size, 8–12 amino acids, and requirement of basic or hydrophobic amino acids at the C-terminal [34]. The favoured amino acids for trimming of peptides by ERAP1 and ERAP2 in the ER are also known [35]. Numerous studies have resulted in the knowledge of the binding preferences of peptides binding into the peptide-binding groove of different HLA-I allomorphs. With all these parameters in mind it is today possible to design peptides that with reasonable probability are immunogenic [36]. We recommend that to heighten this probability the effect of tapasin function on HLA-I maturation

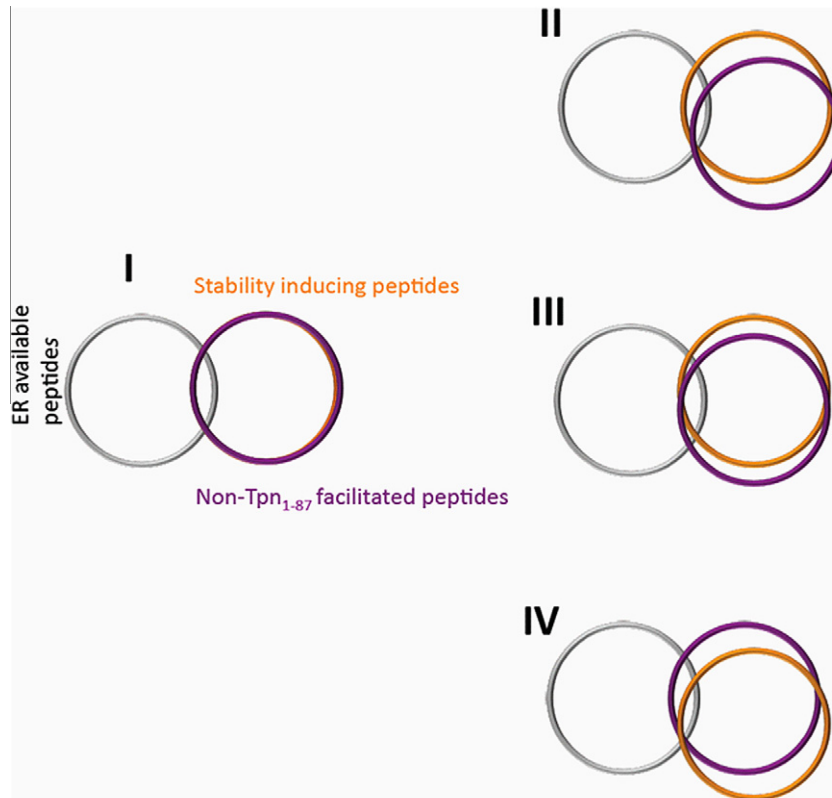

Fig. 4. Schematic model of tapasin-facilitation and pHLA-I stability as criteria for defining immunogenic HLA-I binding peptides. (I) Simplistic perspective, (II), stability gives a more accurate perspective, (III) realistic perspective, (IV) tapasin-facilitation gives a more accurate perspective.

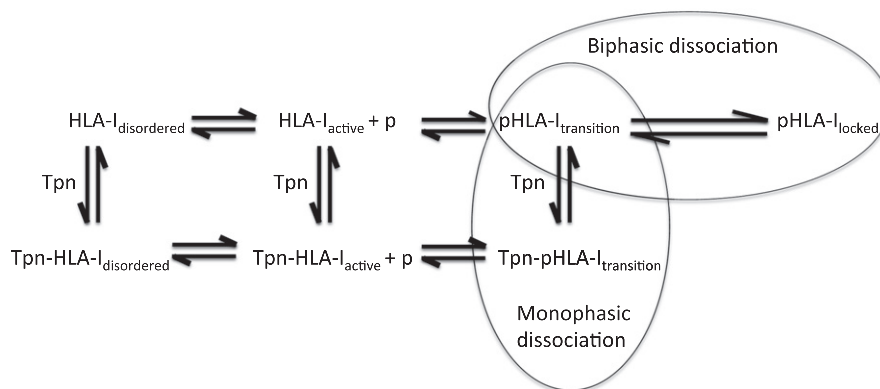

Fig. 5. Scheme of peptide (p)–HLA-I complex formation and dissociation. HLA-I molecules mature through several stages and several equilibria. For HLA-I allomorphs of less tapasin-dependent nature only a low proportion exist in disordered state and peptide binds into the peptide-binding groove, the reactions proceed with high speed to form a mature locked pHLA-I (HLA-I_{locked}). For less tapasin-dependent allomorphs the action of tapasin is less pronounced. HLA-I molecules of more tapasin-dependent nature have been suggested to have a higher propensity for a disordered early state and for these molecules we suggest that tapasin plays an important role in driving the formation to HLA-I forms of intermediary maturation grade, i.e. active HLA-I (HLA-I_{active}), and pHLA-I in transition state (HLA-$I_{transition}$). These molecules have a peptide-receptive conformation and peptide cargo allowing peptide to be exchanged or optimized. After folding, where steady state is reached, dissociation of less tapasin-dependent pHLA-I complexes starts at time point zero with a majority of complexes in pHLA-I_{locked}. Dissociation in the absence of tapasin may follow a bi-phase pattern while dissociation in the presence of tapasin in a peptide-specific manner may drive all or a part of the $pHLA_{locked}$ population to $pHLA_{transition}$ with resulting mono or biphasic dissociation.

should be taken into consideration. To date it is not known exactly how tapasin exerts this HLA-I quality control. HLA-I molecules binding to peptides with the same binding affinities have been shown to be facilitated by tapasin differently [18]. We have earlier shown that stability and tapasin-facilitation are inversely correlated and are both good tools to select immunogenic peptides [18]. Moreover, in this study, we show data suggesting that both tapasin-facilitation and stability of pHLA-I complexes define immunogenic pHLA-I complexes but that the borders defining what stability is and what tapasin-facilitation is differs for different HLA-I allomorphs.

In conclusion, we have here presented data showing that both stability and tapasin-facilitation are HLA-I and peptide allomorph specific features. Since all the peptides used in this study are likely to be immunogenic, and with respect to the unique traits of each HLA-I allomorph, we therefore propose that the low degree of

tapasin-facilitation shown for the pHLA-I complexes studied here further strengthens the concept of Tpn_{1-87} as a highly relevant tool, that together with stability can be used to define immunogenic peptides.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.03.045.

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