HLA class I maturation - in the presence and absence of tapasin

Geironson Ulfsson, Linda

2012

Organization	Document name
LUND UNIVERSITY Department of Biology	DOCTORAL DISSERTATION Date of issue 2012-05-25
Author(s) Linda Geironson	Sponsoring organization

Title and subtitle

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Abstract

Human leukocyte antigen class I (HLA-I) molecules are present on all nucleated cells and present the cell content to cytotoxic T lymphocytes in the form of peptides. Maturation of HLA-I occurs in the endoplasmic reticulum and results in stable peptide-HLA-I complexes, in the presence of proper quality control. For proper peptide binding most HLA-I molecules interact with the peptide-loading complex (PLC), which is a multi-protein complex consisting of the transporter associated with antigen presentation, calreticulin, ERp57 and tapasin. Tapasin integrates HLA-I into the PLC and mediates quality control of the HLA-I maturation. When an optimal peptide is bound, tapasin releases the peptide-loaded HLA-I molecule that is next transported to the cell surface. The mechanisms for tapasin quality control of HLA-I maturation and the criteria defining optimal peptides are not completely known.

Here, a recombinant part of tapasin, the first 87 N-terminal amino acids ($Tpn_{1\text{-}87}$), was produced and shown to facilitate folding of different HLA-I molecules, *i.e*. allomorphs, to different degree in a peptide dependent manner. Folding of HLA-A*02:01 molecules with natural ligands, *i.e*. with peptides purified mainly from HLA-I expressed on the cell surface, was not facilitated by $Tpn_{1\text{-}87}$, while folding of non-natural ligands, *i.e*. not presented at the cell surface, was facilitated. The folding facilitation exerted by $Tpn_{1\text{-}87}$, tapasin-facilitation, inversely correlated with the stability of the peptide-HLA-I complex to some extent. The inverse correlation of these two parameters, tapasin-facilitation and stability, was studied in detail in the third paper. An increased stability was shown to not necessarily be associated with a decreased tapasin-facilitation. In the last paper of this thesis the tapasin-facilitation was studied in a large set of allomorphs folded with peptides of 7 to 13 amino acids in length. The influence of peptide-length for the different allomorphs increased with their tapasin dependence.

In conclusion, $Tpn_{1\text{-}87}$ facilitates folding of HLA-I in a peptide- and allomorph-dependent manner. Based on the above studies and data showing tapasin retention of immature HLA-I molecules, and studies of a suggested peptide-editing role of tapasin, we propose that tapasin keeps HLA-I molecules in a peptide-receptive conformation, which reduces the risk degradation of HLA-I molecules and increases the possibility for binding of optimal peptides.

Key words

HLA-I, tapasin, $Tpn_{1\text{-}87}$, tapasin-facilitation, stability, peptide identity

Classification system and/or index terms (if any)

Supplementary bibliographical information	Language English
ISSN and key title	ISBN 978-91-7473-304-4
Recipient´s notes	Number of pages / Price
	Security classification

Distribution by (name and address)

Signature _____ Date _____

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LUND
UNIVERSITY

AKADEMISK AVHANDLING

som för avläggande av filosofie doktorsexamen
vid naturvetenskapliga fakulteten, Lunds universitet,
kommer att offentligen försvaras i
Segerfalksalen, BMC A10, Sölvegatan 19, Lund,
fredagen den 25 maj 2012, kl. 13.00

Fakultetsopponent: Docent och rådsforskare Adnane Achour,
Instutitionen för medicin, Karolinska Institutet, Huddinge

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LUND UNIVERSITY
Faculty of Science

Department of Biology
Lund University
Sweden

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ISBN 978-91-7473-304-4

Printed by Media-Tryck, Lund 2012

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Gustav Røder, Linda Geironson, Anna Darabi, Mikkel Harndahl, Claes Schafer-Nielsen, Karsten Skjødt, Søren Buus, Kajsa Paulsson

European Journal of Immunology, 2009 Oct;39(10):2682-94

II **Tapasin discriminates peptide-human leukocyte antigen-A*02:01 complexes formed with natural ligands**

Gustav Røder[1], Linda Geironson[1], Michael Rasmussen, Mikkel Harndahl, Søren Buus, Kajsa Paulsson

[1] Both authors contributed equally to this work

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  Manuscript

ABBREVIATIONS

Ab	antibody
ADP/ATP	adenosine di/tri-phosphate
ALPHA	amplified luminescent proximity homogenous assay
APC	antigen presenting cell
ARF	adenosine diphosphate-ribosylation factor
Bap	B-cell receptor-associated protein
β_2m	β-2-microglobulin
BiP	binding immunoglobulin protein
Cnx	calnexin
COP	coat protein complex
CPXV	cowpox virus
Crt	calreticulin
DC	dendritic cell
DRiP	defective ribosomal products
EBV	Epstein-Barr virus
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERAAP	ER aminopeptidase associated with antigen processing
GrpE	growth after phage induction E
H-2	histocompatibility-2
HC	heavy chain
HCMV	human cytomegalo virus
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
IFN	interferon

IPTG	isopropyl-β-D-thio-galactoside
LMP	low molecular weight protein
LOCI	luminescent oxygen channeling immunoassay
MECL	multicatalytic endopeptidase complex-like
MHC	major histocompatibility complex
PDI	protein disulphide isomerase
PLC	peptide-loading complex
SRP	signal recognition pore
TAP	transporter associated with antigen processing
Tapasin	TAP associated glycoprotein
TCR	T cell receptor
TNF	tumour necrosis factor
Tpn	tapasin
VACV	vaccinia virus
VIPR	viral proteins interfering with antigen presentation
WT	wild-type
.220	lymphoblastoid B-cell line 721.220

SHORT INTRODUCTION TO THE IMMUNE SYSTEM

The immune system is responsible for the clearance of invading pathogens like viruses, bacteria and fungi. It can be divided into two major systems of protection, the innate and the adaptive system. The innate immune response is immediate and non-specific, consisting of different kinds of physical barriers, chemical factors and cellular responses that provide a first-line-of-defence. If these innate defences are breached, the adaptive immune system is activated. In contrast to the innate immune response the adaptive response is antigen-specific and leads to immunological memory.

The adaptive immune system includes two major groups of lymphoid cells, the B lymphocytes (B cells) and the T lymphocytes (T cells). Presentation of a specific antigen in the presence of co-stimulatory molecules activates naïve lymphocytes. B cells are responsible for the production of antibodies that bind to and assist in the elimination of extracellular pathogens. In comparison T cells carrying T cell receptors (TCR), only recognize antigens displayed at the cell surface of antigen presenting cells in the form of peptides on major histocompatibility complex (MHC) molecules. T cells expressing the CD4 co-receptor (CD4+ T cells) recognize peptides associated with MHC class II (MHC-II) complexes, resulting in the activation of mechanisms that eliminate extracellular antigens. A second group of T cells, expressing the CD8 co-receptor (CD8+ T cells), learn early in life to differentiate between self and non-self through positive and negative selection, a process that takes place in the cortex and medulla of the thymus, respectively. Naïve CD8+ T cells are activated by professional antigen-presenting dendritic cells (DCs) capable of cross-presentation

and expression of appropriate co-stimulatory molecules. The CD8+ T cells then leave the site of activation/priming, which most often takes place in the secondary lymphoid organs, to circulate in the body. Primed CD8+ T cells scan cells for expression of altered self/non-self peptides and an infected cell is killed if the peptide-MHC-I complexes match the TCRs on CD8+ T cells and certain critical threshold values are reached. A high stability of the peptide-MHC-I complex is of crucial importance and constitutes one of the thresholds. The affinity and number of TCR-peptide-MHC-I interactions formed, and the density per area of stable peptide-MHC-I complexes, reflected by the duration of the interaction between the TCRs and peptide-MHC-I complexes, have also been shown to influence the outcome of the TCR-MHC interaction (Henrickson *et al.*, 2008).

The peptides presented to CD8+ T cells by of MHC-I molecules are derived from all kinds of intracellular proteins, including those synthesized by intracellular pathogens, and are degraded into peptides by the proteasomes. Only a small fraction of the peptides produced binds to MHC-I molecules. The proteasomal generation of peptides is followed by the next limiting step for a peptide-MHC-I complex to form: the capability and possibility of the peptide to bind and to become transported into the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP). Inside the ER the interaction between the MHC-I molecule and a multi-protein complex called the peptide-loading complex (PLC) is of crucial importance for the formation of stable peptide-MHC-I complexes. Stable peptide-MHC-I complexes are subsequently presented at the cell surface of all nucleated cells.

Several factors, including the PLC key protein tapasin, are crucial for a peptide to become presented by MHC-I. The role of the interaction between MHC-I and tapasin for the maturation of MHC-I has been investigated in detail in our studies.

MHC-I AND THE PEPTIDE-LOADING COMPLEX

Background and nomenclature of MHC-I

The MHC was discovered in mice as the genomic region responsible for mediating graft rejection. The genes identified in this region were H-2K, H-2D, and H-2L, which are homologous to the human leukocyte antigen class I (HLA-I) genes HLA-A, -B, and -C. The genes encoding HLA-I are located on the short arm of chromosome 6, and they are known to be the most polymorphic genes present in the whole genome. To date (2012), more than 5500 different HLA-I alleles have been identified (http://hla.alleles.org/nomenclature/stats.html). The majority (>95%) are so called classical HLA-I alleles, with >40% of them being HLA-B alleles, >30% being HLA-A alleles, and around 25% being HLA-C alleles. The non-classical HLA-I alleles HLA-E, -F, and -G constitute only a small fraction of the identified alleles. They have similar sequence and structure as classical HLA-I molecules but they do not show such high levels of polymorphism as the classical molecules. In addition, their expression is tissue specific and at lower levels at the cell surface; they bind also only a limited repertoire of peptides (Rodgers & Cook, 2005).

The name of the HLA-I alleles is constructed in a specific way with the name of the gene followed by up to four sets of digits separated by colon, i.e. HLA-A*01:01:01:01. The digits have been given in the order that the DNA-sequences have been determined. The first set of digits describes the allele group, which most often corresponds to the serological type. The second set of digits refers to the specific HLA-I allele. Alleles with different digits in the first two sets differ from other alleles with at least one nucleotide substitution that changes the amino acid

sequence of the encoded protein. All HLA-I alleles are given at least the first two sets of digits, and alleles that differ by substitutions within the coding sequence but without resulting in another amino acid sequence, so called silent substitutions, are named with the third set of digits. The fourth set of digits tells if there is a nucleotide substitution in a non-coding region. The protein product of an HLA-I allele is called an allomorph.

Structure of MHC-I

The MHC-I molecule consists of a heavy chain (HC) and a light chain called β_2-microglobulin (β_2m). The HC of the mature MHC-I molecule is folded into three α-domains; α1, α2, and α3. During maturation two disulphide bonds, one within the α3-domain and the other one within the α2-domain of the peptide-binding groove, are formed (Tector *et al.*, 1997).

The α3-domain folds into an immunoglobulin-like conformation that is non-covalently attached to β_2m and anchored to the cell by a trans-membrane region continuing in a cytoplasmic part. The α3 domain also contains the main binding site for the CD8+ T cell co-receptor, CD8. The α1 and α2 domains are tightly bound together forming the peptide-binding groove, which preferably binds peptides of 8−11 amino acids and is the most polymorphic part of the MHC-I molecule. When no peptide, or a loosely bound peptide, is bound in the groove, the MHC-I molecule is structurally unstable and the groove has a more open, and thereby peptide-receptive, structure. The N-terminal part of the peptide binds via hydrogen bonds to a cluster of tyrosine residues on one side of the peptide binding groove and the C-terminal region of the peptide binds via three hydrogen bonds to the other end of the peptide-binding groove in the α2-1 helix (Praveen *et al.*,

2010). These bonds are conserved and formed by amino acids in the MHC-I molecule shared by all MHC-I molecules.

The bottom of the groove contains binding pockets where the peptide side chains bind non-covalently. The groove of MHC-I typically consists of six pockets, termed A−F. Different allomorphs prefer different amino acids at specific positions in the peptide; such positions are called anchor positions and the residues at these positions fit into the pockets of the groove (Falk *et al.*, 2006). Many MHC-I molecules define position two and the C-terminal as anchor positions in the peptide (Lund *et al.*, 2004). The restricted peptide binding capability of MHC-I is found in all organisms and is therefore not believed to reflect evolutionary pressure to avoid recognition by CD8+ T cells (a mechanism for microorganisms to survive) or enhance recognition (a mechanism advantageous for the infected individual) (Istrail *et al.*, 2004). The interactions between the amino acids at the anchor positions in the peptide and the pockets in the groove are of crucial importance for the outcome of the stability and affinity of the peptide binding, and thereby the presentation of the peptide at the cell surface.

Maturation of MHC-I

The maturation and assembly of MHC-I molecules with peptides is a sequential process that takes place in the endoplasmic reticulum (ER). Figure 1, on page 21, represents a simplified schematic overview of the maturation. The HC of the MHC-I molecule is translated directly into the ER lumen and the folding of the immature molecule is initially assisted by the ER chaperones immunoglobulin binding protein (BiP) and calnexin (Cnx) (Nossner & Parham, 1995). Binding of Cnx recruits the ERp57 oxidoreductase (Molinari *et al.*, 2004). ERp57 has been

shown to be involved early in MHC-I biogenesis by forming or catalyse formation of the disulfide bond in the α3 domain of the HC (Zhang *et al.*, 2006). The α3 domain of the HC subsequently binds to β_2m, and the membrane bound Cnx is replaced by its soluble homolog calreticulin (Crt) (Sadasivan *et al.*, 1996). At this stage, some MHC-I molecules become loaded with a peptide and are stable enough to exit the ER and progress to the cell surface. However, most of the Crt associated MHC-I molecules are recruited to the peptide-loading complex (PLC) for further maturation. The PLC consists of the transporter associated with antigen processing (TAP), ERp57 and tapasin (Hughes & Cresswell, 1998; Lindquist *et al.*, 1998; Morrice & Powis, 1998).

Proteins to peptides

The proteasome degrades intracellular proteins and is the main producer of peptides that are transported into the ER by TAP. The 20S proteasome is made up of α-type subunits (α1−α7) and β-type subunits (β1−β7) (Lowe *et al.*, 1995; Groll *et al.*, 1997). The β1, β2 and β5 subunits are responsible for the peptide bond cleavage (Fenteany *et al.*, 1995). In an ATP-dependent manner the combination of one 20S proteasome and two 19S regulators on each side forms the 26S proteasome (Ferrell *et al.*, 2000). The 19S regulators help to unfold the substrates (Braun *et al.*, 1999), open the central gate of the proteasome and target the substrates into the core as well as control the product release (Kohler *et al.*, 2001). Like many other components in the MHC-I antigen presentation pathway the proteasome contains interferon (IFN)-γ-inducible subunits. Under conditions of IFN-γ induction, as well as to some extent tumour necrosis factor-α (TNF-α) and IFN-β induction, the β1, β2 and β5 subunits are replaced by immuno-subunits, low-molecular-mass polypeptide (LMP) 2, multi-catalytic endopeptidase complex-like 1 (MECL-1) and LMP7 respectively, resulting in assembly of new proteasomes called immunoproteasomes (Jamaluddin *et al.*, 2001). Antigen

presenting cells (APCs) in the thymus, spleen and lymph nodes constitutively express the IFNγ inducible subunits (Jamaluddin *et al.*, 2001). Immunoproteasomes have an increased capacity to cleave peptides after hydrophobic and basic residues (Gaczynska *et al.*, 1994), thereby generating what will become the C-terminal anchor residue of the MHC-I molecule (Toes *et al.*, 2001). An enhanced proteolytic activity has been observed in the immunoproteasome and the subsequent increased peptide supply has been suggested as the role of the immunoproteasome, rather than the production of specific peptides (Seifert *et al.*, 2010). Upon IFN-γ stimulation another regulator called 11S has been identified and shown to be able to replace one of the 19S regulators (Rock & Goldberg, 1999), thereby forming a proteasome that produces other peptides than the 26S proteasome (Sun *et al.*, 2002). Recently a proteasome called thymoproteasome was identified (Murata *et al.*, 2007). It is similar in structure to the 20S proteasome but has a distinct β5-subunit, β5t. In humans they are specifically found within cortical epithelial cells in the thymus and within a fraction of dendritic cells in thymic cortex (Tomaru *et al.*, 2009). These cells are thought to display a unique repertoire of peptide-MHC-I complexes that leads to a low-intensity TCR signal, which generates immunocompetent CD8+ T cells (Nitta *et al.*, 2010).

CD8+ T cells are able to recognize cultured cells within an hour after viral penetration (Esquivel *et al.*, 1992; Yewdell *et al.*, 1996) but it takes several hours for a virus to replicate in a cell. The capacity for this fast recognition of infected cells is due to production of defective ribosomal products (DRiPs) (Yewdell et al., 1996). A large fraction of the peptides presented on MHC-I molecules at the cell surface are products of what is currently being translated in the cell rather than already expressed proteins (Qian *et al.*, 2006). DRiPs are the products of errors in mRNA generation or in protein synthesis, *e.g.* mis-incorporation or deletion of

amino acids, or premature termination of replication as well as the result of imbalance in synthesis of multi-subunit complexes that are in need of each other to survive. DRiPs are not the single source for antigenic peptides, but most likely a main source.

Inhibition of proteasome activity has been shown to almost completely abolish MHC-I antigen presentation (Rock *et al.*, 1994; Momburg & Hammerling, 1998; Reits *et al.*, 2000). However, it has been suggested that HLA-A*3, HLA-A*11 and HLA-B*35, in contrast to other HLA-I allomorphs, are independent of the proteasome (Benham *et al.*, 1998), which implies that also other cytosolic proteases may be involved in MHC-I antigen processing.

Peptide transport into the ER

The TAP complex consists of TAP1 and TAP2, which form a pore across the ER membrane through which peptides are actively transported from the cytosol into the ER. The transport by TAP occurs in two steps, the first step is the adenosine triphosphate (ATP)-independent binding of peptide, and the second step is the ATP-dependent translocation (Androlewicz *et al.*, 1993; Neefjes *et al.*, 1993). Peptides in the size-range of 8−16 amino acids are the ones most efficiently transported by TAP (van Endert *et al.*, 1994). Human TAP has been suggested to have a preference for basic or hydrophobic amino acids at the carboxyl (C)-terminal of the peptide (Momburg *et al.*, 1994a; Momburg *et al.*, 1994b). However, this preference has been questioned in a study where peptide libraries and competition assays were used to analyse the effect of different amino acids in a peptide (Uebel *et al.*, 1995). No dramatic preferences were seen but a stabilizing effect on peptide binding was demonstrated with the presence of an amino (N)-terminal arginine (R), in addition a C-terminal leucine (L) and phenylalanine (F)

were preferred to alanine (A) (Uebel *et al.*, 1995). TAP-deficient cells show decreased cell surface expression of MHC-I, and the MHC-I molecules expressed at the surface are rapidly degraded (Salter & Cresswell, 1986; Spies & DeMars, 1991; Spies *et al.*, 1992). Peptides presented by such cells are to a large extent signal sequence derived peptides that enter the ER via the signal recognition pore (SRP) (Henderson *et al.*, 1992; Wei & Cresswell, 1992).

ERp57

ERp57 is a protein disulphide isomerase (PDI) family member containing four thioredoxin-like domains (called a, b, b' and a'). The a- and a'-domains have one CXXC motif each giving them their ability to catalyse reduction, oxidation and isomerization of disulphide bonds. ERp57 form transient disulphide bonds between the substrate and the N-terminal cysteine (C) residue in the CXXC motif. The C-terminal C residue in the motif subsequently resolves these bonds, a process called the resolving escape mechanism (Walker & Gilbert, 1997).

In the PLC the C57 residue of ERp57 is bound to the C95 residue of tapasin (Dick *et al.*, 2002). This interaction is in contrast to other PDI family member-mediated disulphide bonds stable because tapasin inactivates the resolving escape mechanism of ERp57 (Dick *et al.*, 2002; Peaper *et al.*, 2005; Peaper & Cresswell, 2008). Mutation of the tapasin C95 residue to A at position 95 (C95A) results in disruption of its interaction with ERp57 (Dick *et al.*, 2002). The role of ERp57 in the PLC has come out contradictory in different studies. The tapasin-deficient human lymphoblastoid B-cell line (LCL)-721.220 (.220), in which the *HLA-A* and *HLA-B* genes have been deleted and HLA-C expression is low, is a commonly used cell line to study transfected HLA-I alleles and different mutations of tapasin. Tapasin-deficient .220.B*44 cells and .220.H-2K^{b} (.220.K^{b}) cells expressing the

tapasin mutant C95A does not interact with ERp57 but are still able to restore MHC-I surface expression quantitatively (Dick *et al.*, 2002; Howarth *et al.*, 2004). The mutation led however to reduced peptide binding to B*44 and reduced amount of TAP-associated MHC-I molecules. MHC-I molecules that associated with C95A tapasin, and hence did not sequester ERp57 to prevent α2 disulphide bond reduction, were partially reduced, while those associating with wild-type tapasin were fully oxidized. This indicated that the ERp57-tapasin conjugate might be involved in maintenance of the oxidized redox state of the α2 peptide-binding domain, a state that is demanded for proper peptide loading. The sensitivity to reduction of the α2 disulphide bond was later shown to reflect the tapasin dependence of MHC-I molecules, in that it turned out that the tapasin dependent allomorph B*44:02 was very sensitive to reduction of the α2 disulphide bond while the tapasin independent allomorph B*44:05 was insensitive to this reduction (Kienast *et al.*, 2007). Opposite to these results, the surface expression of H-2K^{b} on ERp57-deficient B cells in mice was significantly decreased (Garbi *et al.*, 2006) indicating a difference in depletion of ERp57 and depletion of the interaction between ERp57 and tapasin, *i.e.* with C95A tapasin. This might be due to the role of ERp57 in early MHC-I maturation or to a possible difference in the importance of ERp57-tapasin-conjugate formation in human versus mouse systems. In the absence of ERp57 only small amounts of MHC-I were associated with the PLC and the interaction time of MHC-I with the PLC was reduced despite normal tapasin-TAP interaction (Garbi *et al.*, 2006). This suggests that ERp57 increases the affinity of the interaction between tapasin and MHC-I by inducing a conformational change in tapasin (Garbi *et al.*, 2007). In contrast to the study with C95A tapasin and B*44 (Dick *et al.*, 2002), the absence of ERp57 in mice B-cells did not influence the redox state of the MHC-I molecules in this study and suggested a structural rather than a catalytic role of ERp57 in the PLC (Garbi *et al.*, 2006), which is in better accordance with other studies (Peaper & Cresswell, 2008; Zhang *et al.*, 2009).

In a cell-free system, a conjugate of soluble tapasin and ERp57, unlike soluble tapasin alone, was shown to stabilize empty MHC-I molecules and selectively facilitate their loading with high affinity peptides, i.e. peptide editing (Wearsch & Cresswell, 2007). These tasks have been imputed to tapasin alone in other studies (Barnden *et al.*, 2000; Garbi *et al.*, 2000; Barber *et al.*, 2001; Williams *et al.*, 2002; Chen & Bouvier, 2007), but it is possible that the structural role of ERp57 boosts the effects of tapasin by stabilising the interactions in the PLC.

Tapasin

The MHC-I dedicated chaperone tapasin is a 428 amino acid long type I transmembrane protein consisting of three parts: a large N-terminal ER luminal region consisting of two domains, a single transmembrane-spanning domain, and a short cytoplasmic tail. Tapasin has multiple roles, all of them directed at the presentation of peptides on MHC-I molecules at the cell surface. Different regions of tapasin are responsible for the different roles. As discussed above, cysteine 95 of tapasin interacts with ERp57 in the PLC, an interaction with debated functions.

The transmembrane domain (C-terminal part) of tapasin binds to TAP (Koch *et al.*, 2006), thereby forming a bridge between TAP and MHC-I (Li *et al.*, 1997; Ortmann *et al.*, 1997). Tapasin both stabilizes TAP and promotes the binding to and the transport of peptides by TAP (Lehner *et al.*, 1998; Li *et al.*, 2000; Tan *et al.*, 2002; Garbi *et al.*, 2003). In the absence of tapasin, MHC-I is not found in association with TAP (Sadasivan *et al.*, 1996), and the cell surface-expressed MHC-I molecules are less stable. The double lysine motif at the cytosolic C-terminal of tapasin mediates interactions with coat protein type I (COP-I) vesicles

and is involved in the recycling of non-optimal peptide-loaded MHC-I (see further down).

Tapasin incorporates MHC-I into the PLC and the ER-lumenal region of tapasin has been suggested to have several interaction sites for MHC-I (Carreno *et al.*, 1995; Lewis *et al.*, 1996; Peace-Brewer *et al.*, 1996; Yu *et al.*, 1999; Dong *et al.*, 2009). These interactions have a major impact on the outcome of which peptides are presented by MHC-I molecules at the cell surface. Since that concerns the main focus of the present thesis, this topic will be discussed separately later on.

Trimming of peptides inside the ER

The enzyme ER aminopeptidase associated with antigen processing (i.e. ERAAP in mice, and ERAP1 and 2 in humans) is responsible for trimming peptides so that they become suitable for MHC-I binding inside the ER (Saric *et al.*, 2002; Serwold *et al.*, 2002; Saveanu *et al.*, 2005). ERAP1 preferentially binds to peptides with large hydrophobic C-terminals, while ERAP2 preferentially binds basic residues at the C-terminal (Saveanu *et al.*, 2005). Together they remove N-terminal extensions of peptides (Saveanu *et al.*, 2005).

Retention, exit and recycling of MHC-I molecules

The transport molecule called “B cell receptor associated protein 31” (Bap31), target MHC-I molecules to ER-exit sites (Paquet *et al.*, 2004), facilitates the export of MHC-I from the ER and helps in the retrieval of MHC-I from the Golgi (Spiliotis *et al.*, 2000; Paquet *et al.*, 2004; Ladasky *et al.*, 2006). The preferential transport of only mature MHC-I molecules of high stability to the cell surface has

generally been attributed to the retention of immature MHC-I molecules in the ER. Results from different experimental systems have shown that tapasin retains MHC-I molecules in the ER until they have been loaded with optimal peptide (Schoenhals *et al.*, 1999; Grandea *et al.*, 2000). In TAP deficient cells (T2 cells), where there is an absence of optimal peptides in the ER, MHC-I molecules bound to tapasin have been shown to accumulate over long time before ultimate dissociation (Paulsson *et al.*, 2001b; Paulsson *et al.*, 2002; Ladasky *et al.*, 2006).

In addition, a mechanism that recycles MHC-I molecules from late secretory compartments back to the ER has been suggested by several studies (Hsu *et al.*, 1991; Bresnahan *et al.*, 1997; Park *et al.*, 2001; Paulsson *et al.*, 2002). Vesicles with a coat protein complex (COP) I, composed of a coat designated coatomer and the adenosine diphosphate-ribosylation factor (ARF) 1, recognize and bind to C-terminal KKXX motifs in membrane proteins and function as ER retrieval signals for proteins (Cosson & Letourneur, 1994). Tapasin contains a C-terminal KKXX motif (Paulsson *et al.*, 2002), which has been demonstrated to bind to COP-I (Paulsson *et al.*, 2006). In cells expressing tapasin with the KKXX motif mutated to AAXX, neither tapasin nor MHC-I were detected in association with COP-I, indicating a direct role for the tapasin KKXX motif in mediating the MHC-I transport by COP-I coated vesicles. In the same cells, cell surface expression of MHC-I molecules was significantly increased, but MHC-I molecule degradation was also increased, suggesting that immature MHC-I molecules here escape to the cell surface. A similar role as for tapasin in this context has been attributed to Crt, which has been shown to bind to sub-optimally loaded MHC-I molecules and transfer them back to the ER via its KDEL motif (Howe *et al.*, 2009).

Viral interference with the antigen processing machinery

CD8+T cell recognition of viral peptides presented on MHC-I is crucial for the elimination of infected cells. The immune system and pathogens have evolved side by side for millions of years, and invading pathogens have developed several escape mechanisms to cripple the immune system. Among them are viral proteins that interfere with antigen presentation (VIPRs), which target MHC antigen processing in order to skew or totally inhibit a functional immune response against the virus. In this section a couple of VIPRs are exemplified for each key player of the antigen processing machinery. A simplified schematic overview of the viral target sites is shown in Figure 1 on page 21.

Interference with the proteasome

For most allomorphs, the generation of peptides presented on MHC-I molecules starts by proteasomal degradation of proteins into peptides. Mechanisms to avoid proteasomal degradation are therefore one way for viruses to prevent presentation to and recognition by CD8+T cells. The Epstein-Barr virus (EBV) nuclear antigen 1 (EBNA1) contains glycine and alanine in long repeats, which has been suggested to confer its ability to avoid proteasomal degradation (Levitskaya *et al.*, 1995). The transcriptional activator of human immunodeficiency virus 1 (HIV-1) gene expression, i.e. Tat, is another example of a proteasomal interfering virus protein. Tat changes the composition of the immunoproteasome, which results in an increased generation of sub-optimal and cryptic peptides (Gavioli *et al.*, 2004).

Interference with TAP and tapasin

For a peptide to be transported from the cytosol to the ER by TAP, it has to bind to the peptide-binding site on TAP (Arora *et al.*, 2001). Prevention of this binding results in a reduced pool of peptides in the ER available for association with MHC-I, ultimately resulting in a reduced level of MHC-I cell surface expression. Herpes simplex virus type 1 encodes an 88-amino-acid-long cytoplasmic protein called ICP47 that binds strongly to TAP and thereby competes with the binding of peptides to TAP (Ahn *et al.*, 1996; Tomazin *et al.*, 1996). The human cytomegalovirus (HCMV) US6 gene product acts in a different way to inhibit peptide transport by TAP. It makes use of TAP's dependence on ATP-derived energy for the translocation of peptides into the ER, and inhibits the binding of ATP to the nucleotide-binding domain on TAP by arresting TAP in a conformation able to bind peptide and adenosine diphosphate (ADP) but not ATP (Hewitt *et al.*, 2001; Kyritsis *et al.*, 2001). By preventing ATP from binding to TAP, US6 cuts off the energy source required for structural rearrangements and the following peptide translocation into the ER. A related process is illustrated by the EBV protein, BNLF2a, which is post-translationally inserted into the ER-membrane and blocks peptide binding to and transport by TAP by arresting TAP in a conformation unable to transport peptides (Wycisk *et al.*, 2011).

Another key member of the PLC is tapasin, which thereby constitutes a perfect target for viral interference. HCMV encodes an US3 ER-expressed glycoprotein that binds directly to tapasin (Park *et al.*, 2004). It has been shown to decrease the thermostability of tapasin-dependent MHC-I molecules, indicating that tapasin-mediated quality control in the peptide-loading complex is negatively affected by US3 (Park *et al.*, 2004). HCMV has in addition been demonstrated to block tapasin gene transcription, possibly by interfering with the positive regulatory domain 1 (PRDM-1) (Halenius *et al.*, 2011) that has been shown to target the

tapasin promotor and inhibit IFNγ induced transcription of tapasin (Doody *et al.*, 2007).The adenovirus glycoprotein E3-19K is also an inhibitor of the function of tapasin; however, it does not bind directly to tapasin (Bennett *et al.*, 1999). Instead, E3-19K has been suggested to either bind to the tapasin binding-site of TAP, thereby inhibiting tapasin to bind and form the bridge to MHC-I, or induce a conformational change of TAP, thereby inhibiting its interaction with tapasin (Bennett *et al.*, 1999). This protein has also been shown to bind to the ER-luminal domain of MHC-I and to inhibit its transport out from the ER (Andersson *et al.*, 1985; Burgert & Kvist, 1985; Cox *et al.*, 1991). Cowpox virus (CPXV) is another virus encoding a protein, CPXV203, which causes MHC-I retention in the ER (Byun *et al.*, 2007). Even if the MHC-I molecule is able to reach the cell surface, it is still not protected from viral interference. The HIV encoded protein Nef interact with the cytoplasmic tail of the membrane bound MHC-I complex and thereby cause the internalization of the complex by endocytosis, which is followed by degradation (Schwartz *et al.*, 1996).

Thus, interference with members of the PLC prevents virus epitopes from being presented for recognition by CD8+T cells. Even though all MHC-I molecules are to some degree optimized in the PLC (Williams *et al.*, 2002), it is the tapasin dependent allomorphs that are the ones most affected by VIPRs targeting the PLC.

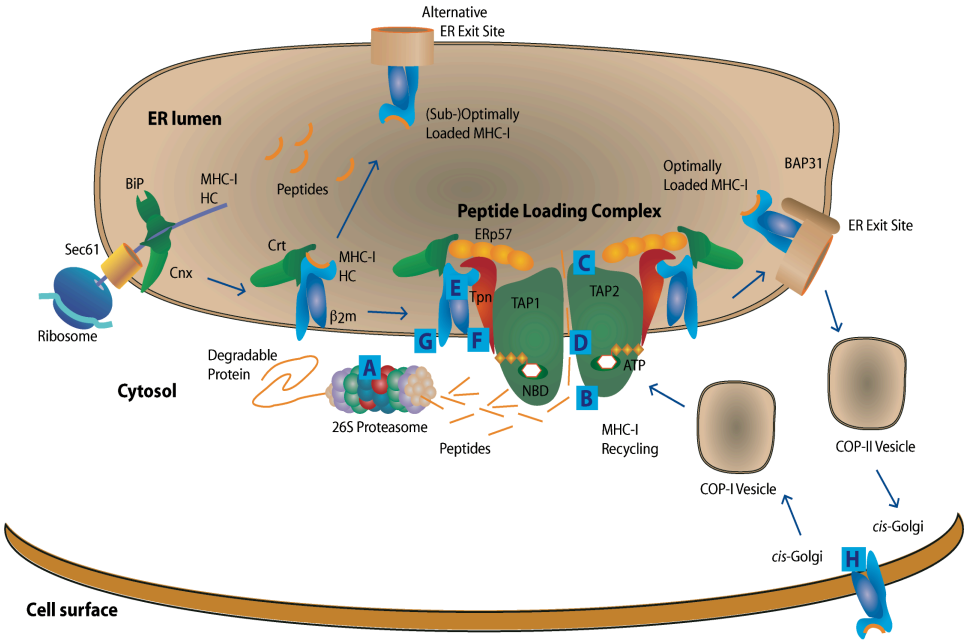

Figure 1. **Viral proteins interfere with MHC-I maturation**. Letters in the figure (A-G) indicate target sites for specific viruses. **A**. The transcriptional activator of human immunodeficiency virus 1 (HIV-1) gene expression, Tat, changes the composition of the immunoproteasome. **B**. Herpes simplex virus ICP47 prevents peptide binding to TAP. **C**. Human cytomegalovirus US6 inhibit TAP transport by preventing ATP binding to TAP. **D**. Epstein-Barr virus BNLF2a prevents both ATP and peptide binding to TAP. **E**. Human cytomegalovirus US3 binds to tapasin and negatively affects its quality control in the peptide-loading complex. **F**. Adenovirus E3-19K inhibits the interaction between TAP and tapasin. **G**. Cowpox virus 203 causes MHC-I retention in the ER. **H**. Human immunodeficiency virus Nef binds to MHC-I and cause endocytosis and degradation of the MHC-I complex. Modified from our mini-review (Roder *et al.*, 2008).

TAPASIN

The discovery of tapasin

In the middle of the 1990s it was shown that transfer of *MHC-I* genes into .220 cells did not result in the complete restoration of surface expression of MHC-I. The expression was shown to be allele specific – some alleles resulted in a MHC-I surface expression at the same level as in control cells, while other alleles showed a significant reduction of surface expressed MHC-I molecules. Fusion of .220 cells with LCL-721.174 cells (lacking TAP1 and 2) restored the surface expression of MHC-I (Greenwood *et al.*, 1994). In addition, fusion with Daudi cells (missing β_2m) restored the surface expression in a similar way. These findings led to the search for additional molecules or/and mechanisms necessary for MHC-I maturation (Grandea *et al.*, 1995).

In 1994 Ortmann and colleagues showed that a 48 kDa glycoprotein co-immunoprecipitated with TAP and suggested that it acts as a bridge between TAP and the MHC-I molecule (Ortmann *et al.*, 1994). Later this 48 kDa protein was given the name TAP-associated glycoprotein, or in short tapasin. It was shown to be expressed in a non-functional (or at least with no function known) form in .220 cells and to be able to restore the reduced MHC-I surface expression when expressed in its functional form in these cells (Ortmann *et al.*, 1997; Copeman *et al.*, 1998).

Interactions between tapasin and MHC-I

In the absence of tapasin there is a lower steady-state level of MHC-I. These MHC-I molecules are less stable at the cell surface and thereby degrade faster, and the export of peptide-receptive MHC-I molecules to the surface is increased due to failure in the ER retention mechanism (Schoenhals *et al.*, 1999; Barnden *et al.*, 2000; Barber *et al.*, 2001; Paulsson *et al.*, 2002; Paulsson *et al.*, 2006). A tapasin interacting allomorph, A*02:01, show almost a three fold longer export time to the cell surface compared to a non-tapasin interacting HLA-I molecule (Lewis & Elliott, 1998). A number of studies demonstrate that the interaction between MHC-I and tapasin increases the stability of cell surface expressed peptide-MHC-I complexes (Barnden *et al.*, 2000; Garbi *et al.*, 2000; Barber *et al.*, 2001; Thirdborough *et al.*, 2008).

The exact interaction site or sites on neither tapasin nor the MHC-I molecule have been determined yet. Many studies have been performed showing the influence of different mutations on MHC-I for its interaction with tapasin (Carreno *et al.*, 1995; Lewis *et al.*, 1996; Peace-Brewer *et al.*, 1996; Yu *et al.*, 1999). Dong *et al* studied the MHC-tapasin interaction by incubating conjugates of eight different tapasin mutants with ERp57 in extracts from .220.B*08:01 cells (Dong *et al.*, 2009). A mutation called TN6 (E185K, R187E, Q189S and Q261S) had the most pronounced effect on both MHC-I interaction, which was completely abolished, and peptide loading activity, which was only 8% of that seen in WT cells. When the TN6 mutant was transduced into .220.B*44:02 cells the cell surface expression of the tapasin dependent allomorph B*44:02 was strongly reduced. The TN6 part of tapasin has been suggested to be responsible for the stabilization of the α2-1 helix in the peptide binding groove (Dong *et al.*, 2009). The stabilization resulted in an open peptide-receptive conformation of the MHC-I molecule until an optimal peptide was bound in the peptide-binding groove (Dong *et al.*, 2009; Van Hateren

et al., 2010). This is in agreement with the most well-studied mutation of MHC-I where threonine at residue 134 is mutated to lysine (T134K) in A*0201 (A*02:01-T134K), which destroys the tapasin interaction (Carreno *et al.*, 1995; Lewis *et al.*, 1996; Peace-Brewer *et al.*, 1996; Yu *et al.*, 1999). The interaction site at tapasin for MHC-I-T134 has been suggested to include R187 (Van Hateren *et al.*, 2010); however, an additional interaction site within the first 87 N-terminal amino acids of tapasin (Tpn_{1-87}) is very likely since Tpn_{1-87} efficiently facilitates peptide binding to A*02:01 and other HLA-I allomorphs (I, II). In addition, the facilitated folding of A*02:01 by Tpn_{1-87} was completely abolished by blocking Tpn_{1-87} with an anti-Tpn_{1-87} antibody (I and unpublished data from our group), which further indicate that an additional interaction site exists within the first 87 N-terminal amino acids of tapasin.

It has been shown that a deletion of the transmembrane region of tapasin does not affect the interaction between tapasin and MHC-I in co-immunoprecipitation experiments (Lehner *et al.*, 1998). On the other hand, tapasin truncated at the N-terminal, does not co-precipitate with MHC-I, which suggest that the 50 most N-terminal amino acid residues of tapasin are crucial for its interaction with MHC-I (Lehner *et al.*, 1998).

Peptide-editing

It has been suggested that tapasin stabilizes MHC-I molecules that bind to suboptimal peptides (Ortmann *et al.*, 1997; Schoenhals *et al.*, 1999; Barnden *et al.*, 2000; Garstka *et al.*, 2011), thereby increasing the number of peptide-receptive MHC-I molecules (Chen & Bouvier, 2007). In addition to its role in stabilizing MHC-I molecules, tapasin has been shown to be involved in the exchange of low affinity bound peptides by higher affinity ones – a process called peptide editing

or peptide optimization (Barnden *et al.*, 2000; Garbi *et al.*, 2000; Barber *et al.*, 2001; Williams *et al.*, 2002; Chen & Bouvier, 2007; Wearsch & Cresswell, 2007; Praveen *et al.*, 2010).

By studying the expression of a set of five peptides with different stability, expressed as mini-genes in the cytosol of .220.K^b and .220.K^b tapasin cell lines, it was shown that the hierarchy of the five cell surface presented peptides differed in the presence and absence of tapasin (Howarth *et al.*, 2004). In the presence of tapasin all peptides were presented in higher amounts and the increases correlated with peptide half-lives. However, in cells lacking tapasin, a peptide with intermediate half-life was dominantly presented. Since all the peptides in this study had similar affinities to K^b, the editing was suggested to be dependent on stability (off-rate) rather than the affinity *per se* (Howarth *et al.*, 2004). In a similar study no difference in average affinity of peptides presented in the presence and absence of tapasin was observed, and it was stated that tapasin is a facilitator stabilising peptide-receptive MHC-I molecules, rather than an editor (Zarling *et al.*, 2003). However, the half-life of the surface expressed allomorphs B8 and A*02:01 differed in the presence and absence of tapasin (Zarling *et al.*, 2003). By including enhancement of stability, and not only affinity, in the expression "peptide editing" this contradiction may not exist. This is also in accordance with other studies where it has been observed that other factors than affinity might have a role in peptide editing (Wearsch & Cresswell, 2007), or the other way around, that stability is not always the only regulating peptide editing factor (Chen & Bouvier, 2007).

Binding of an optimal peptide to a MHC-I molecule results in the dissociation of tapasin (Ortmann *et al.*, 1994; Paulsson *et al.*, 2001b; Rizvi & Raghavan, 2006). The peptide-bound MHC-I molecule has recently however been suggested to

appear in an unstable encounter complex, which converts into a stable complex when an optimal peptide is bound (Praveen *et al.*, 2010). Tapasin is suggested to exert its function as editor of this unstable encounter complex, but how this is performed is still not clear.

The weak interaction between recombinant tapasin and MHC-I makes *in vitro* studies of tapasin function hard to perform. In a study using leucine zippers to tether soluble tapasin and MHC-I molecules together this problem was overcome and several functions of tapasin were studied in detail (Chen & Bouvier, 2007). In addition to stabilising the MHC-I molecule, tapasin accelerated both the dissociation of peptide from B*08:01 and the association of the complex in a peptide dependent manner.

By studying the allomorphs K^b wild type (WT) or K^b (T134K) incubated with a mixture of a high-affinity peptide and a 100-fold higher concentration of a low-affinity peptide, or first incubated with the low-affinity peptide and then added the high-affinity peptide in a 100-fold lower concentration, the high-affinity peptide was shown to be the dominantly bound peptide to K^b WT within short time, while K^b (T134K) mostly bound the low-affinity peptide (Praveen *et al.*, 2010). This further supports an accelerated association and/or dissociation as the mechanism of peptide editing by tapasin.

Tapasin has been demonstrated to disrupt the conserved hydrogen bonds between the peptide C-terminal and the peptide binding groove (F-pocket), as well as disrupting interactions along the entire groove (Wright *et al.*, 2004; Chen & Bouvier, 2007; Praveen *et al.*, 2010). Thereby it was suggested to widen the groove so that a more diversified set of peptides got the chance to bind into the

groove initially and also to allow peptides to associate and dissociate faster (Chen & Bouvier, 2007).

Tapasin dependence

Different HLA-I allomorphs depend to different degree on tapasin for efficient peptide presentation at the cell surface. This in turn requires a sufficiently high stability of the surface expressed peptide-HLA-I complexes. It has been hypothesized that the ionic/hydrophobic environment at the bottom of the F-pocket, which is influenced by several amino acids, affects the conformational flexibility of peptide-free MHC-I molecules (Zernich *et al.*, 2004; Sieker *et al.*, 2008; Garstka *et al.*, 2011). A disordered conformation in the region of the F-pocket in the peptide-binding groove has been suggested to be due to a partially open disulfide bond in the α2 domain of tapasin dependent allomorphs, which makes them unable to bind peptide in the absence of tapasin (Garstka *et al.*, 2011). Tapasin stabilises the MHC-I molecule to an open peptide-receptive conformation, which results in a correct α2 disulphide bond (Garstka *et al.*, 2011).

The influence of amino acids at specific positions

Several studies have indicated the importance of particular amino acids at specific positions in the MHC-I molecule for its dependence on tapasin. The allomorph B*44:02 has been shown to be tapasin dependent for surface expression in several studies (Neisig *et al.*, 1996; Peh *et al.*, 1998; Park *et al.*, 2003; Zernich *et al.*, 2004; Garstka *et al.*, 2011). It has been suggested that the amino acid at position 114 is responsible for this tapasin dependence (Park *et al.*, 2003). Acidic amino acids (aspartic acid and glutamic acid) at position 114 should lead to strong tapasin dependence, while neutral amino acids (aspargine and glutamine) should lead to

moderate dependence and basic amino acids (histidine and arginine) to low tapasin dependence (Park *et al.*, 2003). However, both B*44:02 and B*44:05 have aspartic acid at position 114 and they appear nevertheless to be on opposite ends of the tapasin dependence spectrum.

In a recent study Garstka and colleagues (Garstka *et al.*, 2011) have studied the effect of substitutions in position 116 in B*44:02 and B*4405. B*44:02 has aspartic acid and B*44:05 has tyrosine at position 116, located in the F-pocket of the peptide binding groove. Replacement of aspartic acid at position 116 with histidine (whose shape resembles the one of tyrosine, present in the tapasin independent B*44:05 allele) resulted in a tapasin independent molecule with stable conformation of the F-pocket region. They hypothesized that the presence of the two aspartic acid-residues, one at position 114 and the other one at 116, in B*44:02 results in a conformational disruption of the F-pocket due to excessive hydration. However, in another study where the TAP interaction of different allomorphs was compared, they observed that B*44:03 which only differ from B*44:02 at position 156 (aspartic acid in B*44:02 and leucine in B*44:03) associated very inefficiently with TAP (Neisig *et al.*, 1996).

Another well-studied allomorph regarding tapasin dependence is B*27:05. B*27:05 has, in contrast to B*44:05, been shown to incorporate into the PLC and is quantitatively and qualitatively affected by tapasin despite being relatively tapasin independent with respect to its surface expression (Peh *et al.*, 1998; Purcell *et al.*, 2001; Williams *et al.*, 2002; Park *et al.*, 2003; Zernich *et al.*, 2004). In a study of Peh *et al*, the steady state surface expression of B*27:05 in .220.B*27:05 cells was shown to be similar in the presence and absence of tapasin (Peh *et al.*, 1998). However, when cytotoxicity (*i.e.* cell lysis) was measured after four hours infection with recombinant vaccinia viruses, the absence of tapasin resulted in

only half the cytotoxicity compared to tapasin-positive cells. By twelve hours post infection the difference in percentage cell lysis in the presence and absence of tapasin was no longer observed but an altered peptide repertoire and less stable surface expression were suggested in the absence of tapasin.

The influence of specific amino acids at defined positions of the HLA-I molecule may explain tapasin dependency to some extent, but is not the only regulating factor for this interaction. The peptide identity of the binding peptide is another regulator of the HLA-I-tapasin interaction (Zernich *et al.*, 2004)(I, II, IV). In a study by Zernich *et al*, it was shown that B*44:02 and B*44:05 have different preferences for the P9 side chain of the binding peptide, both aiming at an optimal milieu of the F-pocket in the MHC-I molecule (Zernich *et al.*, 2004).

It is known that the polymorphism in HLA plays a major role in our capability to fight many viral infections. Different degree of dependence with respect to tapasin among HLA-I allomorphs may have evolved in response to evolutionary pressures from viruses and other microorganisms.

THE PROJECT

The main purpose of the project was to elucidate how tapasin mediates the quality control of HLA-I maturation in order to clarify which peptides are selected for presentation at the cell surface. More than one decade of studies around the world has resulted in suggestions of several interesting properties for this peptide editing MHC-I dedicated chaperone. However, despite the clarification of several important aspects there is, as yet, no clear answer of exactly how tapasin mediates MHC-I maturation.

The structure of tapasin did not become known until 2009 (Dong *et al.*, 2009). Prior to that study it was believed that tapasin had two ER-luminal domains, of which the first spanned the initial 85 N-terminal amino acids (Chen *et al.*, 2002). The first 50 amino acids of these were shown to be crucial for stable interaction between tapasin and MHC-I and the restoration of cell surface expression of MHC-I (Bangia *et al.*, 1999). However, a recent report of the protein crystal structure shows that the N-terminal domain, called domain-1, contains amino acids 1-269, while domain-2 spans residues 270-381 of the ER-luminal part of tapasin (Dong *et al.*, 2009). In this project we have produced the first 87 N-terminal amino acids of human tapasin ($Tpn_{1\text{-}87}$) and studied its influence on HLA-I folding.

In the following sections I will not repeat the results and discussions from papers I-IV, but instead try to highlight and clarify specific parts of these papers. Thus they are preferably read as a supplement to papers I-IV, which are attached in the appendix.

Paper I

In this study we established the basis, approaches and tools used in papers II-IV. Our initial intention was to produce Tpn_{1-87} alone. After testing bacterial expression of Tpn_{1-87} by induction with IPTG in several protein expression hosts without any detectable expression, GrpE was inserted upstream of Tpn_{1-87} with a FXa cleavage site between these. GrpE is a nucleotide exchange factor in the Dna/DnaK bacterium chaperone system that can be used to increase the solubility of proteins when fused to them (Wilkinson & Harrison, 1991; Davis *et al.*, 1999). The insertion of GrpE resulted in the expression of GrpE-FXa-Tpn_{1-87}. By using FXa protease, the GrpE may be separated from Tpn_{1-87}. However, FXa digestion destroyed the Tpn_{1-87} therefore, the entire fusion protein, GrpE-FXa-Tpn_{1-87}, was used in all subsequent experiments. GrpE alone showed no effect on the folding of A*02:01 and from now on in this thesis GrpE-FXa-Tpn_{1-87} is referred to as Tpn_{1-87}.

Experiments using proteins purified from eukaryotic cells have shown that an interaction occurs between HLA-I and tapasin even in the absence of other PLC members (Schoenhals *et al.*, 1999; Rizvi & Raghavan, 2006; Thammavongsa *et al.*, 2006) (Kajsa Paulsson, unpublished data). However, due to the low affinity of the binding between tapasin and HLA-I, biochemical studies of this interaction have been difficult, and complementary support tools/chaperones have been shown to be crucial for studying the interaction (Chen & Bouvier, 2007; Wearsch & Cresswell, 2007). Being able to more easily study the interaction between HLA-I and tapasin should help us to better understand the mechanisms whereby tapasin influences HLA-I maturation. By using the anti-HLA antibody (ab) W6/32 bound to protein A beads, we were able to co-precipitate A*02:01 with Tpn_{1-87}. However, this procedure was only successful in a few of several cases, indicating that co-immunoprecipitation is not an optimal method to use for this purpose. Similarly

the use of enzyme-linked immunosorbent assays (ELISAs) and luminescent oxygen channeling immunoassays (LOCIs, commercialized as AlphaScreen^{TM}) with different setups did not allowed us to study the direct binding of Tpn_{1-87} to A*02:01. However, with our AlphaScreen method (see Figure 2) we were able to demonstrate that Tpn_{1-87} increases the amount of W6/32 recognized HLA-I molecules. Thus, the N-terminal part of tapasin has an effect, even though the exact nature of the interaction between this tapasin part and the HLA-I molecule could never be measured.

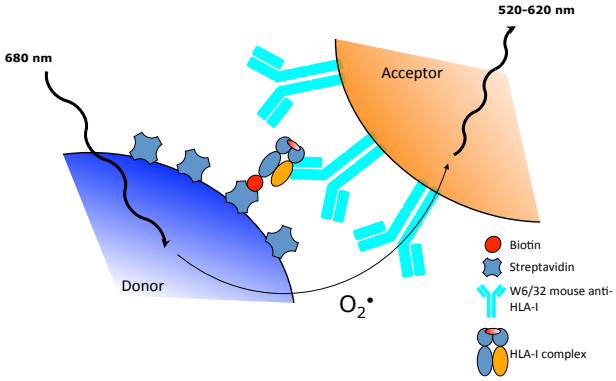

Figure 2. **The principle of our AlphaScreen based peptide-HLA-I folding assay**. The proximity-based assay is a luminescent oxygen channeling immunoassay where the proximity of a donor and an acceptor bead is measured by light emission. The donor beads are streptavidin-coated and bind to the biotinylated HLA-I molecules. The acceptor beads are conjugated with the anti-HLA-I antibody, W6/32, which only recognizes β2m associated HLA-I HC. If W6/32 recognizes the HLA-I molecule the two beads are in close proximity, which upon illumination results in energy transfer from the donor bead to the acceptor bead, ultimately generating a luminescent signal. In the presence of Tpn_{1-87} the amount of W6/32 recognized HLA-I molecules increased.

β2m is a crucial component for the conformational structure and stability of HLA-I (Seong *et al.*, 1988). The mouse monoclonal antibody, W6/32, recognizes β_2m associated HLA-I HC (Brodsky & Parham, 1982). Position 3 of β_2m and position 121 of the HC have been suggested to constitute parts of the epitope for W6/32 (Ladasky *et al.*, 1999). However, also other positions, *i.e.* 44, 89 of β_2m (Kahn-Perles *et al.*, 1987) and positions near the cysteine that forms the disulfide bond of the α2 domain of the HC (Maziarz *et al.*, 1986), have been shown to be of importance for recognition by W6/32. Although W6/32 reacts with all known HLA-I allomorphs (Ladasky *et al.*, 1999), there might still be some difference in its recognition of different allomorphs and differences dependent on the peptide bound to HLA-I.

Tapasin has been suggested to require ERp57 for its stabilization of empty HLA-I molecules, its efficient facilitation of peptide binding and its peptide editing (Wearsch & Cresswell, 2007). Furthermore, tapasin has a critical role in stabilising TAP for proper peptide transport into the ER (Li *et al.*, 2000; Tan *et al.*, 2002). It has been proposed however, that soluble tapasin, which is unable to interact with TAP, may restore the surface expression of B*08 to almost the same level as cells with wild type tapasin (Lehner *et al.*, 1998). On the other hand, studies with murine MHC-I show that both soluble mouse tapasin and human tapasin are unable to restore surface expression of K^d (Simone *et al.*, 2009a; Simone *et al.*, 2009b), which were shown to be at even lower levels than in the complete absence of tapasin (Simone *et al.*, 2009b; Simone *et al.*, 2012). By using $Tpn_{1\text{-}87}$ we have shown that this short soluble fragment on its own facilitates the folding of A*02:01, thereby supporting an effect of soluble tapasin on MHC-I.

In order to study tapasin and tapasin associated MHC-I molecules it was essential to have available an immunoprecipitation-functional tapasin antibody. At the beginning of our study commercial antibodies against tapasin were scarce. For this reason we set out to generate a monoclonal antibody against Tpn_{1-87}. We successfully produced a large set of monoclonal antibodies against tapasin and one of them was found to function in immunoprecipitation. The clone was named αTpn_{1-87}/80 and, in addition to immunoprecipitation of tapasin, it could be used for Western Blot and intracellular staining. The epitope recognized by this antibody was determined by the use of the PepChip3K technology. This approach identified a surface exposed epitope as expected since it could be used in immunoprecipitation of tapasin. Although the original purpose was to obtain an antibody against tapasin that could be used for co-precipitation of MHC-I this aim was not achieved since αTpn_{1-87}/80 binds to the LDPEL sequence of tapasin located in the MHC-I interaction region. On the other hand another valuable application of the antibody is the possibility to block tapasin in cells with a therapeutic purpose. In an unpublished study we have blocked Tpn_{1-87} with αTpn_{1-87}/80, resulting in an inhibited facilitation of A*02:01 folding. This strengthens the idea that the effect of Tpn_{1-87} seen on A*02:01 is specific for Tpn_{1-87}.

Paper II

The results presented in paper I that Tpn_{1-87} facilitates the folding of A*02:01 constituted the basis for the performed in paper II. In this paper we studied the influence of Tpn_{1-87} on an extended number of HLA-I allomorphs. The differences in the Tpn_{1-87} mediated folding facilitation of the five allomorphs were perfectly in accordance with the differences in tapasin dependence seen in previous studies using cellular systems.

In paper II we introduced the phrase “tapasin-facilitation”, which refers to the maximum amount of folded HLA-I obtained in the presence of Tpn_{1-87} (Tpn_{1-87} B_{max}), divided by the maximum amount of folded HLA-I obtained in the absence of Tpn_{1-87} (Ctrl B_{max}). Thus, a facilitation value >1 means that Tpn_{1-87} facilitates the folding of HLA-I. Using a large panel of peptides with different characteristics we were able to demonstrate that also peptides with the same affinity to A*02:01 could differ in their tapasin-facilitation. Two sets of peptides were studied, SYFPEITHI peptides and non-SYFPEITHI peptides. SYFPEITHI peptides are natural HLA-I presented peptides registered in the SYFPEITHI database. Some of the SYFPEITHI peptides are T cell epitopes while others are self-peptides. Depending on the method used for identification of the peptides, there may be a risk that not only peptides presented by MHC-I molecules at the cell surface become included in this group since MHC-I molecules are immunoprecipitated from whole cell lysates in the majority of the ligand identification attempts. Another limitation is that weakly bound peptides may dissociate before the acid elution and, hence, the final pool of peptides dissociated from purified MHC-I molecules may only represent a fraction of the original peptide pool. Provided that the peptide-MHC interaction is stable enough to stand the cell lysis conditions and the washing steps it should also be stable enough to be presented at the cell surface, and if not at there at the moment of cell lysis it might be on its way out. There is also a possibility that non-SYFPEITHI peptides are natural ligands but that they have not been registered in the SYFPEITHI database (yet), and therefore categorized as non-SYFPEITHI/non-natural peptides.

A larger proportion of the SYFPETHI peptides have their anchor positions occupied by preferred amino acids compared to non-SYFPEITHI peptides where several are known to carry less favoured amino acids at these positions. However, among the peptides used in this study approximately half of the non-SYFPEITHI

peptides had a preferred amino acid in at least one of the anchor positions. This indicates the importance of other factors, in addition to anchor residue satisfaction, for the binding of SYFPEITHI peptides, such as amino acids in other positions. Since these additional factors that separate SYFPEITHI from non-SYFPEITHI peptides are unknown, the development of tools allowing definition of criteria that discriminate the two types of peptides is a crucial task for the development of peptide-based vaccines. Indeed, tapasin-facilitation constitutes such a tool since the folding of A*02:01 with SYFPEITHI peptides is not facilitated, or facilitated to only a low degree (*i.e.* tapasin-facilitation <1.5) by Tpn_{1-87}, while folding with non-SYFPEITHI peptides is facilitated (*i.e*. tapasin-facilitation >1.5). The stability of peptide-A*02:01 complexes was shown to be inversely correlated with tapasin-facilitation and to discriminate SYFPEITHI peptides from non-SYFPEITHI peptides in a similar manner as Tpn_{1-87}, which suggests that stability is an additional and at least partly overlapping tool when searching for immunogenic peptide candidates.

The finding that Tpn_{1-87} facilitates folding of non-SYFPEITHI peptides, *i.e*. less stable peptide-A*02:01 complexes, was not expected, since the presence of tapasin has been suggested to result in the presentation of more stable peptide-MHC-I complexes. However, our results are consistent with this and other findings demonstrating that tapasin is released upon binding of high-affinity peptide to MHC-I (Paulsson *et al.*, 2001a), and that tapasin act as a chaperone and thereby increases the number of peptide-receptive MHC-I molecules (Chen & Bouvier, 2007). This means that tapasin does not exert its action directly on already stable peptide-MHC-I complexes; it rather enables such complexes to form by stabilizing sub-optimal peptide-MHC-I complexes whose peptide may be exchanged by an optimal peptide. The way this is achieved is not known in detail, as previously discussed in the tapasin section above.

The question if tapasin solely has a chaperone function for HLA-I, or if it acts as a peptide editor as well, is still debated. Our hypothesis is that these two functions co-exist due to the way tapasin functions as a stabilizer of HLA-I molecules. Tapasin binding to HLA-I results in a stable and more open conformation of the HLA-I molecule that prevents it from degradation and enables peptide association and dissociation. Upon binding of SYFPEITHI peptides to A*02:01 we demonstrate that the binding affinity increases in the presence of Tpn_{1-87}. This suggests that the binding of an optimal peptide may change the conformation of the HLA-I molecule into a locked state, *i.e.* a state with higher affinity between the peptide and the HLA-I molecule, which is followed by release of tapasin. In addition, binding of non-SYFPEITHI peptides to A*02:01 results in a decreased binding affinity in the presence of Tpn_{1-87}. This indicates that the A*02:01 molecule is in a more open state in the presence of Tpn_{1-87}, *i.e*. a state that interacts with tapasin until a peptide that induces a locked conformation binds. In conclusion we hypothesize that the editing function of tapasin is the consequence of its chaperone function.

Paper III

In paper II we demonstrated an inverse correlation between tapasin-facilitation and peptide-HLA-I stability, both constituting reliable tools to screen for immunogenic peptides. Here we set out to investigate if tapasin-facilitation and stability are directly associated, *i.e*. if an increased stability is reflected in a corresponding lower tapasin-facilitation. The aim of this was to get a deeper understanding of the basis for peptide selection performed by tapasin.

High peptide affinity to MHC-I has long been associated with immunogenicity, however the stability of the peptide-MHC-I complex has been shown to correlate with immunogenicity even more strongly (van der Burg *et al.*, 1996)(II). As mentioned in the "Short introduction to the immune system" in the beginning of this thesis, several parameters contribute in concert to immunodominance, *i.e.* the small fraction of peptide-MHC-I complexes that results in a focused attention and activation of the immune system. Figure 3 represents a schematic quantitative overview of the relative importance of the different components that contribute to immunodominance.

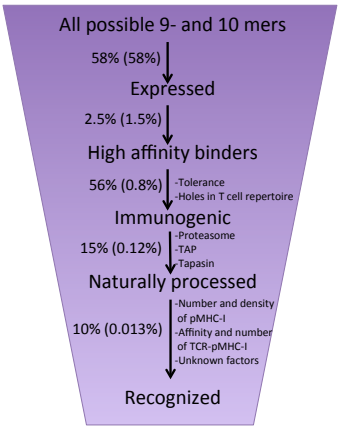

Figure 3. **Relative importance of components that contribute to immunodominance.** Of all 9- and 10-mers encoded in the entire genome of vaccinia virus, approximately 58% are expressed. 2.5% of these, *i.e.* 1.5% of all 9- and 10-mers that potentially could be generated, have a sufficiently high peptide binding affinity (<100 nM) to bind to MHC-I. Due to tolerance and holes in the T cell repertoire only 56% of the high affinity binders are immunogenic. When this peptide pool was tested for natural processing, *i.e.* cleavage by the proteasome, transport by TAP into the ER and quality control by tapasin, only 15% of the immunogenic peptides are produced and allowed to bind MHC-I. Of the naturally processed peptides only 10% cause cytolytic attack by T cells, which indicates that several factors influence immunodominance. Digits within the parenthesis represent % of the potentially generated 9- and 10-mers. Modified from Assarsson *et al.* (Assarsson *et al.*, 2007).

In a study using vaccinia virus (VACV) and A*02:01 (in mice) Sette and colleagues estimated that from the entire VACV genome potentially >115 000 9- and 10-mers could be generated. Of these approximately 58% were expressed (based on the number of 102 VACV-derived antigenic proteins found in the literature) (Assarsson *et al.*, 2007). Approximately 2.5% of the expressed 9- and 10-mers were suggested to bind to MHC-I because of their high binding affinity (*i.e.* IC_{50} <100 nM). More than half of the high-affinity binding peptides were immunogenic, *i.e.* resulted in CD8+ T cell responses upon peptide immunization. In infected DCs, however, only 15% of the immunogenic peptides were generated by natural processing. This pool of peptides contained both dominant and subdominant peptides. Subdominant peptides, *i.e.* naturally processed peptides which only can induce a CD8+T cell response after peptide immunization but not recognized in the context of a natural infection, constituted a majority of the peptides. Of the naturally processed peptides 10% (*i.e.* <1% of the theoretical pool of high affinity peptides) were recognized after infection, *i.e.* were dominant peptides. Of course, this is an approximation and varies to some extent for different models but it gives a clear message that the fraction of peptides with potential to start a cytolytic attack is very small.

The existence of subdominant peptides suggests that in addition to the factors mentioned above additional immunoregulatory mechanisms and factors control immunodominance. The stability of the peptide-MHC-I complex, the number and density of peptide-MHC-I complexes at the cell surface of the infected APC, and the affinity and number of TCR-peptide-MHC-I interactions are all factors that are of importance for immunodominance, but their proportional role has not been elucidated yet. It would be particularly interesting to investigate the influence of tapasin-facilitation in this aspect, possibly increasing the possibility to predict immunodominant peptides. The use of human α1 and α2 domains of A*02:01 in a

mouse setting, as used in the study by Assarsson *et al*. (Assarsson *et al.*, 2007), probably influences the numbers presented here, since the proteasomal processing and PLC effects result from mouse and not human components. Better knowledge about the regulation of immunodominance and how we can influence its effect on the immune response is crucial for understanding the underlying mechanisms of an efficient immune response.

All peptides used in Paper III were SYFPEITHI peptides with high affinity to A*02:01 and B*08:01 respectively, and they resulted in high stability and low tapasin-facilitation. However, the small differences found in the different parameters did not correlate, as the almost two-fold increase in stability for one peptide compared to the other peptides did not correspond to a lower tapasin-facilitation. However, since all peptides studied were SYFPEITHI peptides resulting in peptide-HLA-I complexes of high stability and low tapasin-facilitation, these parameters are suggested to be highly reliable tools in the search of immunogenic peptides as suggested previously (II). It is important to mention that there is an allomorph dependent specificity of the thresholds that discriminate SYFPEITHI from non-SYFPEITHI peptides, which increases the complexity of the tools. To eliminate the risk of using false positives, *i.e.* non-immunogenic peptides with high stability or low tapasin-facilitation, we suggest that the two parameters should be used in a complementary fashion.

The presence of $Tpn_{1\text{-}87}$ did not affect the dissociation of ^{125}I labeled β_2m from A*02:01 and to a minor degree increased its dissociation from B*08:01. This is in accordance with the fact that $Tpn_{1\text{-}87}$ does not affect HLA-I-SYFPEITHI peptide complexes. However, it is not in accordance with a previous study, which demonstrated that tapasin to a significant degree increased the dissociation of the

same peptide from B*08:01 (Chen & Bouvier, 2007). In the study of Chen *et al* the authors used leucine zippers to tether tapasin and B*08:01, which increases the probability to have an interaction between the two molecules, compared to Tpn_{1-87} in solution with B*08:01, used by us. In combination with the difference in temperature in the two assays, *i.e.* 20°C (used by Chen and Bouvier) compared to 37°C (used by us), this circumstance may explain the discrepancy of the effect of tapasin observed here. However, our assay was developed for optimal conditions to examine the stability at physiological temperature and not to investigate the effect of tapasin in this particular situation.

Paper IV

In previous papers (I-III) we demonstrated that tapasin-facilitation depends on the HLA-I allomorph as well as the peptide identity of the binding peptide. In paper IV we examined further both these parameters. A tapasin-dependency spectrum, ranging from very high to almost absent tapasin-facilitation, was performed. The high number of allomorphs tested here has not been studied previously in a single experimental set up, a circumstance that has complicated the scoring of tapasin dependency for different allomorphs. The extremes on each side of the spectrum, *i.e.* the tapasin-dependent allomorph B*44:02 and the tapasin-independent mutant of A*02:01, A*02:01-T134K, were not displaced from their edges. However, other allomorphs like the known less tapasin-dependent allomorph B*27:05 was unexpectedly shown to be more tapasin-dependent than previously suggested (Park *et al.*, 2003).

In addition to studying the tapasin-facilitation of a large set of HLA-I allomorphs we addressed here the relationship between tapasin-facilitation and peptide length. To my knowledge this has not been studied before, instead the generalization that peptides of 8-10 amino acids constitute the optimal lengths is today used when epitopes are predicted in different ways. For allomorphs with low tapasin-facilitation the facilitation was approximately equally low for all peptide lengths tested, *i.e.* 7-13 amino acids. In addition no peptide length was found to be strongly favoured among those longer than 8 amino acids. However, the folding of intermediate tapasin-dependent allomorphs showed a relatively low tapasin-facilitation when folding with 10- and 11-mers. These peptide lengths also form the highest number of peptide-HLA-I complexes in the absence of $Tpn_{1\text{-}87}$. One could therefore hypothesize that peptide lengths of 10 or 11 amino acids are advantageous in scenarios where tapasin function is blocked or ruined, *i.e.* in some viral infections and tumours.

No previous studies appeared to have quantified the proportions of different allomorphs at the cell surface. The reason for this is probably the lack of allomorph specific antibodies for HLA-I. However, in our recombinant system we could show that the less tapasin-dependent allomorphs form a higher number of peptide-HLA-I complexes compared to the more tapasin-dependent allomorphs, both in the presence and absence of $Tpn_{1\text{-}87}$. In the aspect of vaccine development it is highly relevant to investigate if this reflects the situation at the cell surface. If less tapasin-dependent HLA-I allomorphs are expressed in a higher proportion at the cell surface one could hypothesize that this pattern has evolved as a response to evolutionary pressures from viruses and other intracellular organisms interfering with the PLC.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Vårt immunförsvar består av molekyler, celler, och barriärer som har till uppgift att hålla oss friska genom att avlägsna eller stänga ute virus och skadliga mikroorganismer. På alla kärnförsedda celler i vår kropp sitter en stor mängd MHC klass I-molekyler, strukturer som kan liknas vid korgar. I varje korg ligger en peptid som är ett fragment av något som finns inuti cellen, exempelvis delar av kroppsegna proteiner, virusproducerade proteiner och tumörspecifika proteiner. Specifika celler i immunförsvaret, CD8-positiva T-celler, har tidigt lärt sig vad som är kroppseget och vad som är främmande. Om det ligger en peptid i en korg som inte är kroppsegen, känns den igen av CD8-positiva T-celler. T-cellerna aktiveras då och dödar de celler som bär den icke-kroppsegna peptiden i sina korgar.

Den peptid som tillverkats och lagts i korgen (binder till MHC klass I-molekylen) inne i cellen transporteras sedan ut och sätter sig på cellytan. MHC klass I-molekylen är dock väldigt restriktiv i sin bindning och transporteras bara till cellytan om den bär på en peptid som väl passar in i dess peptid-bindningsklyfta. Bindning av peptid sker oftast med hjälp av det så kallade peptidladdnings-komplexet, ett komplex bestående av ett antal medhjälpare där i synnerhet en molekyl vid namn tapasin är av stor vikt.

Av det enorma antal peptider som finns i cellen är det således bara en bråkdel som presenteras på cellytan. Tapasin binder till MHC klass I-molekyler och tros stabilisera dem så att lämpliga peptider får en möjlighet att binda in till peptid-

bindningsklyftan. Om tapasin av någon anledning saknas i cellen transporteras färre MHC klass I-molekyler ut till cellytan och de som finns där är väldigt instabila och faller lätt sönder. Tapasin är alltså med och ser till att stabila peptid-MHC klass I-komplex kan bildas. När detta väl skett så släpper tapasin taget om peptid-MHC klass I-komplexet så det kan transporteras till cellytan. Exakt hur denna process fungerar är ännu inte klarlagt.

Studierna av tapasins funktion inleddes med att vi producerade en liten del av molekylen, Tpn_{1-87}. I vårt första arbete (**I**) redogör vi för hur vi gick tillväga när vi producerade Tpn_{1-87} och visar att vid närvaro av denna tapasin-del under peptid-bindning till MHC klass I i biokemisk modell utanför cellen formas fler peptid-MHC klass I-komplex. I den andra delen av första arbetet (**I**) redogör vi för tillverkningen av antikroppar. Antikroppar känner igen och binder till ett visst ställe på en specifik molekyl, i vårt fall tapasin. Vi demonstrerar och kartlägger olika tillämpningsområden för en av dessa antikroppar och har kartlagt dess exakta inbindningsställe på tapasin. Antikropparna mot tapasin kan användas på många sätt och ge oss mycket kunskap om tapasins funktion.

Det finns en mängd olika MHC klass I-molekyler, så kallade allomorfer, i den mänskliga populationen. De är mer eller mindre beroende av tapasin för att uttryckas på cellytan. I arbete nummer två (**II**) visar vi att Tpn_{1-87} påverkar olika allomorfer olika mycket, genom att studera hur mycket peptid-MHC klass I-komplex som bildas i närvaro respektive frånvaro av Tpn_{1-87} med de olika allomorferna. Resultaten är helt i linje med vad som har visats i andra studier med tapasin. Genom att dividera mängden bildade peptid-MHC klass I-komplex i närvaro av Tpn_{1-87} med mängden bildade i frånvaro av Tpn_{1-87} kan man beräkna tapasin-faciliteringen, och få fram ett mått på hur stor inverkan Tpn_{1-87} har på antalet peptid-MHC klass I-komplex som bildas. Det framkom att komplex-

bildning med peptider som presenteras på cellytan, SYFPEITHI-peptider, har en lägre tapasin-facilitering än icke-SYFPEITHI-peptider, som inte har påvisats på cellytan. Vi föreslår därför att tapasin-facilitering kan användas för att på ett enkelt sätt fastställa vilka peptider det är som presenteras på cellytan, vilket kan vara till stor hjälp vid tillverkningen av peptid-baserade vaccin. Härmed fick vi också en bättre förståelse för tapasins funktion att stabilisera mindre stabila MHC klass I-molekyler och därmed göra dem mer tillgängliga för inbindning av peptid. Våra resultat tyder även på att inbindning av tapasin leder till att stabila peptid-MHC klass I-komplex bildar en än mer stabil låst struktur, medan peptid-MHC klass I-komplex med mindre passande peptid hålls öppna av tapasin så att peptiden snabbt och lätt kan lossna och lämna plats åt en ny peptid. När en optimal peptid har bundit till peptidbindningsklyftan frigör tapasin sig från peptid-MHC klass I-komplexet, vilket tillåter komplexet att uttryckas på cellytan.

I det tredje arbetet (**III**) ville vi undersöka om graden av tapasin-facilitering omvänt korrelerar med stabiliteten på peptid-MHC klass I-komplexen. Det visade sig att de inte alltid följde varandra; en högre stabilitet medförde inte nödvändigtvis en lägre tapasin-facilitering av komplexet. De två parametrarna, stabilitet och tapasin-facilitering, föreslås med fördel kunna användas som komplementära verktyg för att välja ut lämpliga peptider till peptidbaserade vaccin.

I arbete nummer fyra (**IV**) utökar vi antalet allomorfer och jämför tapasin-faciliteringen dem emellan. Detta är första gången någon i en och samma studie tittar på tapasins effekt på ett så stort antal allomorfer samtidigt, något som är till stor fördel om man direkt vill kunna jämföra dem. De mest extrema allomorferna på skalan av tapasin-beroende behöll sin plats i hierarkin medan några allomorfer placerade sig något annorlunda jämfört med tidigare studier. Vi såg också att de

minst tapasin-faciliterade allomorferna formade fler peptid-MHC klass I-komplex än de allomorfer som är mer tapasin-faciliterade, något som möjligen avspeglar proportionerna av de olika allomorferna på cellytan. Dessutom studerade vi effekten av peptiders längd med avseende på tapasin-facilitering och kunde observera att dess relevans ökar med tapasin-faciliteringsgraden för allomorferna.

Dessa studier har lärt oss att antalet korgar innehållande peptid/inbindning av peptid till MHC klass I ökar i närvaro av tapasin. Detta tros bero på tapasins funktion att stabilisera MHC klass I-molekylen, vilket möjliggör inbindning av rätt sorts peptid. Denna peptid ger MHC klass I-komplexet tillräckligt hög stabilitet för att tapasin ska släppa taget och tillåta transport av komplexet till cellytan. Vi kan också konstatera att det finns en stor spännvidd i tapasin-beroende bland olika allomorfer, något som delvis omvänt korrelerar med stabiliteten av MHC klass I-komplexet. Exakt hur och vad tapasin känner av när det binder respektive släpper MHC klass I-molekyler kvarstår att undersöka men en stor del av tapasin-pusslet är nu lagt.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all who have contributed and supported me during all these years of PhD studies.

First of all thank you *Kajsa Paulsson*, my supervisor, for accepting me as a collaborator (2007) and later on as your PhD student. Thank you for being such an inspiring, enthusiastic, positive person with a warm heart and sense of humour. I am truly grateful that you always have taken the time to discuss tapasin, antigen presentation and all other important things in life. Thank you for always understanding and never complaining about my priorities in life, not even about my three years off on maternity-leave.

Gustav Røder, I do not know how to designate you. You have been like an extra supervisor for me, the best colleague I could ever dream of and a good friend. Thank you for taking care of me at Panum, for teaching me all the methods we used, for all valuable discussions about protocols, tapasin and HLA-I, for making all lab days and nights fun and interesting. Thank you for being so generous in all kind of ways, without you I would never be where I am today.

Søren Buus, thank you for letting me work in your lab at Panum. I highly appreciate all your input to this work.

Thank you *Bengt Widegren*, my former supervisor, for accepting me as a PhD student. Thank you for always believing in me, always supporting me and doing your best to make it work out for me. You taught me to never give up, but at a certain point I had to give up and I hope you are not too disappointed with me. You are such a warm and kind person, I wish you all the best.

Bengt Olle Bengtsson, my newest supervisor, thank you for making the effort to become my supervisor when I needed someone from the faculty of Science. Thank you for offering your help and support during the last time of my PhD period and helping me out in the mess of administrators.

Eric Warrant, thank you for always believing in me, and being so positive and helpful in all situations.

Thank you all former and present colleagues and friends at the Section for Immunology, a special thank to the members of the Antigen presentation group: *Camilla*, thank you for being such a nice room mate and helping me out with computer problems with a smile, and always google answers to my sometimes weird questions; *Elna*, thank you for valuable JCs and nice lunchroom chats; *Julia*, thank you for bringing positive energy into our group, and thank you for providing me with Chinese lunches and chocolates; *Jakob*, your ideas and enthusiasm are so inspiring, thank you. Thank you *Gudrun* for all your help with administrative things and for always being nice and understanding.

All past and present members of the Rausing Laboratory, thank you for all nice "tisdagsfikor", and a special thank to *Hans-Olov Sjögren* for helpful scientific advises, and *Leif G. Salford* for providing scientific possibilities.

I wish to express my gratitude to all colleagues at Panum, a special thanks to *Michael Rasmussen* and *Mikkel Harndahl Nord* for performing some of the experiments of this work and always being nice and helpful. Thanks also to all technicians at Panum.

Stefan Stevanovic and *Nina Hillen*, thank you for a nice time in Tübingen, and for introducing me into the world of natural peptide ligand identification.

Jag vill också tacka min familj och mina vänner utanför labbet ….

Lena och *Christine*, vår vänskap började på labbet och jag är så glad och tacksam att den har fortsatt utanför BMCs dörrar. Tack för att ni alltid lyssnar och alltid har stöttat mig. Ni är de enda av mina vänner som verkligen förstår vad denna resa har inneburit, tack för att ni alltid finns där för mig!

Lina och *Ida*, tack för att ni är så fina vänner och att era barn och män utgör en så viktig del av mitt och min familjs liv. Jag saknar verkligen våra mysiga stickkvällar med barn- och inredningssnack, snart är jag med igen.

Johanna, tack för ovärderligt umgänge under våra år som föräldralediga. Din glädje och energi är fantastisk, och tillsammans med latte och scones blev tiden under föräldraledigheten med dig alldeles underbar. Hoppas vi får uppleva en tredje omgång tillsammans .....

Tack till alla nya vänner på Annehem, ni förgyller både vardag och helger för mig och min familj. Framför allt tack till *Stina* och *Malin* för att ni alltid är så gästvänliga och mysiga att umgås med.

Ett stort tack till alla damer som har spelat och spelar i Åkarps IF, framför allt vill jag tacka mina underbara *Kalenderflickor*. Ni har utgjort en så stor och viktig del av mitt liv, vi är så olika och ändå har vi så roligt tillsammans. I er närvaro känner jag mig alltid glad, ni ger mig en otrolig distans till jobbet och det är så skönt att umgås med personer som inte kan uttala ordet disputation :) *Lotta* och *Pernilla*, tänk vad länge vi har känt varandra och hållit ihop. Jag uppskattar verkligen vår vänskap som är så enkel och djup på samma gång, hoppas att jag alltid har er vid min sida.

Tack till *Lena* och *Úlfur*, mina svärföräldrar och barnens farmor och farfar, för att ni är så okomplicerade och hjälpsamma. Jag är så glad för att vi och våra barn har en så bra relation och är innerligen tacksam för all er hjälp i vår vardag med barnen och annat. Dessutom vill jag tacka Úlfur för att du gav mig möjligheten att börja doktorera och för den hjälp du givit mig in i det sista.

Mamma, pappa och *Malin*. Tack för allt ni har gett mig i livet! Tack för att ni alltid har trott på mig och stöttat mig i alla mina val trots att ni inte riktigt förstått vad det är jag har gjort de senaste åren. Ni ställer verkligen ALLTID upp på ALLTING för mig och min familj och det värdesätter vi verkligen högt. Jag är så glad för att ni är mormor, morfar och moster till våra barn, bättre kan det ju inte bli.

Min älskade *Jóhann*, tack för all kärlek, värme och humor du tillför mitt, Viggos och Linnéas liv. Du har varit ett fantastiskt stöd under mina år som doktorand och fått utstå en del ältande, tack för att du alltid är stolt över mig vad jag än gör. *Viggo* och *Linnéa*, mina älskade barn! Ni betyder allt för mig och har gett mig en ovärderlig distans till doktorandlivet. Er kärlek och energi gör det underbart att gå upp på morgonen trots sömnlösa nätter. Nu ska ni äntligen få tillbaka er "9–14-mamma" som har tid att gå till lekplatsen och hitta på roliga saker, jag ser fram emot en underbar sommar och ett härligt liv tillsammans med er. Jag älskar er så, mina tre!

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 DOI 10.1002/eji.200939364 Eur. J. Immunol. 2009. 39: 2682–2694

European Journal of Immunology

The outermost N-terminal region of tapasin facilitates folding of major histocompatibility complex class I

Gustav Roder[1], Linda Geironson[1,2], Anna Darabi[3], Mikkel Harndahl[1], Claus Schafer-Nielsen[4], Karsten Skjødt[5], Søren Buus[1] and Kajsa Paulsson[1,3]

[1] Department of Experimental Immunology, Institute of International Health, Immunology and Microbiology, University of Copenhagen, Panum Institute, Copenhagen, Denmark
[2] The Rausing Laboratory, Division of Neurosurgery, Department of Clinical Sciences, Lund University, Lund, Sweden
[3] Immunology Section, Lund University, Lund, Sweden
[4] Schafer-N, Lersø Parkallé, Copenhagen, Denmark
[5] Department of Immunology & Microbiology, University of Southern Denmark, Odense C, Denmark

Tapasin (Tpn) is an ER chaperone that is uniquely dedicated to MHC-I biosynthesis. It binds MHC-I molecules, integrates them into peptide-loading complexes, and exerts quality control of the bound peptides; only when an "optimal peptide" is bound will the MHC-I be released and exported to the cell surface for presentation to T cells. The exact mechanisms of Tpn quality control and the criteria for being an optimal peptide are still unknown. Here, we have generated a recombinant fragment of human Tpn, $Tpn_{1–87}$ (representing the 87 N-terminal and ER-luminal amino acids of the mature Tpn protein). Using a biochemical peptide–MHC-I-binding assay, recombinant $Tpn_{1–87}$ was found to specifically facilitate peptide-dependent folding of HLA-A*0201. Furthermore, we used $Tpn_{1–87}$ to generate a monoclonal antibody, $\alpha Tpn_{1–87}$/80, specific for natural human Tpn and capable of cellular staining of ER localized Tpn. Using overlapping peptides, the epitope of $\alpha Tpn_{1–87}$/80 was located to $Tpn_{40–44}$, which maps to a surface-exposed loop on the Tpn structure. Together, these results demonstrate that the N-terminal region of Tpn can be recombinantly expressed and adopt a structure, which at least partially resembles that of WT Tpn, and that this region of Tpn features chaperone activity facilitating peptide binding of MHC-I.

Key words: Antibodies · Antigen processing · MHC-I

Supporting Information available online

Introduction

MHC class I (MHC-I, or HLA-I in humans) presents potentially immunogenic peptides to $CD8^+$ T lymphocytes. The assembly and maturation of peptide–MHC-I complexes are integrated processes that take place in the ER [1]. During the late stage maturation of MHC-I, a major proportion of the molecules interacts with the peptide-loading complex (pLC), which at least consists of TAP, tapasin (Tpn), calreticulin, and ERp57 [2]. MHC-I molecules are recruited into the pLC by Tpn, which interacts with MHC-I as well as with TAP, calreticulin and Erp57 [3–6]. Tpn is a multi-functional protein dedicated to MHC-I

Correspondence: Dr. Gustav Roder
e-mail: groder@sund.ku.dk

biosynthesis; it serves as a structural component in the pLC, as a chaperone putatively acting as an active peptide editor and MHC-I quality control mechanism [1, 7, 8], as an ER retention signal for immature MHC-I [9, 10], and as a chaperone stabilizing TAP expression and increasing TAP-performance [11–14]. Furthermore, Tpn has been found outside the ER, where it has been suggested to regulate retrograde transport of escaped immature MHC-I back to the ER from the trans-Golgi compartment [15]. Although the organization of the pLC is not completely understood, it is clear that many of the interactions between pLC components are essential for efficient MHC-I antigen presentation and that Tpn is a key component of the pLC.

The structure of Tpn bound to MHC-I has not yet been determined and no direct structural information is available showing which region(s) of Tpn interacts with which region(s) of MHC-I. The interaction site has been addressed by co-immunoprecipitation of Tpn and MHC-I. Tpn truncated at the C-terminus (deleting the transmembrane region) still co-precipitate MHC-I suggesting that the MHC-I interacting site resides within the remaining N-terminal part of Tpn [16]. In agreement, Tpn truncated at the N-terminus is unable to co-precipitate MHC-I, suggesting that the first 50 N-terminal amino acid residues of Tpn are essential for the interaction between Tpn and MHC-I [16]. Using mild protease treatment to dissect the structure of Tpn further suggested that the N-terminus consists of two ER luminal domains [17]; the first one spanning the first 85 amino acids, *i.e.* including the MHC-I interaction site previously identified by truncation studies [16].

To examine the function of the ER luminal part of Tpn, we generated a construct encoding the first N-terminal 87 amino acids of Tpn, expressed it in *Escherichia coli*, and obtained the corresponding recombinant protein, "$Tpn_{1–87}$". We show that recombinant $Tpn_{1–87}$ specifically facilitates the peptide-dependent folding of HLA-A*0201. To validate the structural integrity of the recombinant $Tpn_{1–87}$ compared with WT, full-length Tpn, we further generated a monoclonal antibody that recognized both recombinant $Tpn_{1–87}$ and WT, full-length Tpn from lysates of human cells. We conclude that recombinant $Tpn_{1–87}$ adopts – at least partially – the structure of native Tpn and that the ER-luminal domain of Tpn exerts chaperone activity.

Results

Expression of $Tpn_{1–87}$ in *E. coli*

It has previously been suggested that the first 85 N-terminal amino acids of human Tpn constitute an ER-luminal domain [17]. This domain also includes the first 50 N-terminal amino acids, which have been shown to bind to MHC-I and stabilize the pLC in the ER [18]. To study the ER-luminal part of Tpn, we cloned $Tpn_{1–87}$ into the pET28a vector and expressed it in *E. coli* (Fig. 1A and Supporting Information Fig. 1). In an attempt to increase expression level, we used an *E. coli* codon optimized construct, and in an attempt to increase solubility of the resulting protein product, we truncated the N-terminal domain at position 87 (being the first hydrophilic residues after position 85). Unfortunately, we failed to express $Tpn_{1–87}$ in several different *E. coli* expression hosts (BL21(DE3), BL21(DE3)-pLysS, and BL21(DE3)-Rosetta-Gami, data not shown). In an attempt to increase protein expression and improve protein solubility, we fused $Tpn_{1–87}$ to the *E. coli* chaperone, GrpE, a nucleotide exchange factor of the bacterial DnaK/DnaJ chaperone system [19]. GrpE has previously been predicted to be highly soluble and subsequently shown to increase the solubility of fused proteins [20, 21]. Another potential advantage of GrpE is its lack of cysteine residues which otherwise might form disulfide bonds complicating expression and folding. We inserted GrpE followed by a Factor-Xa (FXa) cleavage site upstream of $Tpn_{1–87}$ in the pET28a expression vector generating the vector pET28a/GrpE-FXa-$Tpn_{1–87}$ encoding the fusion protein GrpE-FXa-$Tpn_{1–87}$ (Fig. 1A). After IPTG induction, GrpE-FXa-$Tpn_{1–87}$ was successfully expressed in inclusion bodies of BL21(DE3) and BL21(DE3)-pLysS hosts and no expression was detected in the culture supernatants (data not shown).

Preparation of purified GrpE-FXa-$Tpn_{1–87}$

For preparative purposes, recombinant BL21(DE3) transformed with pET28a/GrpE-FXa-$Tpn_{1–87}$ were grown in a lab-scale fermentor and GrpE-FXa-$Tpn_{1–87}$ expression was induced by IPTG and the culture continued for 3 h. In SDS-PAGE analysis, a strong band appeared around 35 kDa corresponding to the expected migration of GrpE-FXa-$Tpn_{1–87}$ (Fig. 1D). Inclusion bodies containing GrpE-FXa-$Tpn_{1–87}$ were obtained by high-pressure cell disruption, washed, and extracted into urea buffer (Fig. 1D). GrpE-FXa-$Tpn_{1–87}$ was purified by column chromatography in urea-containing buffers; first by anion exchange chromatography (AEX) (Fig. 1B and D) and then by size exclusion chromatography (SEC) (Fig. 1C and D). A high-purity preparation of urea denatured GrpE-FXa-$Tpn_{1–87}$ was obtained.

To validate the primary amino acid structure of the purified GrpE-FXa-$Tpn_{1–87}$ protein, it was digested with trypsin and subsequently analyzed by peptide mass spectrometry. All detectable peptide fragments could be assigned to one of the predicted tryptic fragments of the GrpE-FXa-$Tpn_{1–87}$ protein (Supporting Information Table 1). Within the $Tpn_{1–87}$ fragments, there is one native disulfide bond: that is the intramolecular cysteine bond between positions 7 and 71. The mass spectrometry analysis detected the composite mass of the two corresponding tryptic peptide fragments, whereas no composite fragments showing inappropriate intermolecular disulfide bonds (positions 7–7, or 71–71) were found. In agreement, analysis of the purified GrpE-FXa-$Tpn_{1–87}$ protein by SDS-PAGE with or without reduction demonstrated a monomer mobility shift indicating the presence of an intramolecular disulfide bond, and no multimers indicating the absence of intermolecular disulfide bonds (data not shown). We conclude that the denatured, purified GrpE-FXa-$Tpn_{1–87}$ preparation contains intact and correctly disulfide-bonded GrpE-FXa-$Tpn_{1–87}$ (Fig. 1A). This may

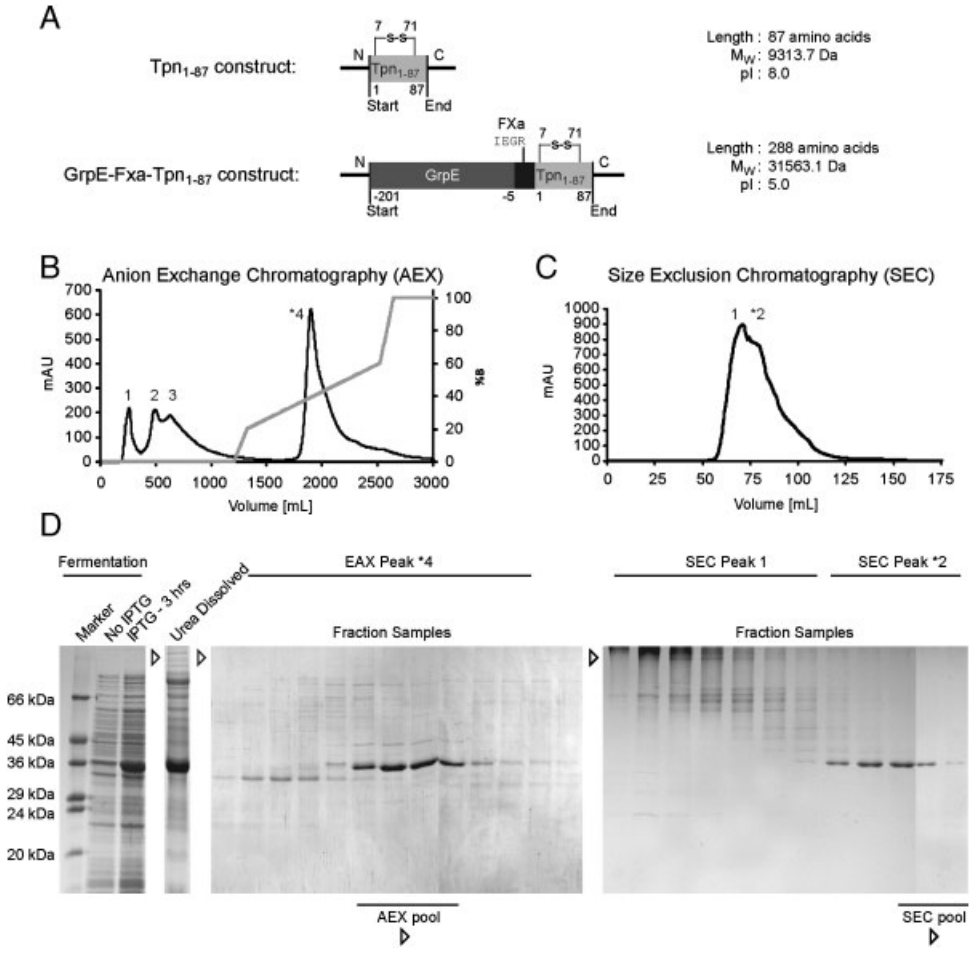

Figure 1. Production of GrpE-FXa-Tpn$_{1–87}$ (A) Schematic illustration of the Tpn$_{1–87}$ and GrpE-FXa-Tpn$_{1–87}$ constructs. Regions are not drawn to scale. Tpn$_{1–87}$ consists of the first 87 amino acids of WT Tpn thus including the native 7–71 disulfide bond. GrpE is inserted upstream of Tpn$_{1–87}$ and there is an FXa cleavage site in between. (B) Purification of recombinantly expressed GrpE-FXa-Tpn$_{1–87}$ by AEX. Urea-solubilized GrpE-FXa-Tpn$_{1–87}$ was purified by gradient segmented AEX on a Q Sepharose Fast Flow column having a volume of 600 mL. Buffer A consists of 8 M urea, pH 8.0, whereas buffer B 8 M urea, 1 M NaCl, pH 8.0. Buffer B was applied over three linear gradient segments. Application of 100% buffer B resulted in a final peak not containing GrpE-FXa-Tpn$_{1–87}$ (data not shown in chromatogram). Peak 4 contained the purified GrpE-FXa-Tpn$_{1–87}$. The AEX purification shown is a representative of multiple similar purifications. (C) Purification of AEX-purified GrpE-FXa-Tpn$_{1–87}$ by SEC. AEX-purified GrpE-FXa-Tpn$_{1–87}$ was pooled and subsequently purified by SEC on a Superdex-200 column having a volume of 200 mL. The buffer consists of 8 M urea, 150 mM NaCl, pH 8.0. Peak 2 contained the purified GrpE-FXa-Tpn$_{1–87}$. The shown SEC purification is a representative of multiple similar purifications. (D) SDS-PAGE visualization of GrpE-FXa-Tpn$_{1–87}$ expression and purification. The first SDS-PAGE shows fermentor-expressed samples lysed in Laemmli buffer. The second SDS-PAGE shows the GrpE-FXa-Tpn$_{1–87}$ exclusion bodies solubilized in 8 M urea buffer. The third and fourth SDS-PAGE are visualizations of the AEX and SEC fractions.

have important implications for the foldability upon dilution of the denatured GrpE-FXa-Tpn$_{1–87}$ as we have previously shown that denatured, but correctly disulfide bonded, molecules fold rapidly and with very high efficiency [22, 23]. *De novo* folding of pre-oxidized denatured GrpE-FXa-Tpn$_{1–87}$ might also benefit from such disulfide-assisted refolding.

GrpE-FXa-Tpn$_{1–87}$ is functionally active and facilitates folding of HLA-A*0201

Initially, we wanted to analyze the effect(s) of folded Tpn$_{1–87}$ in isolation. Denatured GrpE-FXa-Tpn$_{1–87}$ was folded by dilution into various aqueous buffers (testing various pH, salt concentrations and additives, data not shown) and then digested with FXa in an attempt to release Tpn$_{1–87}$ from GrpE. SDS-PAGE analysis revealed a soluble GrpE-FXa-Tpn$_{1–87}$ monomer. Digestion of GrpE-FXa-Tpn$_{1–87}$ with FXa led to the appearance of isolated soluble GrpE (data not shown). However, no isolated soluble Tpn$_{1–87}$ could be detected. For the remaining experiments, we have used the GrpE-FXa-Tpn$_{1–87}$ fusion protein.

The availability of denatured, pre-oxidized recombinant GrpE-FXa-Tpn$_{1–87}$ and MHC-I proteins was used to study the effect of Tpn$_{1–87}$ on folding MHC-I and peptide binding in the absence of other pLC components. The denatured molecules were diluted into a folding buffer containing β_2-microglobulin (β_2m) and peptide and the subsequent generation of peptide–MHC-I complexes was detected in a homogenous assay [24]. One

advantage of such a homogenous assay is that measurements are conducted in the presence of all assay components, *i.e.* with a minimum disturbance of the equilibrium of any interactions. To examine whether GrpE-FXa-$Tpn_{1–87}$ affected folding of MHC-I, a low concentration of HLA-A*0201 was mixed with excess concentrations of β_2m, with or without an excess concentration of GrpE-FXa-$Tpn_{1–87}$, and graded concentrations of a binding peptide. Four different HLA-A*0201-binding peptides were examined; two peptides with high affinity and two peptides with intermediate affinity for HLA-A*0201. After 48 h of incubation at 18°C, the concentrations of folded MHC-I were determined using the homogenous assay (Fig. 2 and Table 1). At the highest concentrations of all peptides, GrpE-FXa-$Tpn_{1–87}$ increased the amount of folded HLA-A*0201. To determine whether the GrpE component of the GrpE-FXa-$Tpn_{1–87}$ fusion protein was responsible for the observed effect on HLA-A*0201, we also folded HLA-A*0201 in the presence of isolated GrpE. No effect of GrpE on the folding of HLA-A*0201 was observed indicating that $Tpn_{1–87}$ is solely responsible for facilitating peptide binding. To further examine whether $Tpn_{1–87}$ was responsible for the observed effect, we generated a HLA-A*0201 mutation, which previously has been reported to disrupt Tpn interaction with MHC-I [25, 26]. In this HLA-A*0201 mutant, termed HLA-A*0201-T134K, the threonine residue in position 134 of HLA-A*0201 has been mutated to lysine. This mutation did not affect the peptide–MHC-I interaction, but it did prevent GrpE-FXa-$Tpn_{1–87}$ from facilitating peptide binding (Fig. 2A and Table 1). GrpE in itself showed no effect on peptide binding to HLA-A*0201-T134K. We conclude that $Tpn_{1–87}$ is the active part of GrpE-FXa-$Tpn_{1–87}$ affecting the folding of HLA-A*0201 and suggest that the specificity of this interaction resembles that of WT, full-length Tpn. Furthermore, the $Tpn_{1–87}$ facilitation occurred independent of the peptide affinity for HLA-A*0201.

Monoclonal antibodies specific for human $Tpn_{1–87}$

To further validate the structural integrity of $Tpn_{1–87}$, and enable future studies of Tpn in the pLC, we generated mouse monoclonal antibodies specific for $Tpn_{1–87}$. GrpE-FXa-$Tpn_{1–87}$ was folded in PBS and purified by SEC (Fig. 3A). Mice were immunized with the folded and purified GrpE-FXa-$Tpn_{1–87}$. After 3 wk, serum samples tested positive for antibodies against GrpE-FXa-$Tpn_{1–87}$ (data not shown). Subsequently, B-cells were isolated and antibody producing B-cell hybridomas were generated. Antibody secreting hybridomas were selected and cloned.

The resulting hybridoma supernatants were tested for monoclonal antibodies recognizing GrpE or GrpE-FXa-$Tpn_{1–87}$ (Fig. 3B). In a direct ELISA, 48 supernatants were found to be positive for GrpE-FXa-$Tpn_{1–87}$, and 15 of these were found to be positive for isolated GrpE (Table 2). Antibody reactivity was also tested in a proximity-based assay in solution thereby avoiding the possible denaturing effect of the immobilization inherent to ELISA (Fig. 3C and Supporting Information Fig. 2). The results of the proximity-based assay confirmed the findings of the ELISA assay.

Detection of WT Tpn by Western blot analysis

Of the 48 antibodies tested, 33 were specific for GrpE-FXa-$Tpn_{1–87}$, but not for GrpE itself. These monoclonal antibodies were of particular interest since they might be directed solely against $Tpn_{1–87}$. To address this issue, we tested the monoclonal antibodies in Western blot against WT, full-length Tpn from lysates of LCL-721.221A2 cells (Tpn positive) and LCL-721.220A2 cells (Tpn negative). All 33 GrpE-FXa-$Tpn_{1–87}$ specific antibodies recognized the same single protein band migrating to a position corresponding to WT Tpn, and this protein was only present in the Tpn positive cells (four representative samples shown and summarized in Fig. 4A and Table 2). We conclude that these antibodies recognize epitope(s) located within the $_{1–87}$ region of WT Tpn. Since the Western blot procedures are likely to denature many protein antigens and destroy discontinuous epitopes in the process, it might be suggested that most of these antibodies recognize linear epitopes.

Intracellular staining of WT Tpn

To further validate the specificity of the $Tpn_{1–87}$-specific monoclonal antibodies, they were used to visualize intracellular WT, full-length Tpn by intracellular fluorescence microscopy of permeabilized and monoclonal antibody-stained LCL-721.221A2 and LCL-721.220A2 cells. Staining was found in the Tpn positive cells, but not in Tpn negative cells (Fig. 4B). To further analyze the staining pattern, we used $\alpha Tpn_{1–87}$/80 and co-stained against ERp57, a known ER-marker protein. Nuclear staining was performed against DAPI. This showed that both the ERp57 and the $\alpha Tpn_{1–87}$/80 monoclonal antibody stained areas around the nucleus in the Tpn positive LCL-721.221A2 cells (Fig. 4B).

Immunoprecipitation of WT Tpn

All of the above assays are prone to detect denatured antigens. In contrast, immunoprecipitation is ideally suited to study antibody reactivity toward native antigens. LCL-721.221A2 and LCL-721.220A2 cells were lysed in NP40 lysis buffer and the lysates were incubated with affinity columns prepared with each of the monoclonal antibodies. Subsequently, the columns were washed extensively and eventually eluted in a high-pH buffer (pH 10.8). The eluate was neutralized and analyzed by SDS-PAGE (Fig. 4C). Of the 33 monoclonal antibodies that could recognize WT Tpn in Western blot, only one, $\alpha Tpn_{1–87}$/80, could affinity purify WT, full-length Tpn.

Mapping the $\alpha Tpn_{1–87}$/80 epitope using overlapping peptides and peptide analogs

Although the above immunoprecipitation assay suggests that $\alpha Tpn_{1–87}$/80 recognizes a surface-exposed epitope, which is

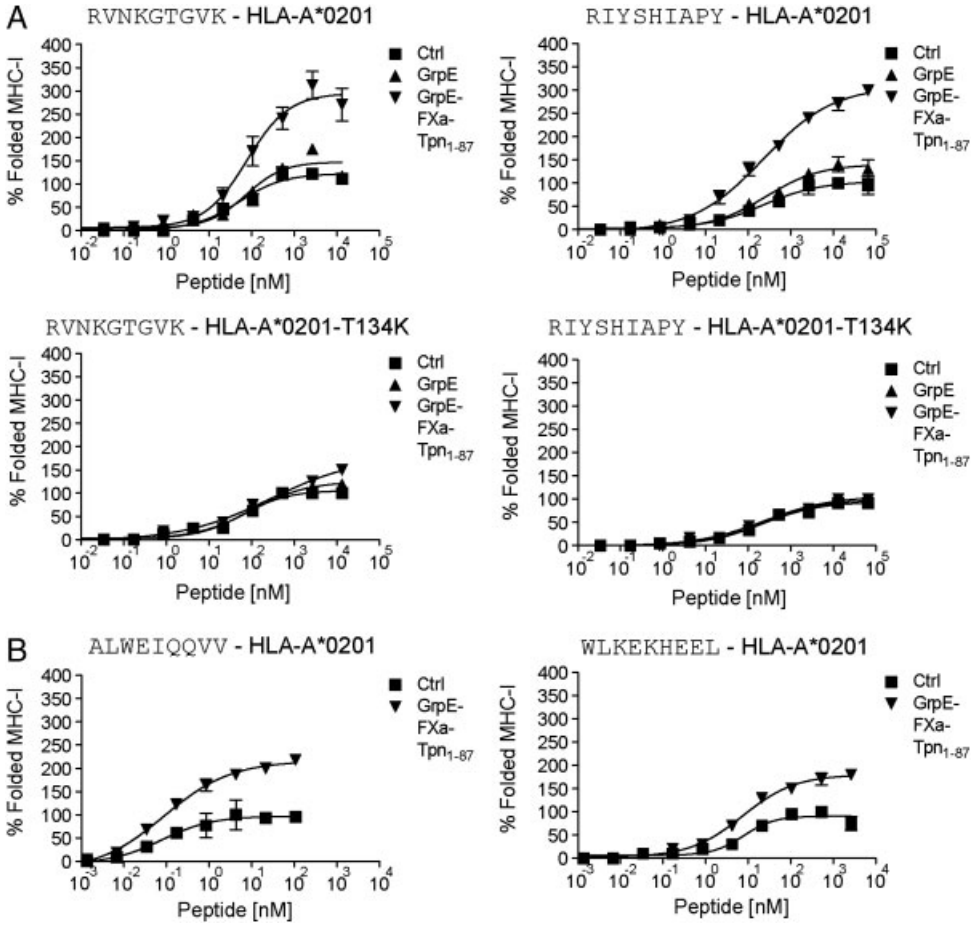

Figure 2. GrpE-FXa-$Tpn_{1–87}$, unlike isolated GrpE, facilitates folding of HLA-A*0201 (A) Folding of recombinant HLA-A*0201 or HLA-A*0201-T134K, with or without GrpE-FXa-$Tpn_{1–87}$. Graded amounts of peptide of intermediate affinity are added to HLA-A*0201 (2 nM) or HLA-A*0201-T134K (12 nM), and recombinant β_2m (30 nM) as reported previously [24]. Urea (Ctrl), 20 nM GrpE (GrpE), or 20 nM GrpE-FXa-$Tpn_{1–87}$ ($Tpn_{1–87}$) was added to the reaction and the mixture was incubated at 18°C for 48 h. Subsequently, W6/32-conjugated acceptor beads and streptavidin donor beads were added to the samples and incubated at 18°C for 18 h. The read-out was converted to % folded HLA-A*0201 complex compared with the plateau of the Ctrl experiment. All points represent data from three or more experiments and the standard deviation is given for each point. (B) Same as in (A), but with peptides of high affinity instead.

Table 1. Summary of peptide–HLA-A*0201 experiments

HLA-I allele	Peptide sequence	Ctrl EC_{50} (nM)	Tpn_{1-87} EC_{50} (nM)	GrpE EC50 (nM)	$Tpn_{1–87}$ facilitation
A*0201	ALWEIQQVV	1	1	n.d.	+
	WLKEKHEEL	8	7	n.d.	+
	RVNKGTGVK	51	61	73	+
	RIYSHIAPY	226	227	262	+
A*0201-T134K	RVNKGTGVK	48	63	60	–
	RIYSHIAPY	221	240	236	–

shared between $Tpn_{1–87}$ and WT Tpn, the fact that $\alpha Tpn_{1–87}$/80 also recognizes WT Tpn in Western blot suggests that this surface-exposed epitope might be linear. We used a peptide microarray to express and test many different peptides simultaneously (PepChip3K, Schafer-N, Copenhagen). A systematic set of 12-mer peptides with 11-mer overlap generating peptides representing $Tpn_{1–87}$ positions 1–12, 2–13, 3–14 and so forth ("sliding truncations") was used to scan through $Tpn_{1–87}$. To analyze the specificity in further detail, each position in each sliding truncation was systematically substituted with an alanine residue ("alanine scan"). The peptide microarray was incubated with protein-A-purified $\alpha Tpn_{1–87}$/80 monoclonal antibody, washed, stained with a Cy3 fluorochrome-labeled secondary goat anti-mouse-IgG antiserum. The sliding

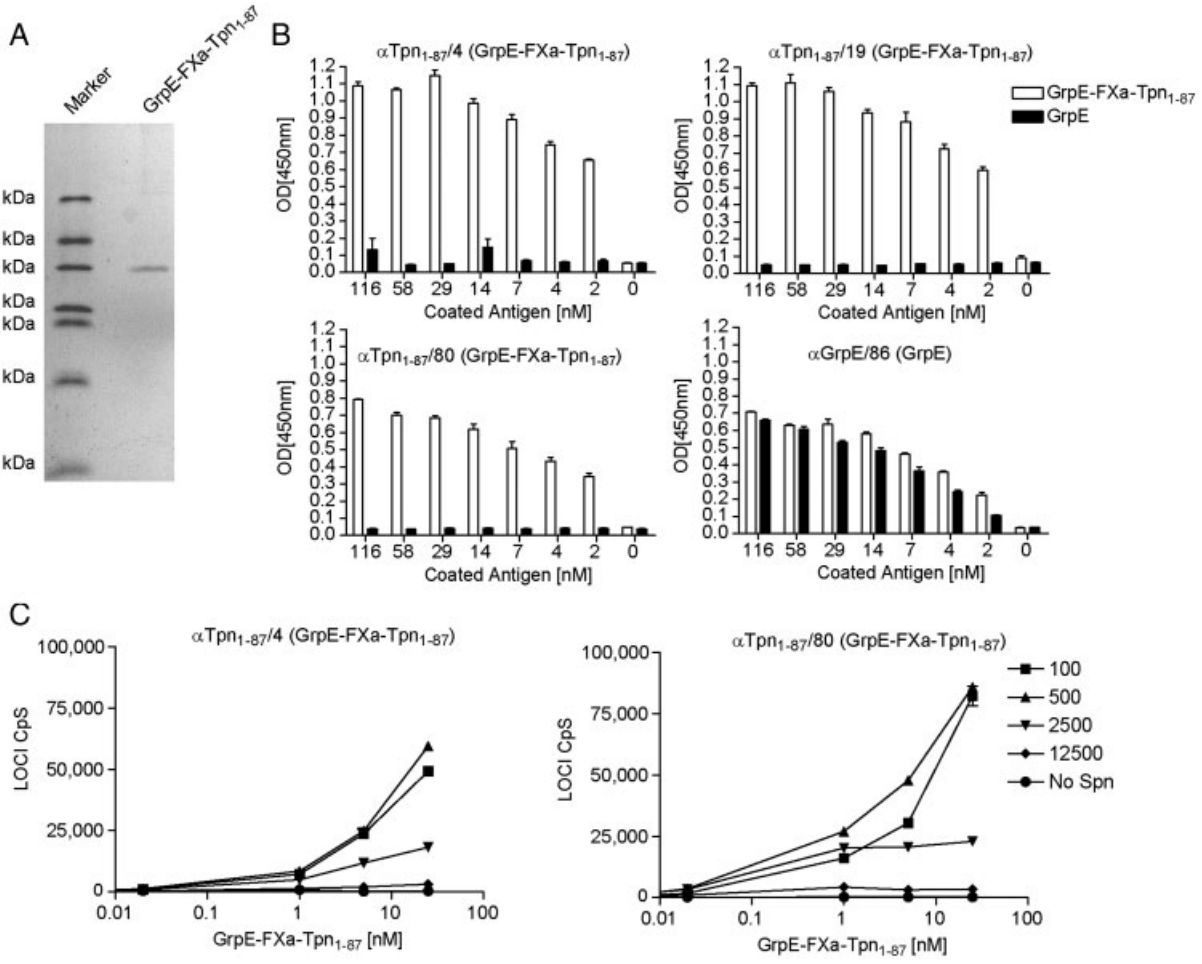

Figure 3. Generation of monoclonal antibodies against GrpE-FXa-$Tpn_{1–87}$ (A) Immunization of mice with 25 μg folded GrpE-FXa-$Tpn_{1–87}$. Mice were immunized with highly pure and folded GrpE-FXa-$Tpn_{1–87}$. (B) Three weeks post immunization B cells were extracted and fused with the SP2/0-AG14 myeloma cell line. Positive clones were selected in an ELISA assay, where coated GrpE or GrpE-FXa-$Tpn_{1–87}$ were recognized by the mouse antibodies in the hybridoma culture supernatant. Anti-mouse-IgG-HRP was used in the detection step. The results are from three independent experiments, and the error bars represent the standard deviation. (C) Graded concentrations of biotinylated BSP-GrpE-FXa-$Tpn_{1–87}$ and culture supernatant were mixed together in a matrix setup and incubated at 18°C overnight. Streptavidin-conjugated donor beads and anti-mouse-IgG-conjugated acceptor beads were used in the proximity-based assay. The results are from three independent experiments, and the error bars represent the standard deviation.

Table 2. Summary of monoclonal antibodies

Clone	Specificity	Western blot	Immunoprecipitation	Intracellular staining	PepChip3K
α$Tpn_{1-87/4}$	$Tpn_{1–87}$	wt-Tpn, $Tpn_{1–87}$	$Tpn_{1–87}$	wt-Tpn	n.d.[a)]
α$Tpn_{1-87/19}$	$Tpn_{1–87}$	wt-Tpn, $Tpn_{1–87}$	$Tpn_{1–87}$	wt-Tpn	n.d.
α$Tpn_{1-87/44}$	$Tpn_{1–87}$	wt-Tpn, $Tpn_{1–87}$	$Tpn_{1–87}$	wt-Tpn	n.d.
α$Tpn_{1-87/80}$	$Tpn_{1–87}$	wt-Tpn, $Tpn_{1–87}$	wt-Tpn, $Tpn_{1–87}$	wt-Tpn	LDPEL
αGrpE/86	GrpE	GrpE	GrpE	-	n.d.

[a)] Not determined.

truncations revealed a single strong signal peak centered on the sequence LDPEL corresponding to human Tpn residues 40–44 (Fig. 5B and Supporting Information Fig. 1D). The alanine scans supported this epitope assignment and extended the epitope to human Tpn residues 37–47, RPDLDPELYLS (Fig. 5D). The corresponding rat sequence, RPDLDPKLYFK (the species difference is underlined), was not recognized by α$Tpn_{1–87}$/80 (data not shown). One of the α$Tpn_{1–87}$/80 interacting peptides,

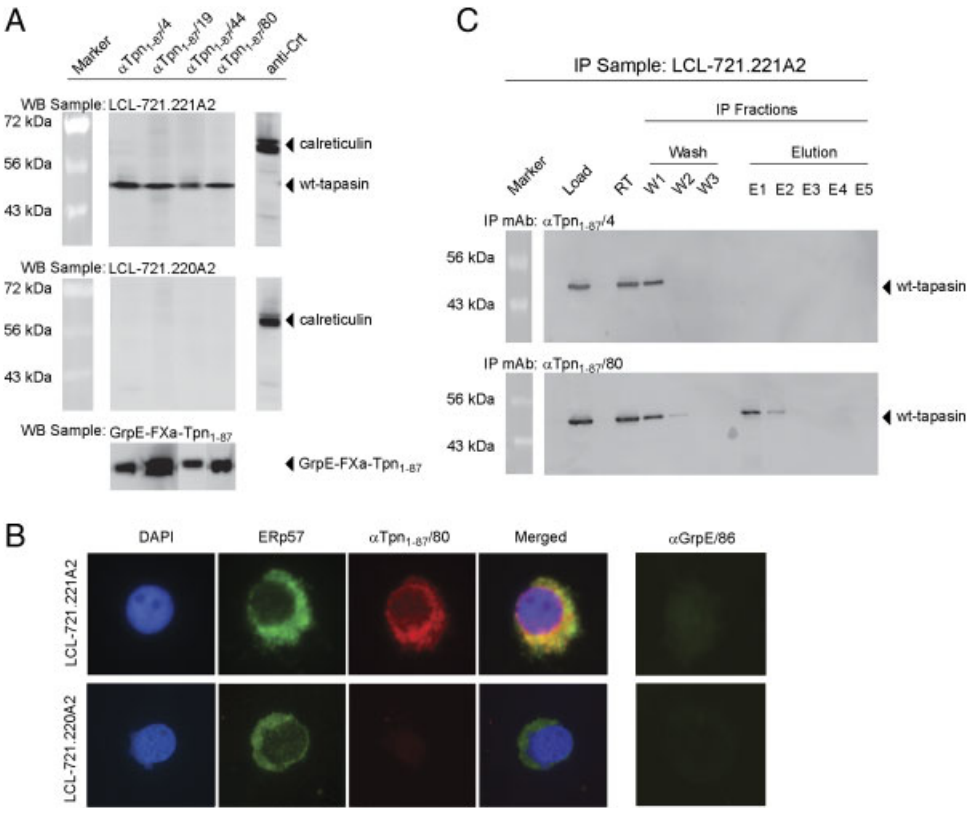

Figure 4. Western blot, intracellular staining, and immunoprecipitation of GrpE-FXa-$Tpn_{1–87}$ and WT Tpn. (A) Western blot analysis of WT Tpn. LCL-721.221A2 and LCL-721.220A2 cells lysed in 1% NP40 lysis buffer and GrpE-FXa-$Tpn_{1–87}$ in PBS were transferred onto nitrocellulose membranes and blotted with mouse anti-Calreticulin (Crt) antibody and the antibody clones as primary antibody. As secondary antibody a HRP-conjugated goat anti-mouse-IgG was applied. One big well was used in the SDS-PAGE and the Western blot was done with a channel separator device. This way, equal amounts of antigen are present for each clone tested. The Western blots are representatives of multiple independent experiments. (B) Intracellular fluorescence staining of WT Tpn. α$Tpn_{1–87}$/80 (directed toward $Tpn_{1–87}$) and αGrpE/86 (directed toward GrpE) were used as primary antibody to stain Tpn in LCL-721.221A2 and LCL-721.220A2 cells in intracellular fluorescence microscopy. Goat anti-mouse-IgG coupled with Alexa-488 was used as secondary antibody. Staining of ERp57 was done using a polyclonal antibody and nuclear staining was done against DAPI. The stainings are representatives of multiple independent experiments. (C) Immunoprecipitation of WT Tpn. The monoclonal antibody was covalently coupled to protein-A agarose and packed into a column. Briefly, 1% NP40 lysate of LCL-721.221A2 cells (lane Load) was incubated with the column matrix at 4°C overnight. The lysate was spun through the column (lane RT). Subsequently, the column was washed with ten column volumes (of 0.5% NP40 in PBS), 25 column volumes (of 0.5% NP40, 0.1% SDS in PBS), and 25 column volumes of (of 0.5% NP40 in PBS) (wash lanes). Bound sample was eluted in a high-pH buffer consisting of 50 mM diethylamine, 150 mM NaCl, pH 10.8 (elution lanes). The LCL-721.221A2 fractions were analyzed by Western blot. The immuno precipitations are representatives of multiple independent experiments.

LDPELYLSVHD, was synthesized and tested as an inhibitor in the described proximity-based assay between α$Tpn_{1–87}$/80 and recombinant GrpE-FXa-$Tpn_{1–87}$. This peptide was able to completely inhibit the interaction between α$Tpn_{1–87}$/80 and GrpE-FXa-$Tpn_{1–87}$ (Fig. 5C). The IC_{50} value of this inhibition was 3 nM suggesting a very strong binding between α$Tpn_{1–87}$/80 and GrpE-FXa-$Tpn_{1–87}$. In conclusion, α$Tpn_{1–87}$/80 recognizes a surface exposed, linear epitope consisting of RPDLDPELYLS with a LDPEL core sequence.

Discussion

We here report on the successful generation of a recombinant protein representing the N-terminal of Tpn, $Tpn_{1–87}$, and the generation of monoclonal antibodies reacting with the corresponding region of native Tpn. This region of Tpn was selected because it has been suggested to be involved in the interaction with MHC-I [18]. Only one antibody, α$Tpn_{1–87}$/80, was capable of recognizing WT, full-length Tpn by immunoprecipitation from cell lysates.

The fact that the α$Tpn_{1–87}$/80 antibody recognizes WT, full-length Tpn both in Western blot (potentially denatured) and in immunoprecipitation (probably native) suggested that it recognizes a surface-exposed, linear epitope. Using a systematic peptide library approach, we demonstrated that the α$Tpn_{1–87}$/80 antibody recognizes a core epitope encompassing Tpn residues 40–44 (LDPEL) generating an interaction that is stable enough to enable the non-equilibrium conditions of several of the detection assays. This strongly suggests that α$Tpn_{1–87}$/80 recognizes a linear epitope although we cannot rule out that the interaction also contains a minor discontinuous component. This analysis also maps the epitope of the α$Tpn_{1–87}$/80 antibody to the Tpn

structure and suggests that residues 40–44 are surface exposed. The crystal structure of Tpn was reported while this manuscript was in preparation (Fig. 5A). Indeed, the 40–44 sequence is located on a surface-exposed loop of the crystal structure (Fig. 5E). In addition, the epitopes of a few other non-immunoprecipitating αTpn_{1-87} antibodies reported here mapped to linear sequences that are not surface exposed according to the recently published crystal structure of Tpn (data not shown). It should also be noted that the surface-exposed Tpn peptide loop 40–44 is part of the proposed MHC-I interaction site.

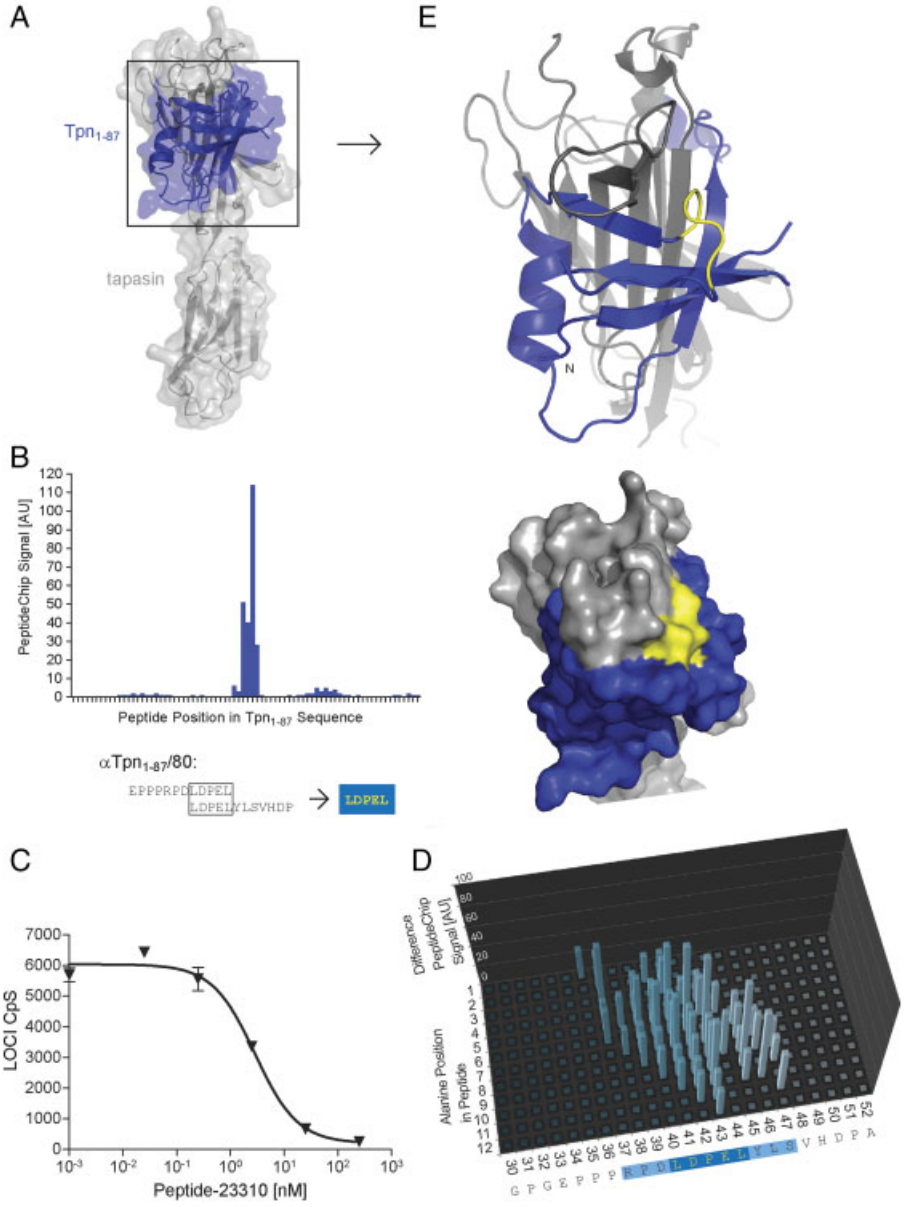

Figure 5. Mapping antibody epitopes overlapping peptide arrays (A) The structure of WT Tpn. Tpn_{1-87} (blue surface) was mapped onto the structure of WT Tpn (PDB ID:3F8U) recently determined [30]. The potential MHC-I contact interface is located inside the Tpn_{1-87} region. (B) Determination of the αTpn_{1-87}/80 epitope. Using the PepChip3K peptide array chip the linear epitope recognized by αTpn_{1-87}/80 was shown to be LDPEL corresponding to the 40–44 residues in the WT Tpn sequence. Similar results were achieved from two separate peptide array chips. (C) The peptide LDPELYLSVHD competes with GrpE-FXa-Tpn_{1-87} for binding to αTpn_{1-87}/80. Graded amounts of the 23310 peptide were added to the proximity-based assay as shown in Fig. 3C. At 2.9 nM this peptide was able to half saturate GrpE-FXa-Tpn_{1-87} binding to αTpn_{1-87}/80. All points represent three or more separate experiments and the standard deviations are shown for each point. (D) Alanine scan on the PepChip3K peptide array chip. Alanine was substituted for the natural residues at every single position in each truncation peptide on the chip. The peptide chip signal represents the difference between the natural peptide and the alanine-substituted version. Thus, a positive signal signifies the importance of the natural occurring residue at the given position in the peptide and the WT Tpn protein sequence. Similar results were achieved from two separate peptide array chips. (E) Mapping of LDPEL onto the Tpn structure shows that the epitope is surface exposed and located in loop (shown in yellow) between β strands 2 and 3. The epitope residues are surface exposed (yellow) as shown in the surface representation of Tpn_{1-87}.

Satisfied that the recombinant product at least partially resembled the corresponding part of WT, full-length Tpn, we turned to the question of whether the $Tpn_{1–87}$ component of the fusion product shared functional characteristics with WT Tpn. One of the advantages of access to recombinant reagents is that they afford more stringent control of the experimental conditions. We have developed an entirely recombinant *de novo* MHC-I refolding and assembly system. Recombinant $Tpn_{1–87}$ was added to this system to assess whether this region of Tpn affected peptide binding (Fig. 2 and Table 1). Interestingly, it appeared to facilitate peptide binding by improving the folding efficiency without changing the affinity of peptide binding. In addition, a mutant HLA-I, with a threonine to lysine substitution at position 134 known to disrupt the interaction with WT Tpn in a cellular system, could not be facilitated by the addition of recombinant $Tpn_{1–87}$. This demonstrated that recombinant $Tpn_{1–87}$ shared a functionally defined specificity with WT Tpn. As a minimum this suggests that $Tpn_{1–87}$ has a pronounced chaperone effect on MHC-I folding and assembly. Based on the limited amount of data available at this point, the $Tpn_{1–87}$ region does not appear to affect the peptide-binding affinity of MHC-I. Also, Tpn_{1-87} facilitated the folding of HLA-A*0201 with four peptides tested independent of their affinity for HLA-A*0201. Clearly, more data are needed to determine whether the $Tpn_{1–87}$ region might affect the peptide-binding specificity and/or peptide editing.

The nature of Tpn-mediated quality control of MHC-I restricted antigen processing and presentation remains to be seen. Access to ample recombinant sources of the different molecules could become instrumental in dissecting the molecular relationships. Tpn is believed to fulfill several structural and functional tasks. A commonly held view is that Tpn acts as a quality control mechanism ensuring that MHC-I molecules present stably bound peptides [1]. Here, we have generated the N-terminal, ER-luminal region Tpn, $Tpn_{1–87}$, and provided evidence that this region of Tpn interacts with MHC-I resulting in facilitated peptide binding. We speculate that this region of Tpn might support empty, open, and receptive MHC-I peptide-binding clefts effectively allowing an otherwise inherently unstable molecule to exchange peptide; *i.e.* this Tpn region might be essential for enabling peptide editing. Others have proposed that ERp57 through a disulfide bond is an integral part for the effect of Tpn on MHC-I [4]. The results presented herein would suggest that ERp57 is not absolutely needed to fulfill the chaperone function of Tpn. Whether the recombinant $Tpn_{1–87}$ will be able to address the specificity, if any, involved in Tpn-mediated peptide editing remains to be found.

Finally, we briefly wish to discuss our findings in relationship to the recently published Tpn structure. Projecting positions 1–87 onto this structure, it becomes obvious that this part of Tpn in isolation misses significant bordering parts of Tpn. This may explain why we failed to express the 1–87 fragment in isolation, and only managed to generate this Tpn fragment in association with a highly soluble fusion tag.

Materials and Methods

Cloning of GrpE-FXa-$Tpn_{1–87}$

Based on the overlapping primers, a construct was made containing a gene encoding the first 87 amino acids ($Tpn_{1–87}$) of the mature human Tpn (NCBI Protein Database, NP_003181). The gene was designed to be codon optimized for bacterial expression and was inserted into the pET28a expression vector (Novagen). The GrpE-FXa-$Tpn_{1–87}$/pET28a construct was made by inserting $Tpn_{1–87}$ into a GrpE/pET28a construct. Briefly, $Tpn_{1–87}$ was amplified by PCR using the primers "ATG-CAT-GGT-CTC-GAA-GGT-CGT-GGT-CCG-GCG-GTT-ATC-GAA-TGC-TGG" and "ATG-CAT-GGT-CTC-AAT-TAA-GAC-GCC-CAT-TTC-GCA-GAC-GC". The GrpE/pET28a construct was amplified with the primers "ATG-CAT-GGT-CTC-GTA-ATA-GCT-CGA-GCA-CCA-CCA-CCA-CCA-CC" and "ATG-CAT-GGT-CTC-GCC-TTC-GAT-CGC-TTT-TGC-TTT-CGC-TAC-AGT-TAC-C". Both PCR products were cut with *Bsa*I to create complementary sticky ends and subsequently ligated together using T4 DNA Ligase. A BirA substrate peptide (BSP) sequence was inserted upstream of GrpE in the GrpE-FXa-$Tpn_{1–87}$/pET28a construct (Supporting Information Fig. 1). All nucleotide sequences were verified by sequencing (ABI, 3100 Avant) using the primers "TAA-TAC-GAC-TCA-CTA-TAG-GG" and "GCT-AGT-TAT-TGC-TCA-GCG-G". All primers were ordered from MWG Biotech.

GrpE-FXa-$Tpn_{1–87}$ and GrpE expression and purification

$CaCl_2$ competent *E. coli* BL21(DE3) cells were transformed with the pET28 constructs. Small-scale protein expression trials using 1 mM IPTG were performed to screen for the highest expressing clone. Cellular lysates were analyzed by SDS-PAGE. Preparative-scale protein expression was done in a 2 L fermentor (Infors) by induction with 1 mM IPTG as described previously [22]. During the expression of BSP-GrpE-FXa-$Tpn_{1–87}$, 0.5 mM biotin was added and attached to the BSP site by co-expressed BirA enzyme. Inclusion bodies were obtained by cell disruption (Constant Cell Disruption Systems) and washed twice with PBS containing 0.5% v/v NonIdet-P40/DOC (Calbiochem, 492016) and dissolved in a buffer containing 8 M urea and 25 mM Tris-HCl, pH 8.0. The urea-dissolved proteins were further purified by standard chromatography techniques (GE Healthcare, Äkta FPLC) at 12°C. The protein to be purified was separated from other *E. coli* proteins by AEX using Q Sepharose Fast Flow (GE Healthcare). Buffer A contained 8 M urea and 25 mM Tris-HCl, pH 8.0, and buffer B was the same but including 1 M NaCl. Segmented gradients were applied as described in "Results". Finally, the protein sample was polished by SEC on a Superdex-200 (GE Healthcare) in a buffer containing 8 M urea, 25 mM Tris-HCl, 150 mM NaCl, pH 8.0, and subsequently stored at −20°C. Protein concentrations were determined by the bicinchoninic acid

method [27]. GrpE was purified from BL21(DE3) in PBS using the same column chromatographic techniques.

Peptide production and purification

The peptides were synthesized by conventional Fmoc chemistry and subsequently purified by reverse-phase HPLC (Schafer-N, Copenhagen). The peptide identities were verified by reverse-phase HPLC followed by ion-trap mass spectrometry (Bruker Daltonics). Their purity was determined to be higher than 90%. The PeptideChip was purchased from Schafer-N and the company carried out the epitope analysis.

Electrophoresis

One-dimensional SDS-PAGE was performed as described by Laemmli [28], by using 1-mm-thick mini gels containing 12% polyacrylamide resolving gels w/v and 5% w/v stacking gels. The SDS-PAGE were run at 180 V and 40 mA for 50 min at room temperature. Protein bands were visualized with Coomassie Brilliant Blue. Protein standards (SDS-7) were from Sigma.

MHC-I folding assay

Biotinylated recombinant HLA-A*0201 molecules were diluted from an 8 M urea buffer into a mixture of peptide and recombinant human β_2m in a Tris-Maleate buffer, pH 6.6, and added to a polypropylene 384-well plate using a liquid handling robot (Hamilton STAR). The HLA-A*0201 concentration was 2 nM, and β_2m concentration was 30 nM. In the Tpn_{1-87} positive samples 20 nM of GrpE-FXa-Tpn_{1-87} was added. For the peptide dose-titrations, eleven fivefold dilutions spanning from 66.6 to 0.01 nM were tested. The reaction mixtures were incubated at 18°C for 48 h to allow for peptide–HLA complex formation reaching steady state.

Donor and acceptor beads were from PerkinElmer (6760002, 6762001). Donor beads were obtained pre-conjugated with streptavidin, acceptor beads were in-house conjugated with the monoclonal anti-HLA-I antibody, W6/32, using standard procedures as described by the manufacturer, except that the antibody concentration was increased to 1 mg/mL during conjugation. The assay was conducted in PBS with 0.1% Pluronic F68 (BASF, 549919) as surfactant. 15 μL of the folding HLA-A*0201 reaction was transferred to 384-well OptiPlate (Perkin Elmer, 6007299) followed by addition of 15 μL of a solution containing W6/32 acceptor beads and streptavidin donor beads (final concentration of beads was 5 μg/mL). The plates were incubated at 18°C overnight. To ensure temperature equilibrium during reading time plates were placed next to the reader (EnVision™, Perkin Elmer) 1 h prior to reading. All handling of AlphaScreen reagents was performed in the dark, or in green light.

Generation of monoclonal antibodies

BALB/c × NMRI mice were subcutaneously immunized three times with 25 μg GrpE-FXa-Tpn_{1-87} adsorbed to $Al(OH)_3$, mixed in 1:1 ratio with Freunds incomplete adjuvant. Four days prior to the fusion the mice received an intravenous injection with 25 μg GrpE-FXa-Tpn_{1-87} administered with adrenaline. The fusion and selection was performed essentially as described by Kohler and Milstein [29]. The SP2/0-AG14 myeloma cell line was used as fusion partner. The experiments involving mice were approved by the National Animal Welfare Committee under the license number 2005/561-954.

IgG supernatant screening

Detection of mouse IgG performed by first coating MaxiSorb plates (Nunc, 464718) with culture supernatant at 4°C overnight. Remaining adsorption capacity was blocked with 5% skim milk in TBS-T. HRP-conjugated goat-anti-mouse-IgG (Sigma Aldrich, 9917) diluted 1:1000 was incubated at room temperature for 1 h. The ELISA was developed with 3,3′5,5′-tetramethylbenzidine hydrogen peroxide (TMB-one; Kem-En-Tec, 4380A) for 5 min at room temperature and the color reaction was read at 450 nm on a MWG Discover HT-R plate reader.

The proximity-based assay (AlphaScreen) was carried out as described in the following. Donor and acceptor beads were from PerkinElmer (6760002, 6762001). Donor beads were obtained pre-conjugated with streptavidin, acceptor beads were in-house conjugated with polyclonal goat anti-mouse-IgG using standard procedures as described by the manufacturer. Various concentrations of biotinylated BSP-GrpE-FXa-Tpn_{1-87} were incubated with various dilutions of hybridoma culture supernatant. An aliquot of 15 μL of this mixture was transferred to a 384-well OptiPlate (Perkin Elmer, 6007299) followed by addition of 15 μL of a solution containing acceptor beads and streptavidin donor beads (final concentration of beads was 10 μg/mL). The plates were incubated at 18°C overnight. To ensure temperature equilibrium during reading time plates were placed next to the reader (EnVision™, Perkin Elmer) 1 h prior to reading. All handling of AlphaScreen reagents was done in the dark, or in green light.

Western blot

The LCL-721.221A2 and LCL-721.220A2 cell lysates were obtained by lysis of 20 million cells in 1 mL of freshly prepared lysis buffer containing 1% NP40 in PBS and one protease inhibitor tablet (Roche Diagnostics, 11697498001). GrpE-FXa-Tpn_{1-87} was diluted to 5 μg/mL in PBS. The samples were diluted 1:1 in Laemmli buffer and run along with a pre-stained marker (Fermentas, SM0671) on SDS-PAGE as described in "Electrophoresis". Subsequently, the proteins were transferred to a nitrocellulose membrane (GE Healthcare, RPN303D)

using a semi-dry electro-blotter (Ancos) applying 6 V and 150 mA for 50 min at room temperature. The blot was blocked for 30 min at room temperature in 5% skim milk in TBS-T. The primary antibody solution was prepared by diluting the culture supernatants 1:4 in 2% skim milk solution. The commercial goat-anti-Tpn (Santa Cruz Biotechnology, sc-14373) and a mouse anti-calreticulin (Abcam, ab22683) antibodies were used as positive controls. The membranes were incubated with primary antibody for 1 h at room temperature and subsequently incubated with HRP-conjugated goat anti-mouse-IgG (Sigma Aldrich, 9917) diluted 1:1000 in 2% skim milk solution for 1 h at room temperature. ECL-plus reagent (GE Healthcare, RPN2132) was applied for 2 min at room temperature. The blot was scanned in a Typhoon 9410 scanner (GE Healthcare) using a fluorescence protocol with the settings: emission filter "520 BP 40 Cy2, Blue FAM", laser "Blue1, 457 nm" and a PMT of 600 V.

Immunoprecipitation

Antibody columns were made by covalently coupling the monoclonal antibody to protein-A agarose beads. Briefly, 10 mL of culture supernatant was mixed with 30 mL of binding buffer consisting of 3.3 M NaCl, 1.65 M L-glycine, 0.2 M NaOH, pH 8.85. 100 μL of washed protein-A agarose beads (Invitrogen, 15918-014) were added and the mix was incubated at 4°C overnight. The beads were washed in a buffer containing 200 mM borate, 3 M NaCl, pH 9.0. The antibody was covalently bound to protein-A by 20 mM dimethyl-pimelimidate, (Sigma Aldrich, D8388) in borate buffer for 2×30 min at room temperature. Remaining dimethyl-pimelimidate was washed away with borate buffer and the beads were washed and incubated in 200 mM ethanolamine buffer, pH 8.0, for 2 h at room temperature. Subsequently, the beads were washed with PBS, followed by 200 mM L-glycine, pH 2.5, and finally PBS.

80 million LCL-721.221A2 cells were lysed in 1 mL 1% NP40 in PBS containing one protease inhibitor cocktail tablet (Roche Diagnostics, 11697498001). GrpE-FXa-Tpn_{1-87} was diluted to 10 μg/mL in PBS. An aliquot of 100 μL sample was incubated with 65 μL column matrix at 4°C overnight. The remaining purification steps were accomplished in micro-spin-columns (Pierce, 89879). The bound sample was washed with ten column volumes of PBS (GrpE-FXa-Tpn_{1-87}) or 0.5% NP40 in PBS (LCL-721.221A2), followed by 25 column volumes of a buffer containing 0.5% NP40, 0.1% SDS in PBS, and finally by 25 column volumes of PBS (GrpE-FXa-Tpn_{1-87}) or 0.5% NP40 in PBS (LCL-721.221A2). Bound sample was eluted from the column using a buffer containing 50 mM diethylamine, 150 mM NaCl, pH 10.8. The elution was done directly into neutralizing buffer containing 1 M Tris-HCl, pH 6.2. The eluted fractions were precipitated by acetonitrile and analyzed either on silver-stained SDS-PAGE or on Western blot.

Antibody purification

Antibodies were purified from culture supernatants using protein-A agarose beads. An aliquot of 10 mL of culture supernatant was mixed with 30 mL of binding buffer consisting of 3.3 M NaCl, 1.65 M L-glycine, 0.2 M NaOH, pH 8.85. 100 μL of washed protein-A agarose beads (Invitrogen, 15918-014) were added and the mix was incubated at 4°C overnight. Subsequently, the beads were washed with binding buffer and the bound antibodies were eluted using an elution buffer containing 0.1 M acetic acid, 0.15 M NaCl. The antibodies were eluted directly into a neutralization buffer containing 0.55 M Tris-HCl, pH 8.2, and the antibodies were stored at 4°C.

Intracellular fluorescence microscopy

All cell lines were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 IU/mL penicillin, 100 mg/mL streptomycin at 37°C in a 5% CO_2 atmosphere. 10 million cells were harvested by spinning down at 1200 rpm at 4°C for 5 min and washed once with ice-cold PBS. The pellets were suspended in 100 μL of PBS containing $CaCl_2$ and $MgCl_2$. A total of 3 μL cells were spread in a single layer on glass slides and dried for 10 min. The dry cells were fixed with acetone for 10 min and washed with 0.1% saponine in PBS. The fixed cells were then permeabilized with 0.1% saponine in PBS for 5 min and blocked with 5% goat-serum for 20 min at room temperature. The slides were incubated for 2.5 h at room temperature with purified monoclonal antibody (diluted 1:5 or 2:3) and washed with PBS. The slides were incubated with secondary antibody goat-anti-mouse coupled with Alexa-488 (Molecular Probes, A-11029) at a dilution of 1:400 for 30 min in the dark at room temperature and analyzed with an OlympusBx60 microscope. All antibodies previously identified as positive for the GrpE-FXa-Tpn_{1-87} (and not GrpE alone) were analyzed in duplicate samples, on two separate occasions (four different slides for each antibody). Antibodies positive for only GrpE were analyzed in duplicates (two slides for each antibody). Two persons analyzed the slides independently. Tpn/ERp57 co-localization experiments were performed using a rabbit anti-ERp57 polyclonal antibody (Abcam, ab10287) and goat-anti-rabbit coupled with Alexa 594 (Molecular Probes, A-11037) as secondary antibody.

PepChip3K epitope mapping

5 μg/mL of the monoclonal antibody in TBO-buffer (0.15 M Tris/acetate pH 8.0, 1 g/L BSA, 1 g/L chicken ovalbumin) was applied directly onto the PepChip3K and incubated at room temperature for 1 h. The PepChip3K was washed in TBO-buffer and 5 μg/mL of Cy3-conjugated goat-anti-mouse-IgG secondary antibody (Sigma Aldrich, C2181) in TBO-buffer was added and incubated at room temperature for 1 h. Subsequently, the PepChip3K was washed in

distilled water, dried, and scanned on a Typhoon 9410 using an excitation laser wavelength of 532 nm, an emission filter of 580 nm and a PMT setting of 500 V. The image was further processed with specialized software from Schafer-N.

Acknowledgements: The authors gratefully acknowledge the expert technical assistance by Lise-Lotte Nielsen, Tasja Ebersole, Hanne Thomadsen, and Anne Schmiegelow. This work was supported by grants from the Alfred Benzon Foundation, Novo Nordisk Foundation (grant A5668-2006), Lundbeck Foundation (grant j.nr 235/05, project.nr. 64806), the Crafoord Foundation, the Swedish Medical Research Council (grant diarie nr. 2006-6500) and EU 6th framework programme (grant ♯ 503231).

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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Abbreviations: **AEX:** anion exchange chromatography · **BSP:** BirA substrate peptide · **FXa:** Factor-Xa · **β_2m:** β_2-microglobulin · **pLC:** peptide-loading complex · **SEC:** size exclusion chromatography · **Tpn:** tapasin

Full correspondence: Dr. Gustav Roder, Laboratory of Experimental Immunology, Faculty of Health Sciences, University of Copenhagen, Panum Bldg 18.3.20, Blegdamsvej 3B, DK-2200 Copenhagen, Denmark
Fax: +45-35-32-76-96
e-mail: groder@sund.ku.dk

Supporting Information for this article is available at www.wiley-vch.de/contents/jc_2040/2009/39364_s.pdf

Received: 24/2/2009
Revised: 15/5/2009
Accepted: 25/6/2009

European Journal of Immunology

Supporting Information

for

DOI 10.1002/eji.200939364

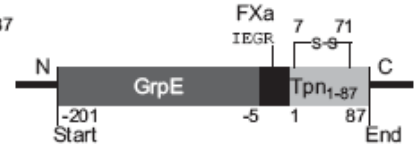
Gustav Roder, Linda Geironson, Anna Darabi, Mikkel Harndahl, Claus Schafer-Nielsen, Karsten Skjødt, Søren Buus and Kajsa Paulsson

The outermost N-terminal region of tapasin facilitates folding of major histocompatibility complex class I

Supporting Information Figure 1

a

Construct GrpE-FXa-Tpn_{1-87}



Length : 288 amino acids
M_W : 31563.1 Da
pI : 5.0

b

Protein GrpE-FXa-Tpn_{1-87}

```
MSSKEQKTPE GQAPEEIIMD QHEEIEAVEP EASAEQVDPR DEKIANLEAQ LAEAQTRERD  60
GILRVKAEME NLRRRTELDI EKAHKFALEK FINELLPVID SLDRALEVAD KANPDMSAMV 120
EGIELTLKSM LDVVRKFGVE VIAETNVPLD PNVHQAIAMV ESDDVAPGNV LGIMQKGYTL 180
NGRTIRAAMV TVAKAKAIEG RGPAVIECWF VEDASGKGLA KRPGALLLRQ GPGEPPPRPD 240
LDPELYLSVH DPAGALQAAF RRYPRGAPAP HCEMSRFVPL PASAKWAS             288
```

c

DNA GrpE-FXa-Tpn_{1-87}

```
ATG AGT AGT AAA GAA CAG AAA ACG CCT GAG GGG CAA GCC CCG GAA GAA ATT ATC ATG GAT   20
CAG CAC GAA GAG ATT GAG GCA GTT GAG CCA GAA GCT TCT GCT GAG CAG GTG GAT CCG CGC   40
GAT GAA AAA ATT GCG AAT CTC GAA GCT CAG CTG GCT GAA GCC CAG ACC CGT GAA CGT GAC   60
GGC ATT TTG CGT GTA AAA GCC GAA ATG GAA AAC CTG CGT CGT CGT ACT GAA CTG GAT ATT   80
GAA AAA GCC CAC AAA TTC GCG CTG GAG AAA TTC ATC AAC GAA TTG CTG CCG GTG ATT GAT  100
AGC CTG GAT CGT GCG CTG GAA GTG GCT GAT AAA GCT AAC CCG GAT ATG TCT GCG ATG GTT  120
GAA GGC ATT GAG CTG ACG CTG AAG TCG ATG CTG GAT GTT GTG CGT AAG TTT GGT GTT GAA  140
GTG ATC GCC GAA ACT AAC GTG CCG CTG GAC CCG AAT GTG CAT CAG GCT ATC GCA ATG GTG  160
GAA TCT GAT GAC GTT GCG CCA GGT AAC GTA CTG GGC ATT ATG CAG AAG GGT TAT ACG CTG  180
AAT GGT CGT ACG ATT CGT GCG GCG ATG GTA ACT GTA GCG AAA GCA AAA GCG ATC GAA GGT  200
CGT GGT CCG GCG GTT ATC GAA TGC TGG TTC GTT GAA GAC GCG TCT GGT AAA GGT CTG GCG  220
AAA CGT CCG GGT GCG CTG CTG CTG CGT CAG GGT CCG GGT GAA CCG CCG CCG CGT CCG GAC  240
CTG GAC CCG GAA CTG TAC CTG TCT GTT CAC GAC CCG GCG GGT GCG CTG CAG GCG GCG TTC  260
CGT CGT TAC CCG CGT GGT GCG CCG GCG CCG CAC TGC GAA ATG TCT CGT TTC GTT CCG CTG  280
CCG GCG TCT GCG AAA TGG GCG TCT TAA TAG                                         288
```

d

```
Human tapasin    1 GPAVIECWFVEDASGKGLAKRPGALLLRQGPGEPPPRPDLDPELYLSVHDPAGALQAAFRRYPRGAPAPHCEMSRFVPLPASAKWAS 87
                   ||  ||||||||| | || | |  |||| ||  |||||||||| ||  | |||| | ||||||| || ||||||||| | |||||||
Mouse tapasin    1 GPEAIECWFVEDAGGGGLSKKPATLLLRHGPRGPPPRPDLDPKLYFKVDDPAGMLLAAFRRYPAGASAPHCEMSRFIPFPASAKWAR 87
                   ||  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||||||| ||||||||||
Rat tapasin      1 GPQTIECWFVEDAGGGGLSKKPATLLLRHGPRGPPPRPDLDPKLYFKVDDPAGMLLAAFRRYPAGAHAPHCEMSSYIPFPASAKWAR 87

AAG33061  = human tapasin, mature full-length = 412 aa
AAC69893  = mouse tapasin, mature full-length = 435 aa
NP_149089 = rat tapasin,   mature full-length = 464 aa
```

The GrpE-FXa-Tpn_{1-87} construct

a) Schematic illustration of the Tpn_{1-87} and GrpE-FXa-Tpn_{1-87} constructs. Regions are not drawn to scale. Tpn_{1-87} consists of the first 87 amino acids of wild-type tapasin thus including the native 7-71

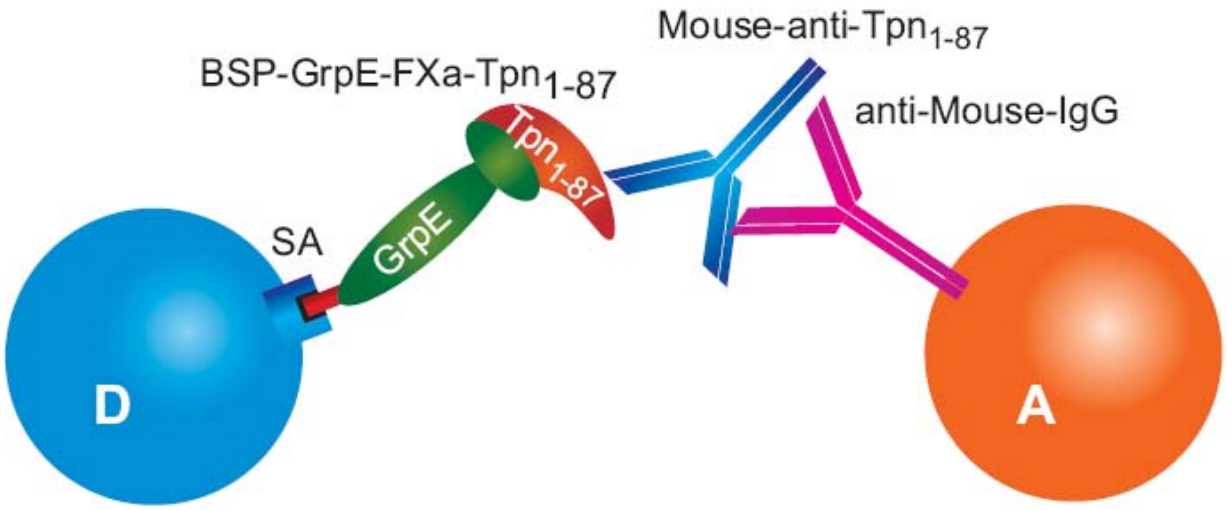
disulfide bond. GrpE is inserted upstream of Tpn_{1-87} and there is an FXa cleavage site in between. The length, molecular weight (M_W) and isoelectric point (pI) are listed to the right.

b) Amino acid sequence of GrpE-FXa-Tpn_{1-87}. GrpE is black, FXa is underlined and Tpn_{1-87} is grey.

c) DNA sequence of the *E.coli* codon optimized GrpE-FXa-Tpn_{1-87} gene in the pET28a expression vector. Same color coding as in the b-panel.

d) A comparison of the human, mouse and rat Tpn_{1-87} amino acid sequence. The vertical bars represent identical amino acids between the sequences. The αTpn_{1-87}/80 linear epitope is colored black. The mouse and rat sequences are identical across the αTpn_{1-87}/80 epitope and different from the human sequence. The GenBank entries are listed below.

Supporting Information Figure 2



Detection of GrpE-FXa-Tpn$_{1-87}$ using a proximity-based assay

The proximity-based assay is a luminescent oxygen channeling immunoassay, where the proximity of a donor and an acceptor bead is measured by light emission. In the illustrated, derived assay, the donor bead is equipped with streptavidin (SA) and the acceptor bead with an anti-Mouse IgG. The mouse anti-tapasin antibody is mixed together with biotinylated GrpE-FXa-Tpn$_{1-87}$ in the presence of both types of beads. If the mouse anti-tapasin antibody recognizes GrpE-FXa-Tpn$_{1-87}$ the two beads are in close proximity.

Supporting Information Table 1

Mass spectrometry fingerprint analysis[a]

	Theoretical			Observed		
Position	**z=1[b]**	**z=2**	**z=3**	**z=1**	**z=2**	**z=3**
-194 to -162	3673.7	1837.8	1225.6	-	1838.9	1225.8
-158 to -145	1527.8	764.9	510.3	1527.9	765.0	-
-142 to -138	573.3	-	-	573.4	-	-
-135 to -129	862.4	432.2	-	862.6	-	-
-126 to -120	847.4	424.7	-	847.6	-	-
-116 to -112	607.3	304.7	-	607.4	-	-
-111 to -98	1643.9	823.0	549.0	1644.1	-	-
-97 to -91	745.4	373.7	-	745.5	-	-
-90 to -74	1818.9	910.4	607.3	1819.0	-	-
-73 to -67	819.4	410.7	-	819.7	-	-
-65 to -26	4217.1	2109.6	1406.7	-	-	1407.3
-25 to -19	780.4	391.2	-	780.5	-	-
-15 to -8	790.4	396.2	-	790.6	-	-
-5 to -1	545.3	-	-	545.4	-	-
1 to 16 (C)[c]	1707.8	854.9	570.3	-	-	-
21 to 28	895.6	448.8	299.5	895.8	-	-
29 to 60	3410.7	1706.4	1137.9	-	1706.6	1138.3
65 to 75 (C)	1155.5	578.8	386.2	-	-	-
76 to 84	929.5	465.8	310.8	929.7	-	-
C-C[d]	2861.3	1431.7	954.8	1431.3	954.6	-

a) This table only includes the observed peptide fragments.
b) Represents the positive charges on the peptide fragments.
c) Cysteine containing peptide fragment capable of forming a disulfide bond.
d) Represents the 2 disulfide linked peptide fragments.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 286, NO. 23, pp. 20547–20557, June 10, 2011

Tapasin Discriminates Peptide-Human Leukocyte Antigen-A*02:01 Complexes Formed with Natural Ligands*[S]

Received for publication, February 17, 2011, and in revised form, April 12, 2011 Published, JBC Papers in Press, April 25, 2011, DOI 10.1074/jbc.M111.230151

Gustav Roder[‡1], Linda Geironson[§1], Michael Rasmussen[‡], Mikkel Harndahl[‡], Søren Buus[‡], and Kajsa Paulsson[‡§2]

From the [‡]Laboratory of Experimental Immunology, Institute of International Health, Immunology and Microbiology, University of Copenhagen, Panum DK-2200 Copenhagen, Denmark and the [§]Immunology Section, Department of Experimental Medical Sciences, Lund University, SE-221 84 Lund, Sweden

A plethora of peptides are generated intracellularly, and most peptide-human leukocyte antigen (HLA)-I interactions are of a transient, unproductive nature. Without a quality control mechanism, the HLA-I system would be stressed by futile attempts to present peptides not sufficient for the stable peptide-HLA-I complex formation required for long term presentation. Tapasin is thought to be central to this essential quality control, but the underlying mechanisms remain unknown. Here, we report that the N-terminal region of tapasin, Tpn_{1-87}, assisted folding of peptide-HLA-A*02:01 complexes according to the identity of the peptide. The facilitation was also specific for the identity of the HLA-I heavy chain, where it correlated to established tapasin dependence hierarchies. Two large sets of HLA-A*02:01 binding peptides, one extracted from natural HLA-I ligands from the SYFPEITHI database and one consisting of medium to high affinity non-SYFPEITHI ligands, were studied in the context of HLA-A*02:01 binding and stability. We show that the SYFPEITHI peptides induced more stable HLA-A*02:01 molecules than the other ligands, although affinities were similar. Remarkably, Tpn_{1-87} could functionally discriminate the selected SYFPEITHI peptides from the other peptide binders with high sensitivity and specificity. We suggest that this HLA-I- and peptide-specific function, together with the functions exerted by the more C-terminal parts of tapasin, are major features of tapasin-mediated HLA-I quality control. These findings are important for understanding the biogenesis of HLA-I molecules, the selection of presented T-cell epitopes, and the identification of immunogenic targets in both basic research and vaccine design.

Mature HLA-I[3] molecules are located on the surface of all nucleated cells where they present peptides to CD8+ T lymphocytes. Before the peptide-HLA-I complex is transported to the cell surface, maturation and assembly with peptides occur in the ER. During late stage maturation, HLA-I molecules are integrated in the peptide-loading complex (PLC), which at least consists of TAP1/2, tapasin, calreticulin, protein disulfide isomerase, ERp57, and HLA-I (1, 2). Integration of HLA-I into the PLC is mediated by tapasin, which structurally bridges HLA-I and TAP (3, 4). Tapasin is a multi-domain protein, which has been suggested to perform multiple functions. Tapasin has been shown to enhance peptide binding to TAP, facilitate peptide loading onto HLA-I, edit the HLA-I bound peptide repertoire, and retain and recycle suboptimally loaded peptide-HLA-I complexes (5–9). Consequently, in the absence of tapasin, cell surface-expressed HLA-I molecules are less stable and present a partly different peptide repertoire than HLA-I on wild-type cells (10–12). The effect of tapasin depends on the HLA-I allomorph where certain allomorphs, such as HLA-A*02:01, are dependent on tapasin for efficient presentation, whereas others are less influenced (10–12).

Recently, we showed that an N-terminal fragment of tapasin, Tpn_{1-87}, assisted folding of peptide-HLA-A*02:01 complexes in the absence of other PLC proteins (13). Here, we set out to identify the peptide-HLA-I targets for the tapasin quality control hidden in Tpn_{1-87} and investigate the mechanisms for the Tpn_{1-87} facilitation of HLA-I. First, we asked whether the Tpn_{1-87} facilitation would depend on the identity of HLA-I heavy chain (HC) and whether the facilitation would correlate with the known tapasin dependence, *i.e.* the differentially facilitated cell surface expression of various HLA-I molecules by tapasin (10–12). Second, with focus on HLA-A*02:01 we asked whether and how peptide identity would influence the degree of Tpn_{1-87} facilitation. Two different large sets of HLA-A*02:01 binding peptides were used: 1) peptides that have been identified as constituents of the natural HLA-I presented peptide repertoire (eluted from HLA-I and present in the SYFPEITHI database of natural HLA-I binding peptides, whereof a major proportion also been identified as T-cell epitopes (14)) and 2) other HLA-I binders, identified in an unrelated biochemical epitope screening effort (15) but not up to present date qualified to be part of the SYFPEITHI database. We also analyzed the influence of specific amino acids on HLA-A*02:01 binding

* This work was supported, in whole or in part, by National Institutes of Health Grant HHSN266200400025C. This work was also supported by the Novo Nordisk Foundation, the Lundbeck Foundation, the Wedell-Wedellsborgs Foundation, the Benzon Foundation, the Copenhagen Cluster of Immunology, Swedish Medical Research Council Grant 2006-6500, the Crafoord Foundation, the Royal Physiographic Society in Lund, and the Thelma Zoegas, Magnus Bergvalls, Groshinskys, and Greta and Johan Kocks Foundations.

[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3 and Figs. S1–S5.

[1] Both authors contributed equally to this work.

[2] To whom correspondence should be addressed: Immunology Section, BMC-D14, Department of Experimental Medical Sciences, Lund University, SE-221 84 Lund, Sweden. Tel.: 46-46-222-4167; E-mail: kajsa_m.paulsson@med.lu.se.

[3] The abbreviations used are: HLA, human leukocyte antigen; MHC, major histocompatibility complex; PLC, peptide loading complex; TAP, transporter associated with antigen processing; HC, heavy chain; β2m, β2-microglobulin; ER, endoplasmic reticulum; PSCPL, positional scanning combinatorial peptide libraries; RB, relative binding; ROC, receiver operating curve; AUC, area under the curve; Ctrl, control.

using positional scanning combinatorial peptide libraries (PSCPL), each with only one fixed amino acid at a certain position and the rest of the positions with random amino acids.

The findings in this paper show that the effect of Tpn_{1-87} varies with the identity of the HLA-I molecule in a manner that correlates perfectly with the described tapasin dependence of different HLA-I molecules (10–12). We also show that Tpn_{1-87} recognizes and facilitates the folding of HLA-A*02:01-peptide complexes of low intrinsic stability but does not change the peptide binding function in a way that affects the specificity. Finally, the data show that the degree of Tpn_{1-87} facilitation (*i.e.* increased HLA-I B_{max} Tpn_{1-87}/Ctrl) with high specificity and sensitivity separates HLA-A*02:01 SYFPEITHI ligands from other HLA-A*02:01 peptide binders. Based on these results, we would ascribe Tpn_{1-87} independency as a striking discriminator of natural ligands to other HLA-A*02:01 binding peptides of similar affinity. This provides a tool with high potential for improved predictions of immunogenicity of peptides. We propose that the chaperone function located in the N-terminal part of tapasin, together with a possible peptide editing function, involving tapasin Cys-95 and with retention/recycling of immature MHC-I molecules mediated by the C-terminal double lysine motif of tapasin, equip the multifunctional tapasin with a unique set of tools to assist in the MHC-I quality control and shape the resulting repertoire of presented CD8+ T-cell epitopes.

EXPERIMENTAL PROCEDURES

Peptide Synthesis—All of the peptides were purchased from Schafer-N (Copenhagen, Denmark) with a purity of 95% or higher. The HLA-I peptide panels were designed to be HLA-I binders. In each panel, some of the peptide sequences were derived from the SYFPEITHI database, which represent immunogenic peptides that bind stably to HLA-I and get presented on the cell surface (14). The non-SYFPEITHI peptide sequences did not exist in the SYFPEITHI database and have not been found to be immunogenic. The peptide sequences used in this work are listed in supplemental Table S1.

Positional Scanning Combinatorial Peptide Libraries—PSCPLs were purchased from Schafer-N (Copenhagen, Denmark). We used a previously reported method capable of determining the HLA-I peptide binding specificity using PSCPLs (17). In brief, peptides are synthesized with completely random amino acids at all positions in the peptide except at a chosen position. This position is then substituted with a fixed amino acid. Consequently, a complete PSCPL 9-mer peptide library would have 20 amino acid substitutions for each position and systematically address all nine positions of a 9-mer peptide resulting in 180 unique sublibraries. Relative binding (RB) values are defined as X_9 = EC_{50}/sublibrary EC_{50}, the ratio between the affinity of the completely random library with no fixed amino acid residues and the affinity of the specific sublibrary. Amino acid substitutions leading to RB values below 0.5 are considered disfavored, whereas substitutions leading to RB values above 2.0 are considered favored. All of the experiments were done four times, and Student's *t* tests were used to determine significant differences between RB values in the absence and presence of Tpn_{1-87}.

Protein Production—The recombinant GrpE-FXa-Tpn_{1-87} protein, here termed Tpn_{1-87}, was produced as described previously (13). Briefly, a gene encoding the Factor Xa cleavage site, FXa, and the first 87 amino acids of the mature human tapasin (NP_003181) was inserted into a GrpE/pET28a vector. Expression of Tpn_{1-87} was done in *Escherichia coli* BL21(DE3) cells as described previously (35). Urea-dissolved Tpn_{1-87} was purified by anion exchange and size exclusion chromatography (GE Healthcare, Äkta FPLC). HLA-I HCs and human β_2-microglobulin (β_2m) were produced as described previously (35). HLA-I HC and GrpE-FXa-Tpn_{1-87} was stored individually in 8 M urea at −20 °C until use.

HLA-I Folding Assay—The peptide-HLA-I folding assays were done as described previously (13, 36). Briefly, 2 nM of biotinylated recombinant HLA-I HC was diluted into a mixture of peptide and 30 nM recombinant human β_2m in the absence or presence of 20 nM Tpn_{1-87} in PBS containing 50 mM Tris and maleic acid, pH 6.6. The reaction mixtures were incubated at 18 °C for 48 h to allow the peptide-HLA-I complex formation to reach steady state. Detection of folded peptide-HLA-I complexes was done by adding 15 μl of the folding reaction to 15 μl of PBS containing 10 μg/ml each of AlphaScreen donor beads (PerkinElmer Life Sciences, 6760002; conjugated with streptavidin) and Acceptor beads (PerkinElmer Life Sciences, 6762001; in-house conjugated with W6/32, a conformation-specific monoclonal anti-HLA-I antibody (37)). The peptide-HLA-I complex formation allows for a proximity-based signal transfer between the donor and acceptor beads. The plates were incubated at 18 °C overnight and then equilibrated for 1 h to reader temperature and read (EnVision™; PerkinElmer Life Sciences). The conversion of AlphaScreen signal to peptide-HLA-I complex concentration was done using a preformed peptide-HLA-A*02:01 complex as standard. All of the experiments were done in duplicate on the same day and repeated on separate days, and standard deviations for each folding reaction were calculated and visualized in the graphs. Tpn_{1-87}-mediated folding facilitation was defined as the ratio between the maximum concentrations of folded peptide-HLA-I complexes obtained in the presence or absence of Tpn_{1-87} (Tpn_{1-87} B_{max}/Ctrl B_{max}). Student's *t* test was used to determine whether the means were significantly different.

HLA-I Stability Assay—The assay has been described elsewhere (38). Briefly, 50 nM biotinylated HLA-A*02:01 HC, ^{125}I-labeled β_2m (final specific activity of 250 cpm/μl), and 1 μM of a binding peptide was folded in the presence or absence of 500 nM Tpn_{1-87} in PBS containing 50 mM Tris and maleic acid, pH 6.6. The folding reactions were incubated in a streptavidin-coated FlashPlate (PerkinElmer Life Sciences, SMP103) at 18 °C for 24 h. Dissociation of the peptide-HLA-I complex was initiated by adding 1 μM unlabeled β_2m and incubating the plate at 37 °C. HLA-I-bound ^{125}I-labeled β_2m was continuously read at 37 °C (TopCount NXT; PerkinElmer Life Sciences). All of the experiments were done at least two times with double setups. The half-lives were calculated from the dissociation curves using the exponential decay equation in Prism 5 (GraphPad).

Statistics—The AlphaScreen peptide dose-response experiments were done two times on separate days with fresh stock material. Each experiment was done in duplicate using the

same stock material. Sigmoidal curve graphs were calculated from all of the data belonging to the same peptide-HLA-I experiment. The curves are calculated based on the mean values, and standard deviations are shown in both vertical directions. Only peptide-HLA-I experiments from which saturated sigmoidal curves could be calculated are included in this work. To determine the underlying mechanism of Tpn_{1-87} facilitation, the ability of the B_{max}, EC_{50}, peptide-HLA-I stability, and Tpn_{1-87} facilitation parameters to select between SYFPEITHI and non-SYFPEITHI peptides was measured using receiver operating characteristic (ROC) statistics. Here, a ROC curve was constructed from which the ability of the parameters to select with high sensitivity and specificity is demonstrated by the area under the curve (AUC). An excellent parameter demonstrates an AUC close to 1. To determine any significant differences between the AUCs, a jack knife-based ROC analysis was made in which the entire data set was partioned, and different partitions were included in different rounds of the ROC analysis, resulting in different AUCs for the same parameter. The AUCs were averaged, and a Student's *t* test was applied to determine statistical differences between the parameter AUCs.

RESULTS

Tpn_{1-87} Facilitates Folding According to Both HLA-I HC and Peptide Identity—Efficient antigen presentation of different HLA-I molecules differentially depends on tapasin, so that some HLA-I molecules are expressed at the cell surface at normal levels in the absence of tapasin, whereas the expression of other HLA-I molecules is dramatically decreased. In addition, not only the quantity but also the quality of these peptide-HLA-I complexes is altered (11). The reason behind these differences in tapasin dependence is not known.

We recently observed that Tpn_{1-87} facilitates folding of HLA-A*02:01, but not the tapasin-independent HLA-A*02:01-T134K (13), suggesting that Tpn_{1-87} facilitation may be coupled to the tapasin dependence for various HLA-I molecules. We therefore produced recombinant versions of HLA-B*27:05, HLA-A*02:01, HLA-A*02:01-T134K, HLA-B*08:01, and HLA-B*44:02 and studied their folding in the presence and absence of Tpn_{1-87}. These HLA-I molecules cover a wide spectrum of tapasin dependence as defined by decreased cell surface expression in the absence of tapasin (10, 12). Studies took place under equilibrium conditions, and titrated amounts of peptides were offered to the folding HLA-I complexes in the presence or absence of Tpn_{1-87}. The resulting formation of peptide-HLA-I complexes and the maximum obtainable concentration of folded peptide-HLA-I complexes (B_{max}) were initially determined for a few peptide-HLA-I combinations (Fig. 1*A* and supplemental Fig. S1) and then extended to larger allomorph-specific peptide sets (Fig. 1*B*). The analysis showed that the folding of the peptide-HLA-I complexes was differentially facilitated by Tpn_{1-87} according to the identity of the HLA-I HC (Fig. 1*B*). Underlining the relevance of our *in vitro* system, the results were in perfect accordance with previous studies of tapasin in cellular models: the facilitation was very pronounced for HLA-B*44:02, intermediate for HLA-B*08:01 and HLA-A*02:01, and very low or absent for the HLA-B*27:05 and HLA-A*02:01-T134K molecules (10, 12).

These experiments also showed that for the tapasin-dependent allomorphs, there was a large variation of tapasin facilitation that seemed to depend on the different peptides. For a more extensive study of the relation of Tpn_{1-87} facilitation to peptide identity, we chose to study HLA-A*02:01 in detail and used a panel of 88 HLA-A*02:01-specific peptides. In contrast to many other HLA allele families, HLA-A2 is frequent in all studied ethnic groups, making it a strong candidate for development of peptide-based vaccines. HLA-A*02:01 is both the most prominent member of the HLA-A2 family and one of the most examined HLA-I molecules; hence many of the known HLA-A*02:01-restricted peptides are registered in the SYFPEITHI database of naturally occurring ligands. We have also previously identified a large body of HLA-A*02:01 binding peptides that are not in the SYFPEITHI database (termed non-SYFPEITHI peptides) and have not yet been observed to be presented on the cell surface as T-cell epitopes (15, 16). We examined folding with 44 SYFPEITHI and 44 non-SYFPEITHI peptides. Tpn_{1-87} facilitated folding of HLA-A*02:01 with SYFPEITHI peptides to a strikingly lesser degree than with non-SYFPEITHI peptides (Fig. 1*C*). As expected, the folding of the mutant HLA-A*02:01-T134K, which cannot interact with tapasin, was not facilitated with either the studied SYFPEITHI or non-SYFPEITHI peptides. Together, these results suggested that both the identity of the HLA-I HC and the peptide determines the degree of Tpn_{1-87} facilitation.

To clarify whether the difference between SYFPEITHI and non-SYFPEITHI peptides in terms of Tpn_{1-87} facilitation could also be observed for other HLA-I molecules, we analyzed the binding of SYFPEITHI and non-SYFPEITHI peptides restricted to HLA-B*08:01, HLA-B*27:05, or HLA-B*44:02 (Fig. 1*D*). The folding of HLA-B*08:01 resembled folding of HLA-A*02:01 because folding with SYFPEITHI peptides was less facilitated than folding with non-SYFPEITHI peptides. In contrast, the tapasin-independent HLA-B*27:05 resembled HLA-A*02:01-T134K and was not or was only slightly facilitated irrespective of peptide identity. HLA-B*44:02 is by far the most tapasin-dependent allomorph and showed a large Tpn_{1-87} facilitation of folding with both SYFPEITHI and non-SYFPEITHI peptides.

Tpn_{1-87} Increases the Binding Affinity of Natural Ligands for HLA-A02:01—In an attempt to explain the facilitation mechanism, we tested whether Tpn_{1-87} would alter the peptide binding affinity to HLA-I. Therefore, we calculated the affinities from the folding of multiple peptide-HLA-I combinations in peptide dose-response experiments in the absence or presence of Tpn_{1-87}. For a majority of peptide-HLA-I complexes containing HLA-B*08:01, HLA-B*44:02, and HLA-B*27:05 molecules, Tpn_{1-87} decreased the affinity (increased EC_{50}) of the peptide-HLA-I interaction (Fig. 2*A*). Although there seemed to be a small average decrease also in HLA-A*02:01-peptide affinity, the situation was more complex *per se*, because Tpn_{1-87} also increased the affinity of a large number of peptide-HLA-A*02:01 complexes (Fig. 2*A*). A closer analysis showed that the peptides with increased affinity in the presence of Tpn_{1-87} to a large extent were contributed from the SYFPEITHI group, whereas the peptides with decreased affinities were predominantly non-SYFPEITHI peptides (Fig. 2, *B* and *C*). The affinities of the same HLA-A*02:01 binding peptides on the HLA-A*02:

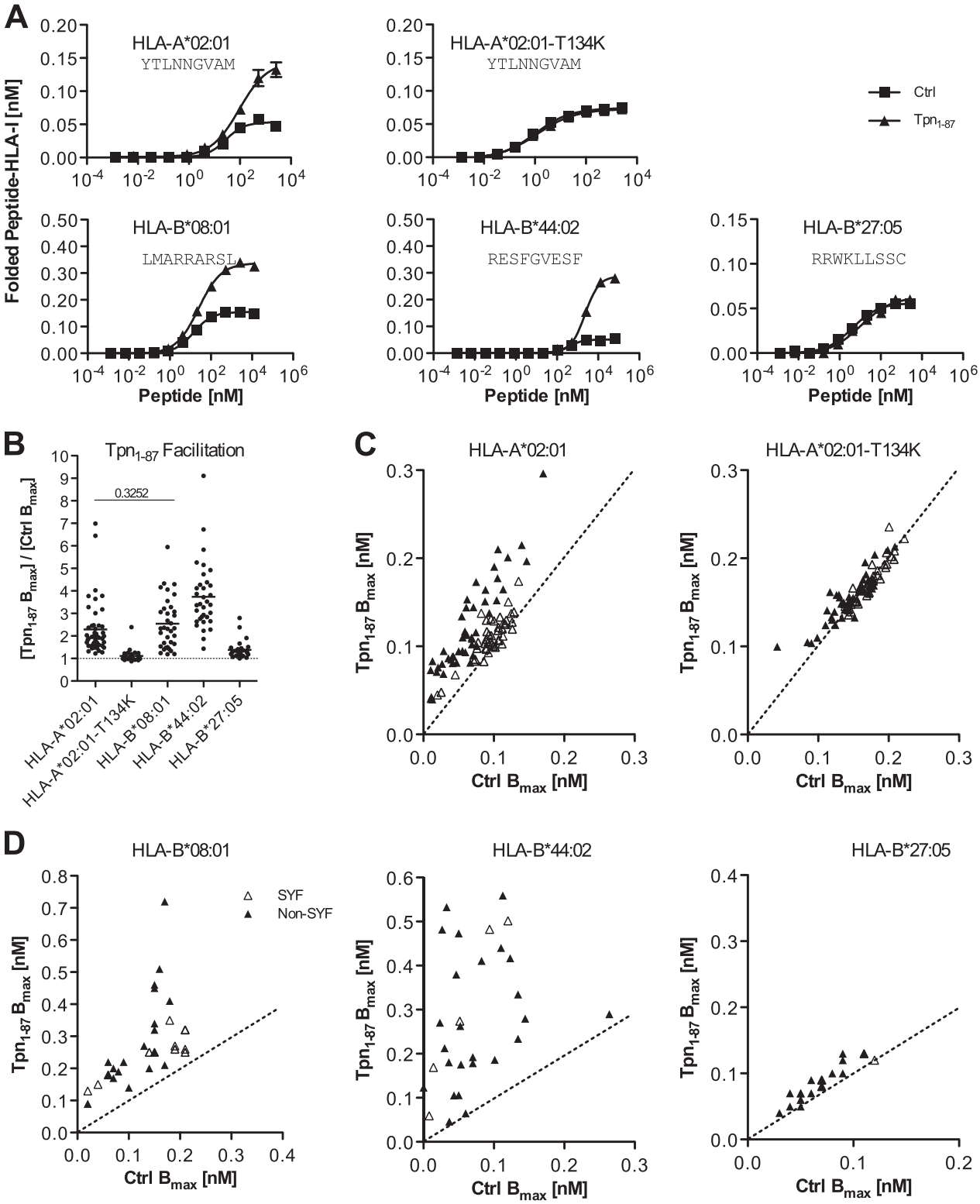


FIGURE 1. **Tpn_{1-87} facilitates folding of peptide-HLA-I complexes according to peptide and HLA-I identity.** *A*, folding of peptide-HLA-I complexes in single peptide dose-response experiments. Fixed concentrations of β_2m and HLA-I HCs were mixed with titrated concentrations of peptide in the presence (▲) or absence (■) of Tpn_{1-87}. The mixtures were incubated at 18 °C for 48 h, and folded peptide-HLA-I complexes were detected by the HLA-I conformation-specific W6/32 monoclonal antibody in a homogenous assay (36). *B*, a study of Tpn_{1-87} facilitation based on the maximum amount of folded peptide-HLA-I complexes, B_{max}. Peptide dose-response curves were made by offering each peptide in different concentrations to the folding reaction. The saturation plateaus were calculated as B_{max} from the curves. Binding curves were made in the presence (Tpn_{1-87} B_{max}) and absence (Ctrl B_{max}) of Tpn_{1-87} with SYFPEITHI (△) and non-SYFPEITHI (▲) peptides. *B*, the degree of Tpn_{1-87} facilitation for each of the tested HLA-I molecules is shown. *C*, the B_{max} values with and without Tpn_{1-87} for the binding of each of the tested peptides to HLA-A*02:01 and HLA-A*02:01-T134K are plotted. *D*, the B_{max} values for the binding of peptides specific for HLA-B*44:02, -B*08:01, and -B*27:05 with and without Tpn_{1-87} are plotted. All of the experiments were done in quadruplicate, and standard deviations for each folding reaction were calculated and visualized in the graphs. A Student's *t* test was applied to determine whether the means were significantly different. All of the means were significantly different ($p < 0.001$), except for the one with the *p* value shown in the graph.

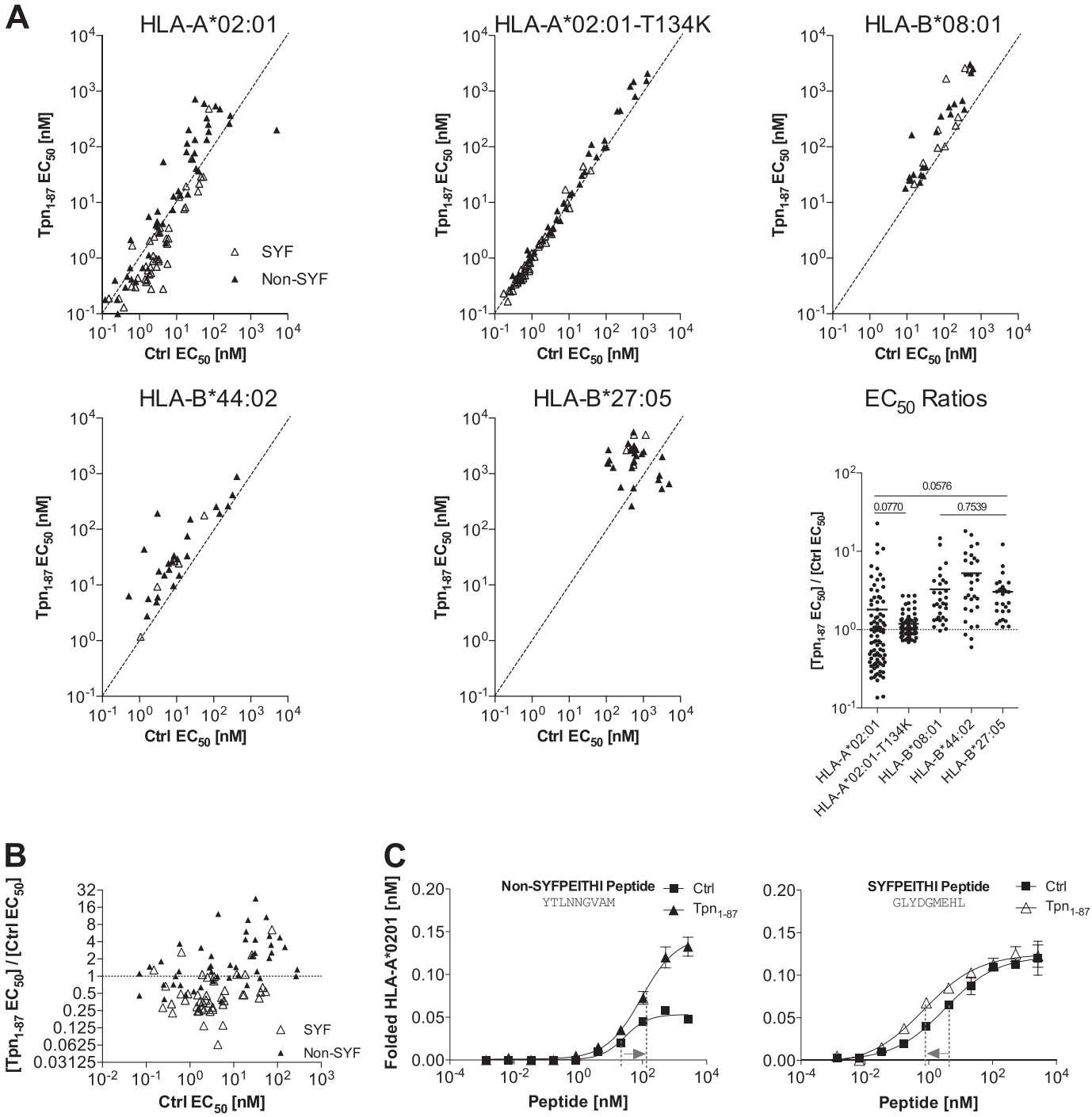


FIGURE 2. **Tpn_{1-87} influences the peptide binding affinity to HLA-I.** *A*, Tpn_{1-87} influences the peptide affinity to HLA-I. Multiple binding curves were made, and the peptide concentration resulting in half-saturation was calculated as EC_{50}. Binding curves were made in the presence (Tpn_{1-87} EC_{50}) and absence (Ctrl EC_{50}) of Tpn_{1-87}. 44 SYFPEITHI (△) and 44 non-SYFPEITHI (▲) peptides were tested on HLA-A*02:01. The same peptides were tested on HLA-A*02:01-T134K. Other peptide panels were tested on HLA-B*08:01, HLA-B*44:02, and HLA-B*27:05. The EC_{50} ratios (Tpn_{1-87} EC_{50}/Ctrl EC_{50}) are shown in the EC_{50} ratios graph. A Student's *t* test was used to determine whether the means were significantly different ($p < 0.05$) between the HLA-I molecules. The *p* values are shown in cases where no significant differences were found (for a complete list see supplemental Table S2). *B*, the EC_{50} ratios on HLA-A*02:01 were plotted against Ctrl EC_{50} and grouped in non-SYFPEITHI and SYFPEITHI peptide groups. *C*, peptide dose-response curves representing the non-SYFPEITHI and SYFPEITHI peptide-HLA-A*02:01 complexes were analyzed for shifts in EC_{50}. For the non-SYFPEITHI peptide, the EC_{50} values increased in the presence of Tpn_{1-87} corresponding to a decrease in affinity. For the SYFPEITHI peptide, the EC_{50} values decreased in the presence of Tpn_{1-87}, corresponding to an increase in affinity.

01-T134K mutant were not influenced by Tpn_{1-87} (Fig. 2*A*), correlating this effect on affinity to tapasin dependence (18).

Tpn_{1-87} Discriminates Affinity-paired Natural and Non-natural Peptides—Although Tpn_{1-87} increased the binding affinity of SYFPEITHI peptides to HLA-A*02:01, the number of the HLA-A*02:01 complexes folded with SYFPEITHI peptides were not at all or only slightly increased by Tpn_{1-87} (*i.e.* Tpn_{1-87} facilitation), whereas those with non-SYFPEITHI peptides in general were much more facilitated (Fig. 1*B*). We next set out to determine whether the effects of Tpn_{1-87} on SYFPEI-

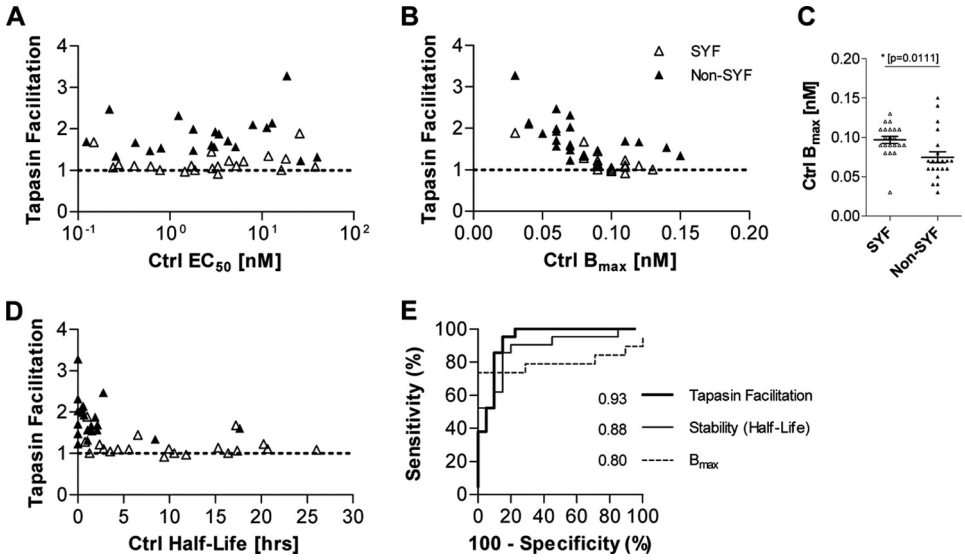


FIGURE 3. **Tpn_{1-87} facilitates folding and discriminates immunogenic peptides independent of peptide affinity to HLA-A*02:01.** 21 SYFPEITHI and 21 non-SYFPEITHI peptides were paired, based upon affinity to HLA-A*02:01. Fixed concentrations of β_2m and HLA-A*02:01 HC were mixed with various concentrations of peptide in the presence or absence of Tpn_{1-87}. *A*, the peptide affinities (EC_{50}) to the HLA-I molecules were calculated as the peptide concentration required to reach the half-saturation point on the sigmoidal dose-response curve. The Tpn_{1-87} facilitation was plotted against EC_{50}. *B*, the Tpn_{1-87} facilitation was plotted against the saturation plateau, B_{max}. *C*, the B_{max} values for the SYFPEITHI and non-SYFPEITHI peptides in the absence of Tpn_{1-87} were plotted in a vertical scatter diagram. *D*, the Tpn_{1-87} facilitation was plotted against measured stabilities of the peptide-HLA-A*02:01. *E*, ROC analysis was performed for the ability of each parameter (B_{max}, stability, and Tpn_{1-87} facilitation) to discriminate between SYFPEITHI peptides and non-SYFPEITHI peptides. The AUC values are shown. To determine whether significant differences exist between the areas under the ROC curves, a jack knife analysis was performed on the ROC areas. A Student's *t* test was used to determine statistically significant differences ($p < 0.05$) between the parameters tested. All of the AUCs differed significantly in the *t* test. ***, $p < 0.0001$.

THI *versus* non-SYFPEITHI peptide-HLA-I complexes reflected differences in peptide-HLA-I binding affinities or not. To this end, we analyzed the Tpn_{1-87} facilitation using a selection of SYFPEITHI and non-SYFPEITHI peptides, which had been paired according to affinity to HLA-A*02:01, thus eliminating peptide affinity differences between SYFPEITHI and non-SYFPEITHI peptides. Strikingly, the Tpn_{1-87} facilitation was much higher for non-SYFPEITHI peptides than for SYFPEITHI peptides, even though there was no difference in affinity, and the two peptide groups were clearly defined based on the Tpn_{1-87} facilitation (Fig. 3*A*). Hence, the degree of Tpn_{1-87} facilitation did not depend on the peptide affinity to HLA-A*02:01, at least not for the high affinity binding interactions studied here. These results also suggested that Tpn_{1-87} is able to distinguish natural ligands from other binders in a manner not dependent on peptide affinity.

Tpn_{1-87} Does Not Facilitate Folding of HLA-I Complexes with SYFPEITHI Peptides—Because peptide-HLA-I affinity could not explain the Tpn_{1-87}-based discrimination of SYFPEITHI from non-SYFPEITHI peptides, some other mechanisms must exist. We observed that each peptide-HLA-I combination is unique regarding the maximum concentration of folded peptide-HLA-I complex obtainable (Ctrl B_{max}) (Fig. 1*B*). Consequently, we used the affinity-paired peptide panel to analyze whether the Tpn_{1-87} facilitation correlated with Ctrl B_{max}. The resulting Tpn_{1-87} facilitation inversely correlated with the maximum concentration of folded peptide-HLA-I complexes, suggesting that Tpn_{1-87} in general facilitated the folding of HLA-A*02:01 with peptides otherwise unable to efficiently support folding (low Ctrl B_{max}; Fig. 3*B*) and that SYFPEITHI peptides could be characterized as being able to support HLA-I folding (exhibiting a high Ctrl B_{max}) with little or no direct need for Tpn_{1-87} (Fig. 3*C*). For the mutant HLA-A*02:01-T134K, which is known to be unable to interact with tapasin, the SYFPEITHI peptides gave a slightly higher B_{max} than the non-SYFPEITHI peptides (supplemental Fig. S5*A*), but folding of T134K was not assisted by Tpn_{1-87}, and there was naturally no correlation between Tpn_{1-87} facilitation with neither affinity nor B_{max} (supplemental Fig. S5, *B* and *C*).

Another parameter that might characterize peptide HLA-I complexes involving SYFPEITHI peptides is peptide-HLA-I stability, which has been suggested to be a better indicator of peptide immunogenicity than affinity (19, 20). Thus, we speculated whether Tpn_{1-87} would affect the stability of peptide-HLA-I complexes and whether the Tpn_{1-87} facilitation would depend upon the peptide-HLA-I stability. To answer these questions, we first measured the stability of peptide-HLA-A*02:01 complexes folded with the affinity-paired peptides in the presence or absence of Tpn_{1-87} (supplemental Fig. S3). In general, we observed no differences in the stability of the peptide-HLA-A*02:01 complexes in the absence or presence of Tpn_{1-87}, suggesting that Tpn_{1-87} does not affect the stability of already folded HLA-A*02:01 complexes, at least not for the high affinity interactions studied here. To discover whether differences in stability still could explain Tpn_{1-87} dependence, we measured the stability of the affinity-paired peptide-HLA-A*02:01 complexes. The results showed that the stability varied remarkably across the different peptide-HLA-A*02:01 complexes, where complexes folded with SYFPEITHI peptides were more stable than those folded with non-SYFPEITHI peptides (Fig. 3*D*). For the studied peptides here, Tpn_{1-87} facilitation inversely correlated with the intrinsic stability of the peptide-HLA-A*02:01 complexes (Fig. 3*D*). Thus, stable peptide-HLA-A*02:01 complexes (exemplified by those folded with SYFPEI-

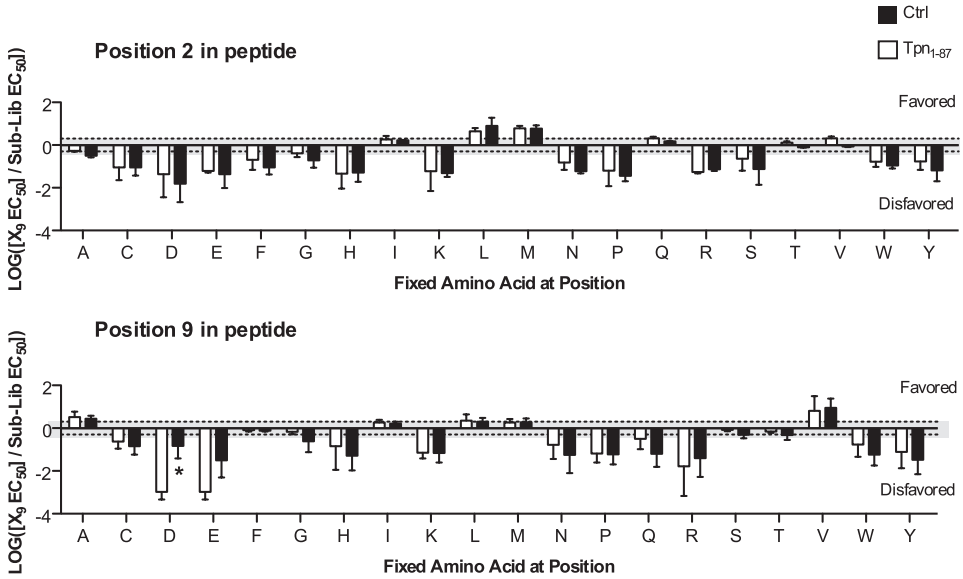


FIGURE 4. **Tpn_{1-87} alters the peptide binding specificity of HLA-A*02:01 to a minor extent.** The peptide binding specificity of HLA-A*02:01 was tested using PSCPLs and shown for substitution positions 2 and 9 in the peptide. Log values of RB values (X_9 EC_{50}/sublibrary EC_{50}) are plotted on the *y* axis, and the amino acid substitutions are shown on the *x* axis. Amino acid substitutions leading to RB values above 0.3 (log(2)) are considered favored, and values below −0.3 (log(0.5)) are considered unfavored (these boundaries are indicated by the *gray shading*). Significant differences ($p < 0.05$) are marked with an *asterisk*.

THI peptides) were only slightly or not at all facilitated by Tpn_{1-87}. These results suggest that the intrinsic stability of peptide-HLA-A*02:01 complexes is of importance for the degree of Tpn_{1-87} facilitation.

Tpn_{1-87} Facilitation Accurately Identifies Immunogenic Peptides—Improved prediction of the immunogenicity of epitopes is highly desirable for a variety of purposes including selection of peptide-based vaccine candidates. To increase the proportion of correctly identified immunogenic HLA-I presented peptides, not only the HLA-I allomorph specific peptide binding motif must be considered but also other parameters such as the influence of the antigen processing machinery components and CD8+ T-cell receptor features. The studies of SYFPEITHI and non-SYFPEITHI peptides above showed that the maximum concentration of folded peptide-HLA-I complex (Ctrl B_{max}), peptide-HLA-A*02:01 stability, and/or Tpn_{1-87} facilitation could be used to separate the two groups of peptides. The majority of peptides present in the SYFPEITHI database has been demonstrated to activate CD8+ T-cells and is considered immunogenic. To statistically evaluate the accuracy of the three parameters in correctly identifying SYFPEITHI from non-SYFPEITHI peptides, a ROC analysis was performed on the affinity-paired peptides binding to HLA-A*02:01. Using a sliding threshold, the *y* axis depicts the sensitivity (the ability to find SYFPEITHI peptides), and the *x* axis depicts 1 − the specificity (equivalent to the risk of including non-SYFPEITHI peptides) (Fig. 3*E*). A random nondiscrimination would follow the $y = x$ diagonal, whereas useful discriminations would be shifted up and to the left. The AUC is a performance measurement of the discrimination parameter, and the higher the AUC, the better the parameter performs. The ROC analysis of the paired peptides showed that all three parameters performed well, but that Tpn_{1-87} facilitation was the best parameter to discriminate the SYFPEITHI peptides (Fig. 3*E*). The intrinsic stability performed second best, again suggesting it to be a vital part of the underlying mechanism of Tpn_{1-87} facilitation. We also measured affinity, stability, and Tpn_{1-87} facilitation on the entire peptide panel tested on HLA-A*02:01 (supplemental Fig. S4). Even though the peptides were not affinity-paired and the affinity range included high-to-medium affinity peptides, the ROC analysis showed that Tpn_{1-87} facilitation and intrinsic stability were the best parameters in discriminating SYFPEITHI from non-SYFPEITHI peptides, whereas B_{max} and affinity were inferior.

*Tpn_{1-87} Alters the Peptide Binding Specificity to Only a Minor Extent for HLA-A*02:01*—Tpn_{1-87} has a minor effect on HLA-A*02:01 peptide binding specificity. Only certain peptides bind to any given HLA-I molecule, and these peptides share common amino acid features. The peptide binding specificity of a given HLA-I is determined by favoring or disfavoring of certain amino acids in certain positions of the peptide. An unbiased method has previously been reported capable of determining the peptide binding specificities of HLA-I molecules using PSCPLs (17). To investigate whether Tpn_{1-87} alters or shows a preference for facilitation based on the peptide binding specificity of HLA-A*02:01, *i.e.* the peptide-HLA-I complex formation based on occupancy of certain amino acids at specified positions of the peptide, we here set out to use PSCPL in the presence or absence of Tpn_{1-87}. The peptide binding specificity was analyzed for the HLA-A*02:01 anchor positions 2 and 9 (Fig. 4) and showed that the peptide specificity was largely unaltered when Tpn_{1-87} was present during the peptide-HLA-I folding. Expanding the PSCPL analysis to the nonanchor positions showed that Tpn_{1-87} only to a minor extent altered the amino acid preferences at these positions (supplemental Fig. S2), suggesting that more complex features of peptides and HLA-I molecules are the major denominators of the degree of tapasin facilitation. Analysis of the SYFPEITHI and non-SYFPEITHI peptides used in our folding assay showed a larger fraction of suboptimal anchors, *i.e.* in position 2 (p value 0.0004)

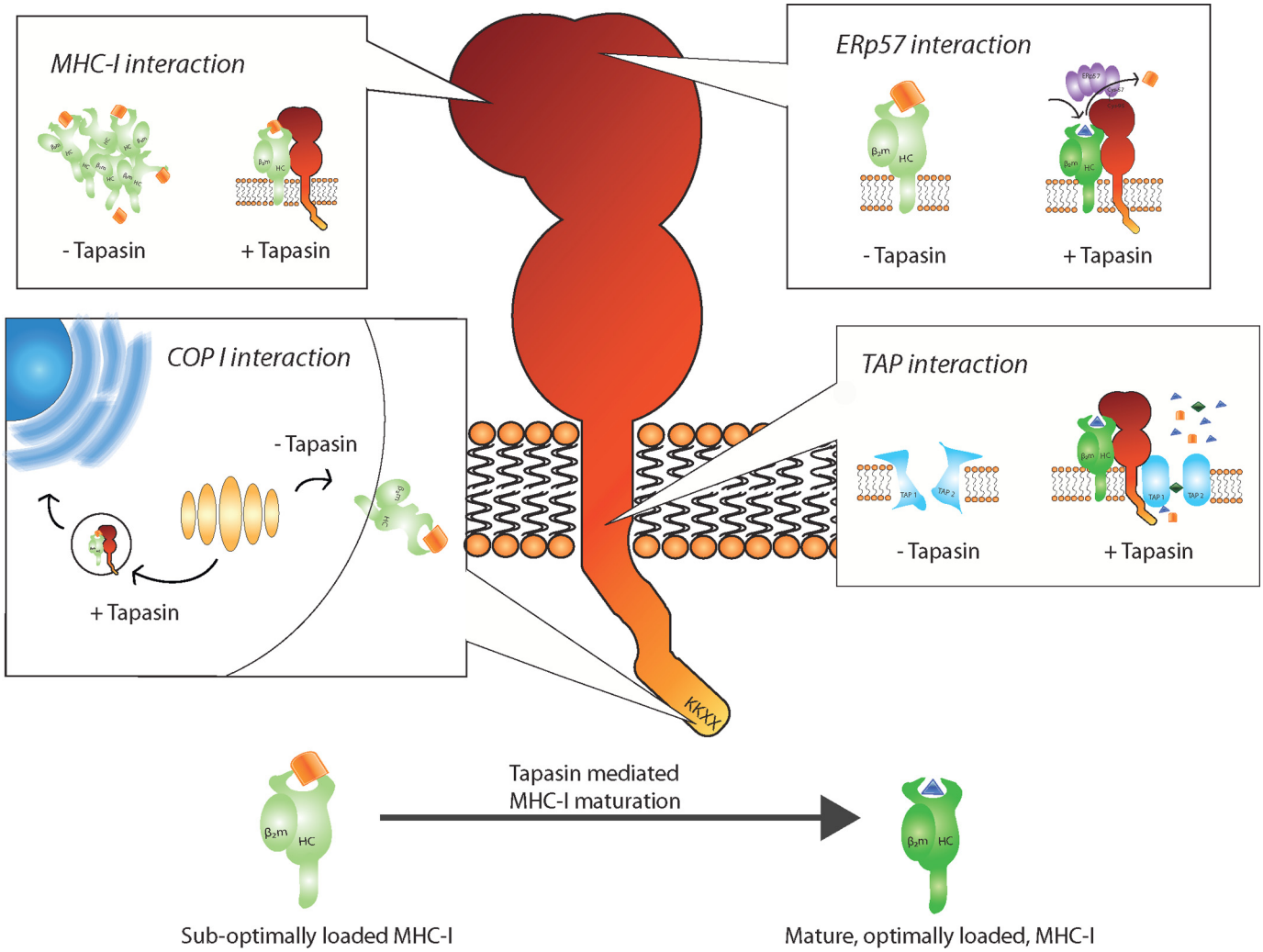


FIGURE 5. **Different sites and regions of tapasin work in concert to quality control MHC-I.** *Top left box*, entire regions and single residues in tapasin, from the cytoplasmic tail to the most N-terminal region, have been suggested to be involved in MHC-I binding. Tapasin incorporates MHC-I into the PLC and brings it into close proximity of the TAP transported peptides. Major MHC-I binding sites are located in the ER luminal part of tapasin. In the first 87 amino acids of tapasin, a chaperone function is located that is suggested to keep the MHC-I in a peptide-receptive state and prevent MHC-I aggregation and degradation. *Top right box*, Cys-95 in tapasin forms a disulfide conjugate with Cys-57 in ERp57, which was suggested to allow tapasin to function as a MHC-I peptide editor. *Bottom right box*, sites in the cytosolic and transmembrane region of tapasin are important for binding to TAP1 and TAP2. Tapasin both stabilizes TAP and promotes binding of peptides to TAP before the ATP-dependent peptide translocation across the ER membrane. *Bottom left box*, a double lysine motif is located in the C-terminal of tapasin and mediates interaction with coat protein type I (*COP I*) vesicles. Coat protein type I vesicles have been proposed to recycle immature/peptide-receptive MHC-I molecules from the Golgi back to the ER. Binding of optimal peptide releases MHC-I from tapasin, allowing efficient antigen presentation on the cell surface.

and the C terminus (p value 0.0103) in the non-SYFPEITHI peptides (supplemental Table S3), suggesting this to be one property that separates the SYFPEITHI- from non-SYFPEITHI-HLA-A*02:01 complexes.

DISCUSSION

The regulation of MHC-I maturation is complex and controversial. In particular, the interactions of the PLC proteins and the mechanisms and functions of tapasin are debated. Because of the multi-functionality of tapasin, it seems plausible that different domains or regions of the protein contribute with different functions. Indeed, it has been shown that an ER retention/recycling motif and TAP interaction interface are located at the C-terminal part of tapasin (21, 22). The cysteine in position 95 in the ER luminal part of tapasin forms a disulfide conjugate with ERp57, and this conjugation has been proposed to be required for tapasin peptide editing (23). Furthermore, the ER luminal part of tapasin associates with MHC-I but not with TAP (1, 24). We recently demonstrated that the N-terminal region of tapasin, Tpn_{1-87}, which is contained in the ER luminal domain, facilitates folding of HLA-A*02:01 in the absence of other ER proteins (13) (Fig. 5).

Here, we demonstrate that the Tpn_{1-87} assisted folding of peptide-HLA-I complexes varies according to both the HLA-I molecule and the peptide identity. It is noteworthy that the Tpn_{1-87} facilitation was in agreement with the established tapasin dependence hierarchy of HLA-I molecules in cellular contexts (10, 12). The reason for the HLA-I molecules being differentially affected by tapasin is to date not well understood, but one possibility is that the primary sequence of the HLA-I HC affects the HLA-I stability or directly modulates the three-dimensional conformation of the tapasin interaction site on

HLA-I. The tapasin interaction site has been suggested to be located near the HLA-I HC α_2-helix, close to the C-terminal part of the peptide when situated in the peptide-binding groove (18). This explanation is supported by the observation that mutation of the threonine to lysine in position 134 (T134K) on an exterior loop near the α_2-helix renders HLA-A*02:01 tapasin-independent (18, 25, 26). Consistently, we observed here that the folding of HLA-A*02:01-T134K could not be facilitated by Tpn_{1-87}. All of the wild-type HLA-A*02:01, HLA-B*44:02, HLA-B*08:01, or HLA-B*27:05 molecules have a threonine at position 134, but there are other structural differences between these HLA-I molecules, some of which might directly affect the tapasin binding. Single amino acid mutations in positions 114 and 116 in the HLA-I HC have been reported to influence tapasin dependence (12, 27). We here observed that the peptide identity was also a determinant for the Tpn_{1-87} facilitation for the HLA-A*02:01, HLA-B*44:02, and HLA-B*08:01 molecules (Fig. 1). Tpn_{1-87} did not facilitate the folding of the tapasin-independent HLA-B*27:05 and HLA-A*02:01-T134K molecules regardless of peptide offered. Hence, the effect of Tpn_{1-87} on peptide binding specificity differences could not be studied with these HLA-I molecules. PSCPLs were tested on HLA-A*02:01 to determine whether Tpn_{1-87} would alter the HLA-A*02:01 amino acid preferences at any of the nine positions of the binding peptide. No major differences were detected, and there were only two significant differences ($p = 0.05$), showing that at position 7 histidine was more disfavored in the peptides in the absence of Tpn_{1-87}, and at position 9 aspartic acid was more disfavored in the peptides in the presence of Tpn_{1-87} (Fig. 4 and supplemental Fig. S2). Therefore, the Tpn_{1-87} facilitation in this *in vitro* setting cannot be said to allow a greater number of different peptides identities to bind to the HLA-A*02:01 molecule because of an alteration of the HLA-A*02:01 peptide binding specificity. Importantly, the PSCPL analyses are based on average affinities of a large (if not infinite) population of peptides varying at all positions, except in the position analyzed. Analyses of singular peptides with defined primary sequences differ, in that the affinities are not "average" but "exact." Differences between all of the singular peptides (an infinite high number) in the PSCPL pool (and subpool) could pull the observed average affinity of a sublibrary in different directions, and the average direction may not be changed by the presence of Tpn_{1-87}.

To explain the affinity alterations in peptide-HLA-I binding, we thoroughly examined the binding at different peptide concentrations. The decreased peptide affinities could be explained by the absence of Tpn_{1-87} facilitation at lower peptide concentrations and Tpn_{1-87} facilitation at higher peptide concentrations, whereas increased peptide affinities could be explained by an increased Tpn_{1-87} facilitation at lower peptide concentrations, but not necessarily a simultaneous increase in B_{max} (Fig. 2*C*). For the majority of the peptide-HLA-I complexes tested, Tpn_{1-87} decreased the affinity (increased EC_{50} value) of the peptide to the HLA-I molecules, but for HLA-A*02:01, Tpn_{1-87} increased and decreased the affinity in a peptide-dependent manner separating the SYFPEITHI and non-SYFPEITHI peptides (Fig. 2*A*). This could indicate that although Tpn_{1-87} has no major effect on the number of formed complexes, it catalyzes the induction of a locked, stable conformation of HLA-A*02:01 with SYFPEITHI peptides.

Intact, stable peptide-MHC-I complexes at the cell surface are of major importance both to allow for proper signaling in case of infection and to prevent false signaling by uninfected cells whose surface MHC-I molecules could inadvertently pick up peptides in the surrounding if a MHC-I molecule would become reactivated (*i.e.* peptide-receptive) upon peptide dissociation. HLA-I molecules are known to be very unstable in the absence of HLA-I binding peptides. Here, we suggest that the intrinsic stability of the peptide-HLA-I complex is of importance for determination of the tapasin dependence, because the intrinsic stability of peptide complexes formed with HLA-A*02:01 inversely correlated with the Tpn_{1-87} facilitation (Fig. 3*D* and supplemental Fig. S4). Moreover, the significantly larger fraction of suboptimal amino acids in the anchor positions in the non-SYFPEITHI peptides (supplemental Table S3) could indicate an increased need for chaperoning by tapasin, which is also suggested by the lower stability of these peptide-MHC-I complexes (Fig. 3 and supplemental Fig. S4). We suggest that a complex combinatorial effect of amino acids on several positions, including the anchor positions, dictates facilitation by Tpn_{1-87}.

Having analyzed the peptide repertoire presented by MHC-I on the cell surface, a previous study suggested that tapasin controls the peptide repertoire, resulting in the presentation of more stable peptide-MHC-I complexes (28). At first glance, our finding that the Tpn_{1-87} facilitation was high for unstable peptide-HLA-A*02:01 complexes seems to contradict the suggested role of tapasin as a mediator of cell surface expression of stable peptide-HLA-I complexes. However, findings from several different experimental systems suggest that tapasin selectively associates with peptide-receptive MHC-I molecules: 1) a direct interaction between recombinant tapasin and peptide-empty HLA-A*02:01 was demonstrated, and this interaction was sensitive to and could be disrupted by MHC-I binding peptide (29); 2) when using purified microsomes, the addition of high affinity peptides efficiently released MHC-I from tapasin (30); 3) in the absence of suitable peptides, *i.e.* in the TAP-deficient T2 cells, MHC-I molecules accumulate bound to tapasin for over 40 min before dissociation (6, 30); 4) another study using recombinant tapasin and HLA-B*08:01 showed that tapasin acts directly on HLA-B*08:01 as a chaperone increasing the number of peptide-receptive MHC-I molecules (31); and 5) finally, tapasin was demonstrated to increase the average affinity of the peptides to MHC-I presented at the cell surface (32) and to increase the stability of peptide-MHC-I complexes (20). Hence, inside the PLC, tapasin is thought to retain and keep MHC-I molecules in a peptide-receptive state until trimming of suboptimal peptides or replacement with optimal peptides allows the release of stable peptide-MHC-I complexes from the PLC (33). We propose that by using both the ER retention and a chaperone function, tapasin would be able to exert key quality control of HLA-I by promoting the presence in the ER of a high number of suboptimally loaded peptide-HLA-I complexes (Fig. 5).

It is debated whether tapasin functions as a chaperone for MHC-I or a peptide editor in the sense of removing nonoptimal

peptides during peptide loading of MHC-I. We believe that both functions may co-exist in the sense that tapasin may act as a chaperone keeping MHC-I in a peptide-receptive state. Using a large set of peptides, we observed Tpn_{1-87} to have chaperone activity and no peptide editing capabilities in terms of direct removal of unstably bound peptide. Rather, this most N-terminal part of tapasin may have an indirect peptide editing function in maintaining a peptide-receptive conformation of empty HLA-I molecules, a state that the HLA-I molecule would have to assume at least briefly during peptide exchange, in line with the model of MHC-I encounter complexes, as recently suggested (34). Our data on the effect of Tpn_{1-87}, however, do certainly not exclude the possibility that wild-type tapasin in its natural environment of the PLC in the ER has a peptide-editing function, although our data would argue that additional requirements to such functionality would reside outside the Tpn_{1-87} region.

Identifying peptides that are suitable as targets in basic research or clinical applications is a critical question, and it is not always possible to determine what peptides will be presented, when such analysis is based only on the affinities between peptide and the MHC-I molecules or the stability of the resulting complex. We have here introduced a completely novel tool, Tpn_{1-87}, with high potential for prediction of immunogenicity, and we have used large sets of HLA-A*02:01 binding peptides allowing comparisons and analysis of groups with statistically significant numbers of peptides. The relevance of discrimination between SYFPEITHI and non-SYFPETHI peptides is corroborated by the large proportion of the SYFPEITHI peptides that have been demonstrated to activate T-cells (in our here used affinity-paired data set 14:21). However, the proportion of immunogenic peptides from a SYFPEITHI set is supposed to be even higher, because all SYFPEITHI peptides have not been studied in T-cell activation assays and were consequently not identified as immunogenic or not, but all studied SYFPEITHI peptides are eluted from HLA-I molecules expressed and purified from cells. Hence, we here present a both specific and sensitive tool of high relevance for identification of T-cell epitopes. Moreover, the results presented here allow interpretation in more generalized terms than previous studies based on small numbers of peptides. In addition to being able to study large numbers of peptides, our *in vitro* model has several advantages, *i.e.* the effect of defined molecules or even specific parts of molecules (*i.e.* Tpn_{1-87}), and only these, are studied, the assay is run in a format allowing large numbers of peptides and quadruplicate to be run in each experiment, and the assay is standardized and highly reproducible. Crucially, the functionality of the *in vitro* system developed perfectly reflects *in vivo* observations of the tapasin dependence of the studied HLA-I molecules. A previous study showed that mild cleavage of tapasin suggested an N-terminal proximal domain within the first 85 amino acids (39). However, with the more recent publication of the tapasin structure published by Dong *et al.* (40), it is clear that the Tpn_{1-87} is only the most N-terminal part of the larger Tpn_{1-270} domain. Our results in this paper demonstrate that some, if not all, HLA-I folding facilitation activity is preserved in the Tpn_{1-87} part of the domain. Importantly, perfectly in line with our results for Tpn_{1-87}, the *E. coli* expressed Tpn_{1-271} fragment not only facilitates peptide-HLA-I complex formation but also shows similar ability for discrimination of SYFPEITHI *versus* non-SYFPEITHI peptides (data not shown).

For HLA-B*08:01, the facilitation effect of Tpn_{1-87} was indicated to discriminate SYFPEITHI from non-SYFPEITHI peptides, similarly to HLA-A*02:01 (Fig. 1 and supplemental Fig. S1). However, for proper conclusions and statements to be made, the number of peptides studied for binding to HLA-B*08:01 is too low, and the data sets need to be significantly extended. For HLA-A*02:01-T134K and HLA-B*27:05, Tpn_{1-87} does not facilitate neither SYFPEITHI nor non-SYFPEITHI complexes, which is in agreement with the tapasin independence of these allomorphs (Fig. 1 and supplemental Fig. S1). Because HLA-B*44:02 is the most tapasin-dependent allomorph studied so far, we included it in this study, but we could not detect any difference in Tpn_{1-87} facilitation of complexes formed with SYFPEITHI or non-SYFPEITHI peptides (Fig. 1 and supplemental Fig. S1). Unfortunately, in the assays used in this work, HLA-B*44:02 is difficult to work with because it does not fold or generate signal above background level, with the majority of peptides tested by us. We speculate that the reason why we do not see a difference in the facilitation of Tpn_{1-87} on SYFPEITHI *versus* non-SYFPEITHI peptide-HLA-B*44:02 complex formation is due to HLA-B*44:02 being intrinsically less stable than many other HLA-I allomorphs, and even SYFPEITHI peptides may not, at least not with the conditions used in our *in vitro* assays, induce a locked conformation that renders the peptide-HLA-I complex independent of Tpn_{1-87}.

Finally and very importantly, we demonstrate that the N-terminal region of tapasin in the absence of other PLC proteins selectively with high specificity and sensitivity facilitates folding of, but does not dissociate, peptide-HLA-I complexes and thereby discriminates between natural and non-natural HLA-A*02:01 binding peptides (Figs. 1 and 3). This opens up possibilities to further disseminate features of and responses to peptides presented and not presented on the surface of human cells in cancer, autoimmune diseases and during positive and negative selection in the thymus. Our results also present a novel tool relevant for the development of predictors of peptide immunogenicity and of candidates for peptide-based vaccines. Based on these findings, we propose that tapasin prevents suboptimally loaded HLA-I molecules from aggregating, keep them peptide-receptive, and that both peptide and HLA-I HC identity are important parameters for the tapasin quality control.

Acknowledgments–We acknowledge the technical assistance of Lotte Nielsen, Tasja Ebersole, Mette Olsen, and Sara Pedersen. We thank Camilla Thuring for help with the illustrations and David Liberg for critical reading of the manuscript.

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Supporting Information Table 1

HLA-A*02:01 (and T134K) **All Peptides SYFPEITHI**	1	YLEPGPVTA
	2	YLNKIQNSL
	3	ALSNLEVKL
	4	KVAELVHFL
	5	TLNAWVKVV
	6	SLSRFSWGA
	7	FLWGPRAYA
	8	ALFDGDPHL
	9	HLIDYLVTS
	10	VLVKSPNHV
	11	ILDKKVEKV
	12	HLGNVKYLV
	13	GLHCYEQLV
	14	GLYDGMEHL
	15	RLMKQDFSV
	16	YMNGTMSQV
	17	SLLPAIVEL
	18	FLDGNELTL
	19	TLWVDPYEV
	20	ILDTGTIQL
	21	VLFSSDFRI
	22	RLNMFTPYI
	23	ILMEHIHKL
	24	QVCERIPTI
	25	HLSTAFARV
	26	RLPRIFCSC
	27	SLDQSVVEL
	28	KIFGSLAFL
	29	LLMDCSGSI
	30	MLGTHTMEV
	31	LLIENVASL
	32	MVDGTLLLL
	33	LLGATCMFV
	34	KLVANNTRL
	35	RLTRFLSRV
	36	GILGFVFTL
	37	KLLEPVLLL
	38	YLSGANLNL
	39	ILAKFLHWL
	40	RLLQETELV
	41	SLLLELEEV
	42	ALCRWGLLL
	43	FLWGPRALV
	44	ILFGHENRV
HLA-A*02:01 (and T134K) **All Peptides Non-SYFPEITHI**	1	ILSPHNVVT
	2	AMHYIRHRA
	3	KIFEYGFTF
	4	ALWEIQQVV
	5	FTFDNSKFV
	6	MMFDAMGAL
	7	KMVGTVQRV
	8	KLAEIFQPF
	9	VVYKEAKIK
	10	WLKEKHEEL
	11	FGKWRPVQL
	12	HLKRTILAL
	13	TMLYNKMEF
	14	IFRRDQIWF
	15	YTLNNGVAM
	16	GLAGGAATA
	17	KVRGRLLAL
	18	QLAFTYCQV
	19	WMDMWESPM
	20	ALEEGRKYV
	21	YLPEDSDIL
	22	ELADQLIHL
	23	NVWATHACV
	24	LLLGGTSEI
	25	DLYDYITRI
	26	FLYGWLFIL
	27	PLNEGIMAV
	28	FLFLYWPHY
	29	SGFGGETPV
	30	SILEYAKSI
	31	KMYEYVFKG
	32	SMFYGIFPS

	33	WFMTWQPNI
	34	SLFGAAVSL
	35	YLLLTTNGT
	36	SIFFDYMAI
	37	YQIEGAWRA
	38	FQWHEAMFL
	39	TLKPGTMSV
	40	YIITCCLFA
	41	GLYSLPHDL
	42	RQPLNIQAI
	43	SVFSRPLPL
	44	GIYGAVIPL
HLA-A*02:01 Subset **EC_{50} Pairs SYFPEITHI**	1	HLIDYLVTS
	2	VLVKSPNHV
	3	ILDKKVEKV
	4	HLGNVKYLV
	5	RLMKQDFSV
	6	YMNGTMSQV
	7	SLLPAIVEL
	8	FLDGNELTL
	9	TLWVDPYEV
	10	RLNMFTPYI
	11	HLSTAFARV
	12	SLDQSVVEL
	13	LLIENVASL
	14	LLGATCMFV
	15	GILGFVFTL
	16	KLLEPVLLL
	17	YLSGANLNL
	18	ALCRWGLLL
	19	FLWGPRALV
	20	ILFGHENRV
	21	HLIDYLVTS
HLA-A*02:01 Subset **EC_{50} Pairs Non-SYFPEITHI**	1	AMHYIRHRA
	2	FTFDNSKFV
	3	MMFDAMGAL
	4	KMVGTVQRV
	5	WLKEKHEEL
	6	GLAGGAATA
	7	ALEEGRKYV
	8	NVWATHACV
	9	LLLGGTSEI
	10	FLYGWLFIL

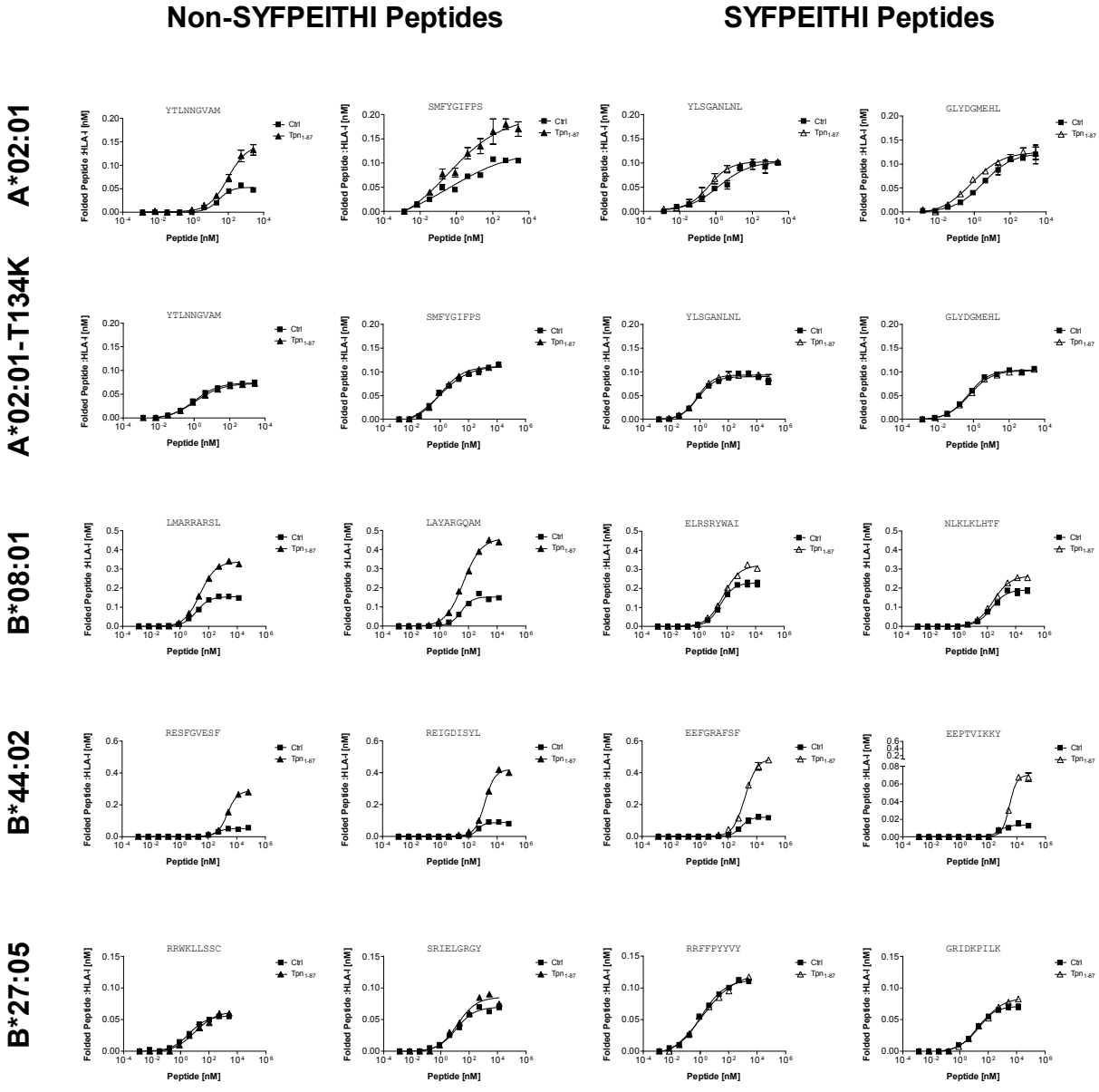
	11	PLNEGIMAV
	12	SILEYAKSI
	13	KMYEYVFKG
	14	SLFGAAVSL
	15	YLLLTTNGT
	16	SIFFDYMAI
	17	FQWHEAMFL
	18	TLKPGTMSV
	19	YIITCCLFA
	20	GLYSLPHDL
	21	GIYGAVIPL
HLA-B*08:01 **All Peptides SYFPEITHI**	1	DLERKVESL
	2	ELRSLYNTV
	3	EIKDTKEAL
	4	EIKDTKEAL
	5	NLKLKLHTF
	6	YLKVKGNVF
	7	ELRSRYWAI
	8	RAKFKQLL
	9	FLRGRAYGL
	10	QAKWRLQTL
	11	ELRSRYWAI
	12	LPHNHTDL
HLA-B*08:01 **All Peptides Non-SYFPEITHI**	1	TLRRRFAVA
	2	FPRYPLNVL
	3	FIKDRATAV
	4	QLSLRMLSL
	5	QLSLKMLSL
	6	YRRKLTNPA
	7	VPRPRFSAL
	8	FARERRLAL
	9	LAYARGQAM
	10	YGLERLAAM
	11	EAILRRFPL
	12	LARLFLYAL
	13	WLRAHPVAI
	14	LMARRARSL
	15	VLRRRRRDA
	16	RLRLLLKQM
	17	MTRRRVLSV
	18	MEQRVMATL
	19	EAKLFFQVI
	20	LAARKARAA

HLA-B*44:02 **All Peptides SYFPEITHI**	1	EEFGRAFSF
	2	EEPTVIKKY
	3	EENLLDFVRF
	4	EEKRGSLHVW
	5	KEFEDDIINW
HLA-B*44:02 **All Peptides Non-SYFPEITHI**	1	RERIRYFHY
	2	AEHFENQVL
	3	HEGDIVPLF
	4	IEAGDEVFF
	5	REWGWRIPF
	6	LEHGLYPQL
	7	AEALLADGL
	8	REMGIVDLL
	9	REIGDISYL
	10	AELGAFFSI
	11	YEGDLRVTF
	12	QEGAMHTAL
	13	SETQGTEKL
	14	REMHHLVEF
	15	AEIESATLF
	16	AESICSYWL
	17	AETESATLF
	18	KEAVNHFHL
	19	REGGGAVRL
	20	REAGMAATL
	21	IENIDFASL
	22	CELSSHGDL
	23	WEMRAGREI
	24	TEMYIMYAM
	25	GEGPGINPI
	26	RESIVCYFM
HLA-B*27:05 **All Peptides SYFPEITHI**	1	ARLFGIRAK
	2	GRIDKPILK
	3	GRNSFEVRV
	4	IRHNKDRKV
	5	RRFFPYYVY
HLA-B*27:05 **All Peptides Non-SYFPEITHI**	1	NRRFVNVVP
	2	RRVFHGVAK
	3	SRLTYQWHK
	4	RRFGGTVIR
	5	RQWAQDLTL
	6	RRTAAGIMK
	7	RRWCFDGPR
	8	KRWGFRSGV
	9	RRLHRLLLM
	10	RRWKLLSSC
	11	SREGMFLPK
	12	RREGGGAVR
	13	KRLGDVISV
	14	KRYKNRVAS
	15	FRQCTGRPK
	16	RRRVLSVVV
	17	SRIELGRGY
	18	WRMGYRTHN
	19	GRVTVSTKR

Supporting Information Table 2

A0201/T134K	0.0770	()
A0201/B0801	0.0253	(*)
A0201/B4402	<0.0001	(***)
A0201/B2705	0.0576	()
T134K/B0801	<0.0001	(***)
T134K/B4402	<0.0001	(***)
T134K/B2705	<0.0001	(***)
B0801/B4402	0.0499	(*)
B0801/B2705	0.7539	()
B4402/B2705	0.0260	(*)

Supporting Information Figure 1

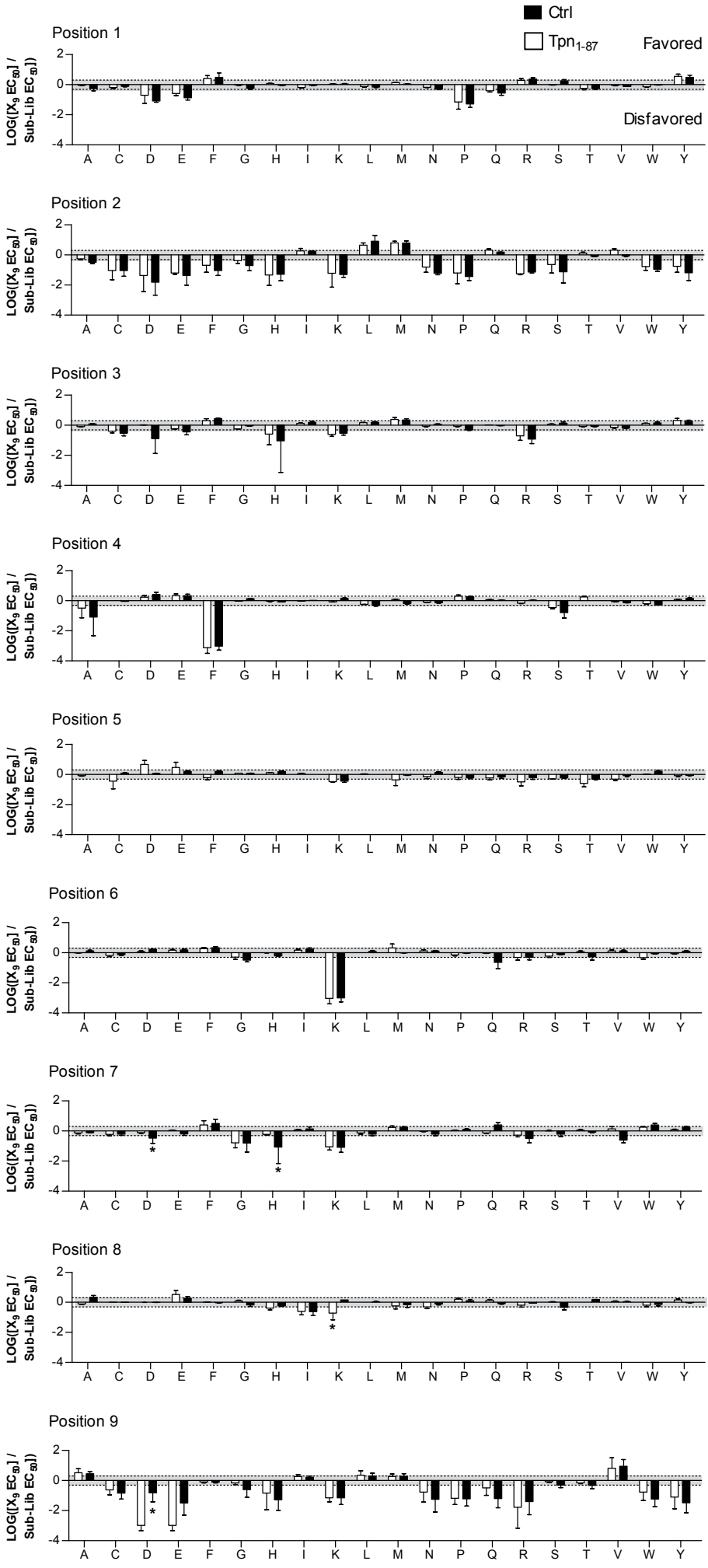


Supporting Information Figure 1

$Tpn_{1\text{-}87}$ differentially facilitates folding of peptide-HLA-I complexes.

Fixed concentrations of β_2m and HLA-I heavy chains were mixed with titrated concentrations of peptide in the presence or absence of $Tpn_{1\text{-}87}$. The mixtures were incubated at 18 ˚C for 48 hrs, and folded peptide-HLA-I complexes were detected by the W6/32 monoclonal antibody in a homogenous assay (Harndahl et al, 2009).

Supporting Information Figure 2

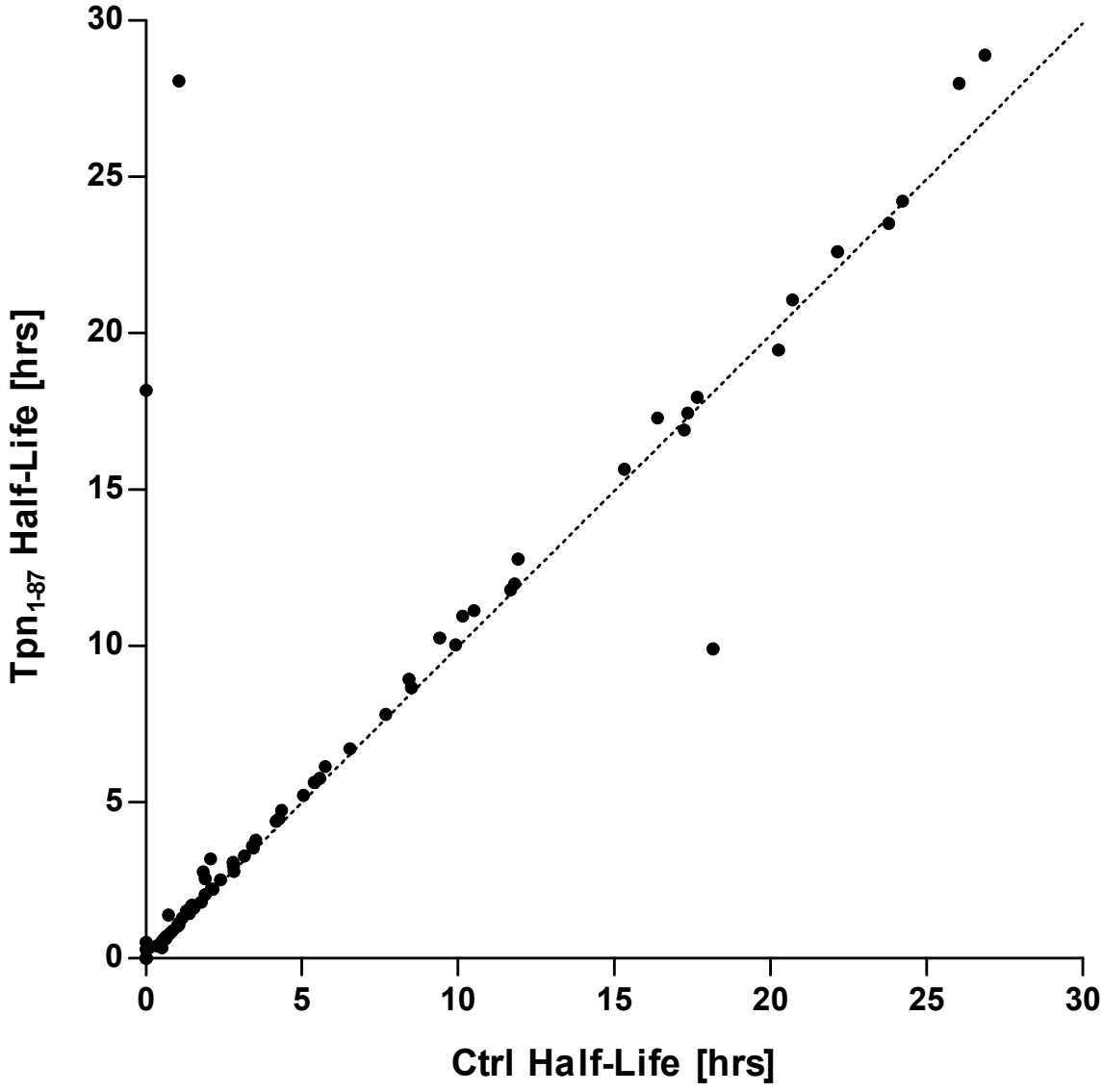


Supporting Information Figure 2

*Tpn_{1-87} does not grossly alter the peptide binding specificity of HLA-A*02:01; a complete amino acid substitution scan across the entire peptide.*

Fixed concentrations of β_2-microglobulin and HLA-A*02:01 heavy chain were mixed with titrated concentrations of each 9-mer peptide sub-library in the presence or absence of Tpn_{1-87}. The mixture was incubated at 18 ˚C for 48 hrs, and subsequently folded peptide-HLA-A*02:01 complexes were measured in a biochemical assay. Each sub-library has one amino acid fixed in a position in the 9-mer peptide, and thus for a 9-mer peptide library there are 180 different sub-libraries and a library containing random amino acids at all positions (X_9). Relative binding (RB) values were calculated as the ratio between the affinity for the X_9 library and the affinity of each sub-library. The RB values were normalized to the total sum of 20 for each position in the peptide, and the log values of the normalized RB values are shown along the y-axis. The amino acid substitutions are shown along the x-axis. Each experiment was done four times, and the average and standard deviations are shown in the graph. Significant differences between RB values in the presence and absence of Tpn_{1-87} were determined by unpaired t tests, and are marked with an asterisk. Only differences between RB values outside the grey area are marked, since only these are relevant for the peptide binding specificity.

Supporting Information Figure 3

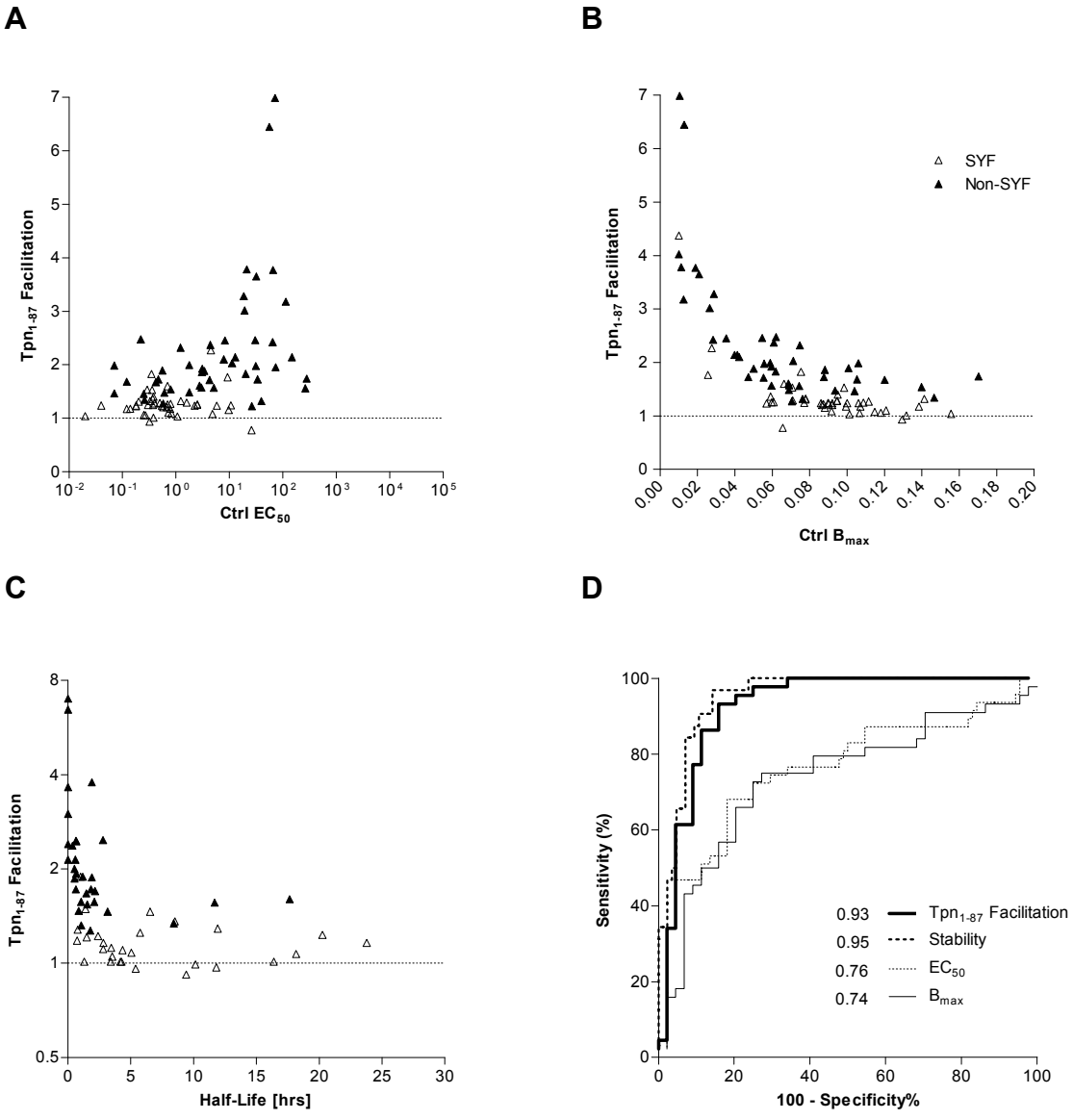


Supporting Information Figure 3

*$Tpn_{1\text{-}87}$ does not alter the stability of the peptide-HLA-A*02:01 complex*.

Peptide and HLA-A*02:01 heavy chain in the absence (Ctrl) or presence ($Tpn_{1\text{-}87}$) of $Tpn_{1\text{-}87}$ were incubated with ^{125}I labeled β_2-microglobulin. After 18 hrs of incubation at 18 ˚C, an excess amount of cold β_2-microglobulin was added, and the reaction was incubated at 37 ˚C, and read at regular intervals. The stabilities of the peptide-HLA-I complexes were calculated as half-lifes from the read-out using one-phase dissociation kinetics.

Supporting Information Figure 4



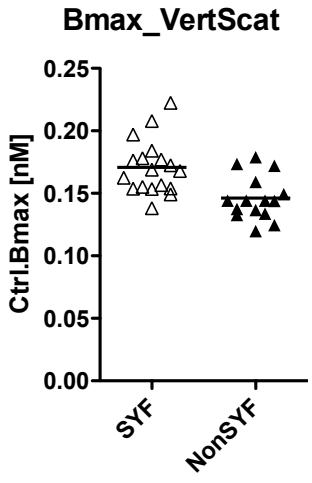
Supporting Information Figure 4

*Tpn_{1-87} facilitates folding of peptide-HLA-A*02:01 complexes, and the facilitation inversely correlates with the intrinsic stability of the peptide-HLA-A*02:01 complex*.

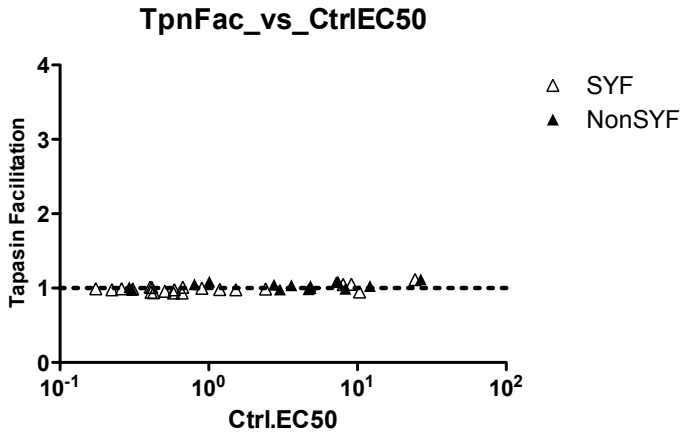
The Tpn_{1-87} facilitation was analyzed using the same set of peptides tested on HLA-A*02:01 in Fig. 1. (**A**) The Tpn_{1-87} facilitation was plotted against Ctrl EC_{50}, the affinity measured in the absence of Tpn_{1-87}. (**B**) The Tpn_{1-87} facilitation was plotted against the Ctrl B_{max}, the saturation plateau measured in the absence of Tpn_{1-87}. (**C**) The Tpn_{1-87} facilitation was plotted against the intrinsic stability of the peptide-HLA-A*02:01 complex. (**D**) A ROC analysis was used on all four parameters (EC_{50}, B_{max}, stability and Tpn_{1-87} facilitation) to determine which parameter best correlates with the folding facilitation. To determine whether significant differences exist between the areas under the ROC curves a jack-knife analysis was performed on the ROC areas. A student's t test was used to determine statistical significant differences between the parameters tested.

Supporting Information Figure 5

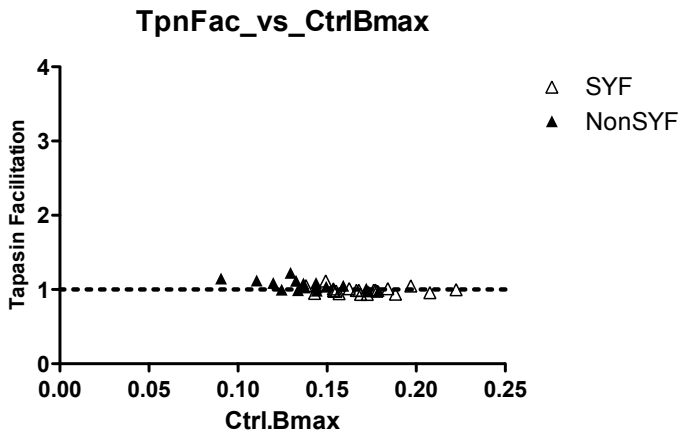
A



B



C



Supporting Information Figure 5

*Tpn_{1-87} does not facilitate folding of HLA-A*02:01-T134K.*

Twenty-one SYFPEITHI and 21 non-SYFPEITHI peptides were paired, based upon affinity to HLA-A*02:01. Fixed concentrations of β_2m and HLA-A*02:01-T134K HC were mixed with various concentrations of peptide in the presence or absence of Tpn_{1-87}. (**A**) The B_{max} values for the SYFPEITHI and non-SYFPEITHI peptides in the absence of Tpn_{1-87} were plotted in a vertical scatter diagram. (**B**) The peptide affinities (EC_{50}) to the HLA-I molecules were calculated as the peptide concentration required to reach the half-saturation point on the sigmoidal dose-response curve. The Tpn_{1-87} facilitation was plotted against EC_{50}. *(***C**) The Tpn_{1-87} facilitation was plotted against the saturation plateau, B_{max}.

Supporting Information Table 3

SYFPEITHI peptides			
Batch Number	Sequence	Pos 2	C-term
4179	YLEPGPVTA	L	A
4183	YLNKIQNSL	L	L
4184	ALSNLEVKL	L	L
4185	KVAELVHFL	V	L
4186	TLNAWVKVV	L	V
4187	SLSRFSWGA	L	A
4189	FLWGPRAYA	L	A
4190	ALFDGDPHL	L	L
4193	HLIDYLVTS	L	S
4194	VLVKSPNHV	L	V
4196	ILDKKVEKV	L	V
4197	HLGNVKYLV	L	V
4198	GLHCYEQLV	L	V
4199	GLYDGMEHL	L	L
4200	RLMKQDFSV	L	V
4201	YMNGTMSQV	M	V
4202	SLLPAIVEL	L	L
4203	FLDGNELTL	L	L
4204	TLWVDPYEV	L	V
4205	ILDTGTIQL	L	L
4207	VLFSSDFRI	L	I
4211	RLNMFTPYI	L	I
4213	ILMEHIHKL	L	L
4216	QVCERIPTI	V	I
4217	HLSTAFARV	L	V
4220	RLPRIFCSC	L	C
4221	SLDQSVVEL	L	L
4223	KIFGSLAFL	I	L
4224	LLMDCSGSI	L	I
4226	MLGTHTMEV	L	V
4227	LLIENVASL	L	L
4228	MVDGTLLLL	V	L
4229	LLGATCMFV	L	V
4231	KLVANNTRL	L	L
4232	RLTRFLSRV	L	V
4233	GILGFVFTL	I	L
4234	KLLEPVLLL	L	L
4235	YLSGANLNL	L	L
4239	ILAKFLHWL	L	L
4244	RLLQETELV	L	V
4245	SLLLELEEV	L	V
4251	ALCRWGLLL	L	L
4253	FLWGPRALV	L	V
4254	ILFGHENRV	L	V
Suboptimal anchors		5	5
		44	44
		11%	**11%**

Non-SYFPEITHI peptides			
Batch Number	Sequence	Pos 2	C-term
6411	ILSPHNVVT	L	T
6436	AMHYIRHRA	M	A
6438	KIFEYGFTF	I	F
6945	ALWEIQQVV	L	V
8711	FTFDNSKFV	T	V
8712	MMFDAMGAL	M	L
8718	KMVGTVQRV	M	V
9585	KLAEIFQPF	L	F
9929	VVYKEAKIK	V	K
10042	WLKEKHEEL	L	L
10051	FGKWRPVQL	G	L
10055	HLKRTILAL	L	L
10179	TMLYNKMEF	M	F
10570	IFRRDQIWF	F	F
10621	YTLNNGVAM	T	M
10851	GLAGGAATA	L	A
10886	KVRGRLLAL	V	L
11076	QLAFTYCQV	L	V
11083	WMDMWESPM	M	M
11091	ALEEGRKYV	L	V
11250	YLPEDSDIL	L	L
11503	ELADQLIHL	L	L
11514	NVWATHACV	V	V
11689	LLLGGTSEI	L	I
11866	DLYDYITRI	L	I
13533	FLYGWLFIL	L	L
14600	PLNEGIMAV	L	V
16321	RSLYNTVAVL	S	L
16556	SGFGGETPV	G	V
16682	SILEYAKSI	I	I
16683	KMYEYVFKG	M	G
16782	RSLYNTIATL	S	L
16784	RSLFNTVAVL	S	L
17803	SLFGAAVSL	L	L
17917	YLLLTTNGT	L	T
17920	SIFFDYMAI	I	I
18458	YQIEGAWRA	Q	A
18475	FQWHEAMFL	Q	L
18928	TLKPGTMSV	L	V
18949	YIITCCLFA	I	A
20123	GLYSLPHDL	L	L
20126	RQPLNIQAI	Q	I
20139	SVFSRPLPL	V	L
20142	GIYGAVIPL	I	L
20154	FLFLYWPHY	L	Y
20163	SMFYGIFPS	M	S
20165	WFMTWQPNI	F	I
Suboptimal anchors		21	16
		47	47
		45%	**34%**

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

Linda Geironson[*], *Gustav Røder*[‡], *and Kajsa Paulsson*[*,‡]

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

Running title: Stability and tapasin folding facilitation of MHC-I

Corresponding author: Kajsa Maria Paulsson, Department of Experimental Medicine, Immunology Section, BMC-D14, Lund University, SE-221 84 Lund, Sweden. Phone: +46 46 222 4167. E-mail: kajsa_m.paulsson@med.lu.se

Keywords: Tapasin, MHC-I, peptide, stability, vaccine

Abstract

Only a small fraction of the peptides generated inside the cell end up on the cell surface presented by HLA-I. 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 suggest that the stability of immunogenic peptide-HLA-I complexes vary above an HLA-I allomorph dependent lower limit (e.g. >2 hours 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.

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 sub-optimally loaded HLA-I molecules back to the ER [4, 12-14], and, to act as a peptide-editor, allowing exchange of sub-optimal 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):

$$K_D = k_d / k_a \qquad (I)$$

Under conditions of limited HLA-I (receptor) concentration and high peptide (ligand) concentration EC_{50} is a reasonable approximation of KD (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 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 $Tpn_{1\text{-}87}$ (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 $Tpn_{1\text{-}87}$, 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 $Tpn_{1\text{-}87}$ 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-SYFPEITHI 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 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.

Materials and methods

Peptides

All peptides were purchased from Schafer-N (Denmark). Peptides intended to be used in set ups 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$_{2\text{-}10}$ (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.

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$_{1\text{-}87}$. 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.

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.

Results

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 $Tpn_{1\text{-}87}$. 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 $Tpn_{1\text{-}87}$ allowing folding of pHLA-I complexes [23]. Under conditions of limited receptor concentration ([HLA] $\leq K_D$), 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 $Tpn_{1\text{-}87}$ 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 figure 1)[18]. We have previously defined the ratio between the maximum amount of folded complexes, B_{max}, in the presence and absence of $Tpn_{1\text{-}87}$ as the *$Tpn_{1\text{-}87}$ 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 $Tpn_{1\text{-}87}$ [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.

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 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 $Tpn_{1\text{-}87}$. 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).

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 for 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_2m and the samples incubated at 37°C, 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 the tapasin-facilitation degree (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].

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 sub-optimal 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 $Tpn_{1\text{-}87}$ facilitation and vice versa [18].

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 $Tpn_{1\text{-}87}$ (i.e tapasin-facilitation to very low degree (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 $Tpn_{1\text{-}87}$.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 $Tpn_{1\text{-}87}$ 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 $Tpn_{1\text{-}87}$ 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 $Tpn_{1\text{-}87}$ on pHLA-B*08:01 stability in a dissociation assay at 37°C was seen. A destabilizing effect of $Tpn_{1\text{-}87}$ 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.).

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 to, 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 $Tpn_{1\text{-}87}$ influence on dissociation, which is likely due both to the temperature difference and the absence of binding forces strong enough to allow the truncated $Tpn_{1\text{-}87}$ 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 $Tpn_{1\text{-}87}$ 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 highten this probability the effect of tapasin function on HLA-I maturation 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 $Tpn_{1\text{-}87}$ as a highly relevant tool, that together with stability can be used to define immunogenic peptides.

Acknowledgements

We sincerely thank professor Søren Buus and Michael Rasmussen for critical reading and discussions. We also thank Michael Rasmussen for help in performing experiments and analyzing data. We thank David Liberg for critically reading the manuscript. We thank the following foundations and councils for financial support: Novo Nordisk foundation, Lundbeck foundation, the Swedish Medical Research Council - grant 2006-6500, the Crafoord foundation, the Royal Physiographic Society in Lund, Thelma Zoegas, Magnus Bergvalls, Österlunds, Groschinskys, Greta & Johan Kocks foundations.

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Figure Legends

Figure 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. EC50), 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 β2m-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.

Figure 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/abscent tapasin-facilitation for A*02:01, defining the upper limit indicating immunogenic peptides.

Figure 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.

Figure 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.

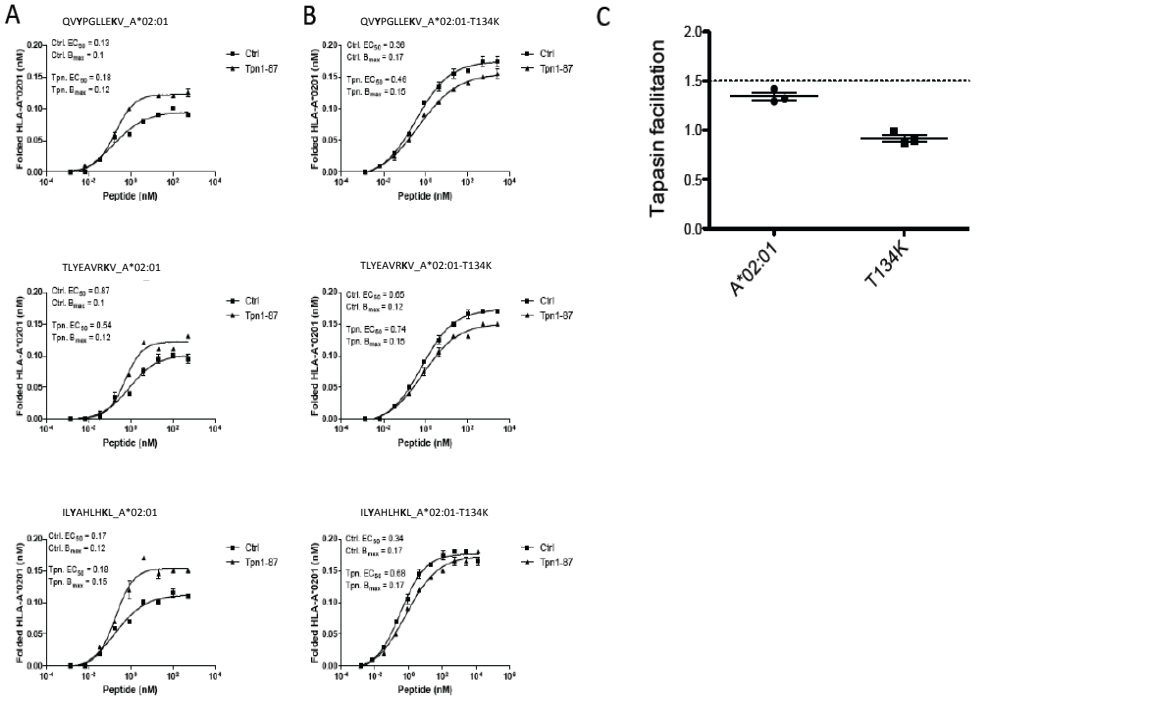
Figure 5

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 the right and 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 optimised. 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 biphase pattern, while dissociation in the presence of tapasin in a peptide-specific manner may drive all or a part of the pHLA-I_{locked} population to pHLA-$I_{transition}$ with resulting mono or biphasic dissociation.

Supplementary figure 1

*Tpn_{1-87} facilitates folding of peptide-HLA-A*02:01 complexes with a non-SYFPEITHI peptide.* Recombinant HLA-A*02:01 heavy chain and β_2m were mixed with different concentrations of the indicated peptide. 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 HLA-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 obtained, B_{max}, was calculated from the plateau at high peptide concentrations.

Figure 1.


A
QVYPGLLEKV_A*02:01
B
QVYPGLLEKV_A*02:01-T134K
C
Tapasin facilitation
A*02:01
T134K
TLYEAVRKV_A*02:01
TLYEAVRKV_A*02:01-T134K
ILYAHLHKL_A*02:01
ILYAHLHKL_A*02:01-T134K
Ctrl
Tpn1-87
Peptide (nM)

D

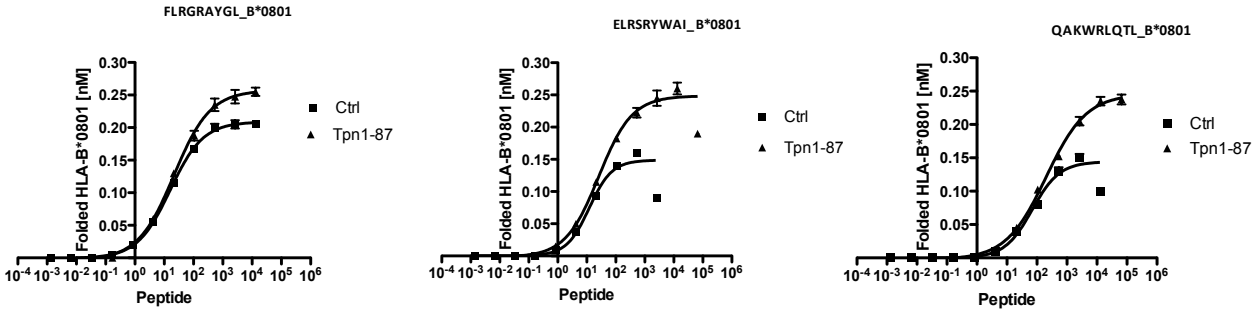

FLRGRAYGL_B*0801
ELRSRYWAI_B*0801
QAKWRLQTL_B*0801
Folded HLA-B*0801 [nM]
Peptide
Ctrl
Tpn1-87

Figure 2.

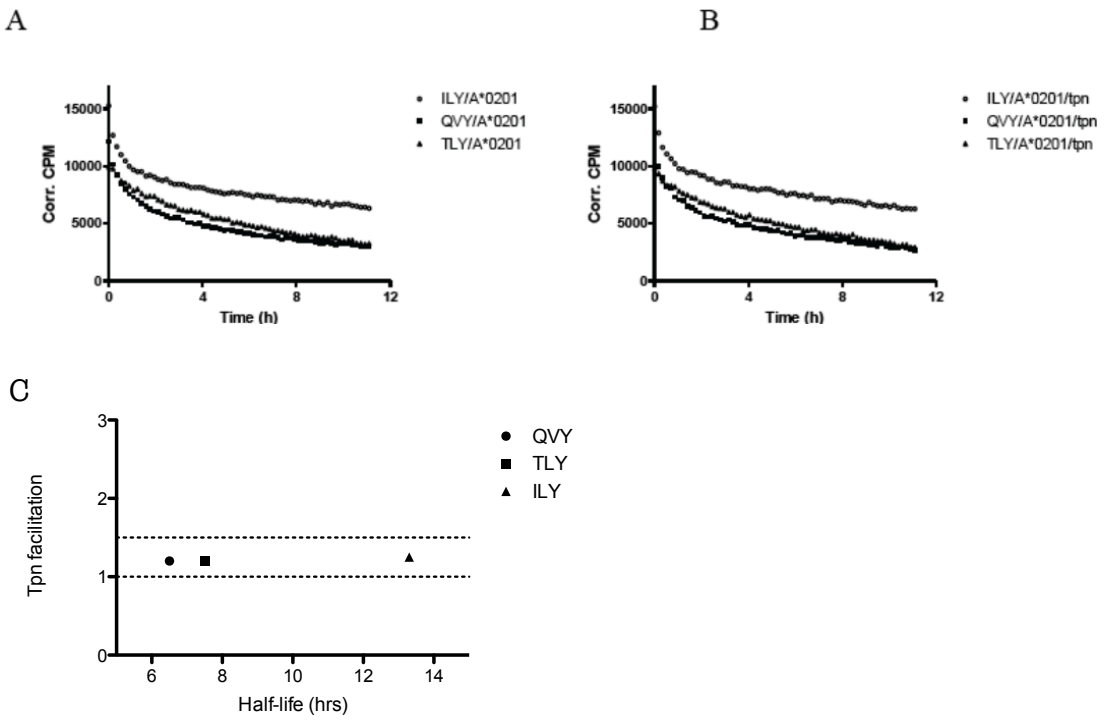
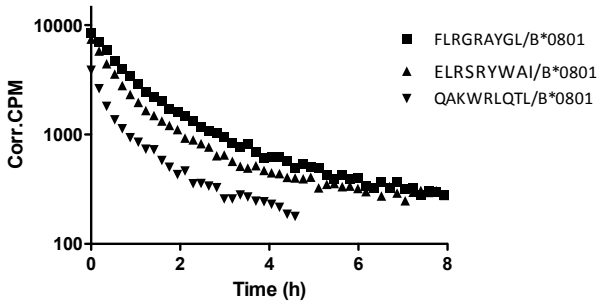
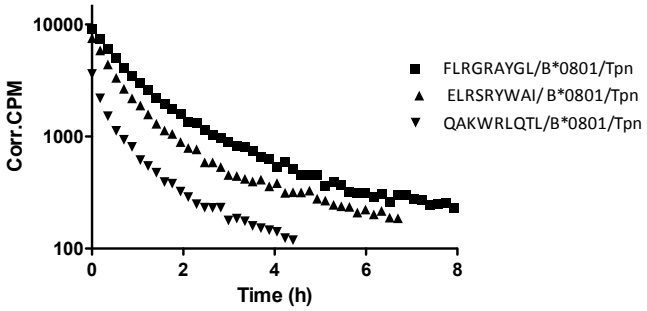

A
B
Corr. CPM
Time (h)
ILY/A*0201
QVY/A*0201
TLY/A*0201
ILY/A*0201/tpn
QVY/A*0201/tpn
TLY/A*0201/tpn
C
Tpn facilitation
Half-life (hrs)
QVY
TLY
ILY

Figure 3.

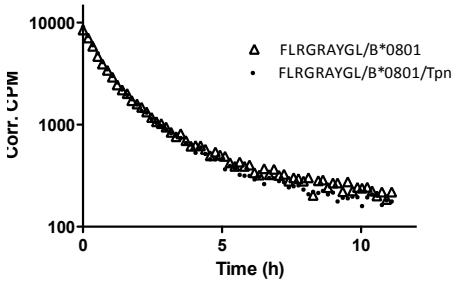
A

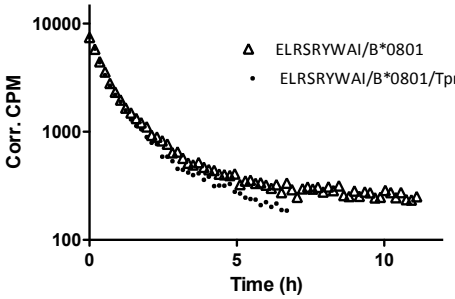


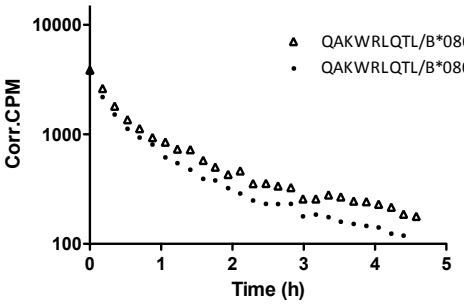
B



C







D

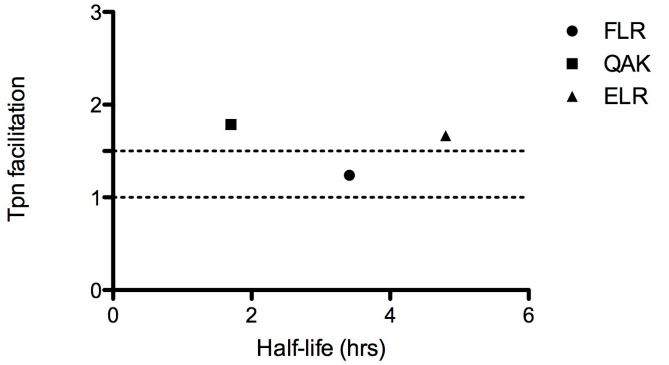


Figure 4.

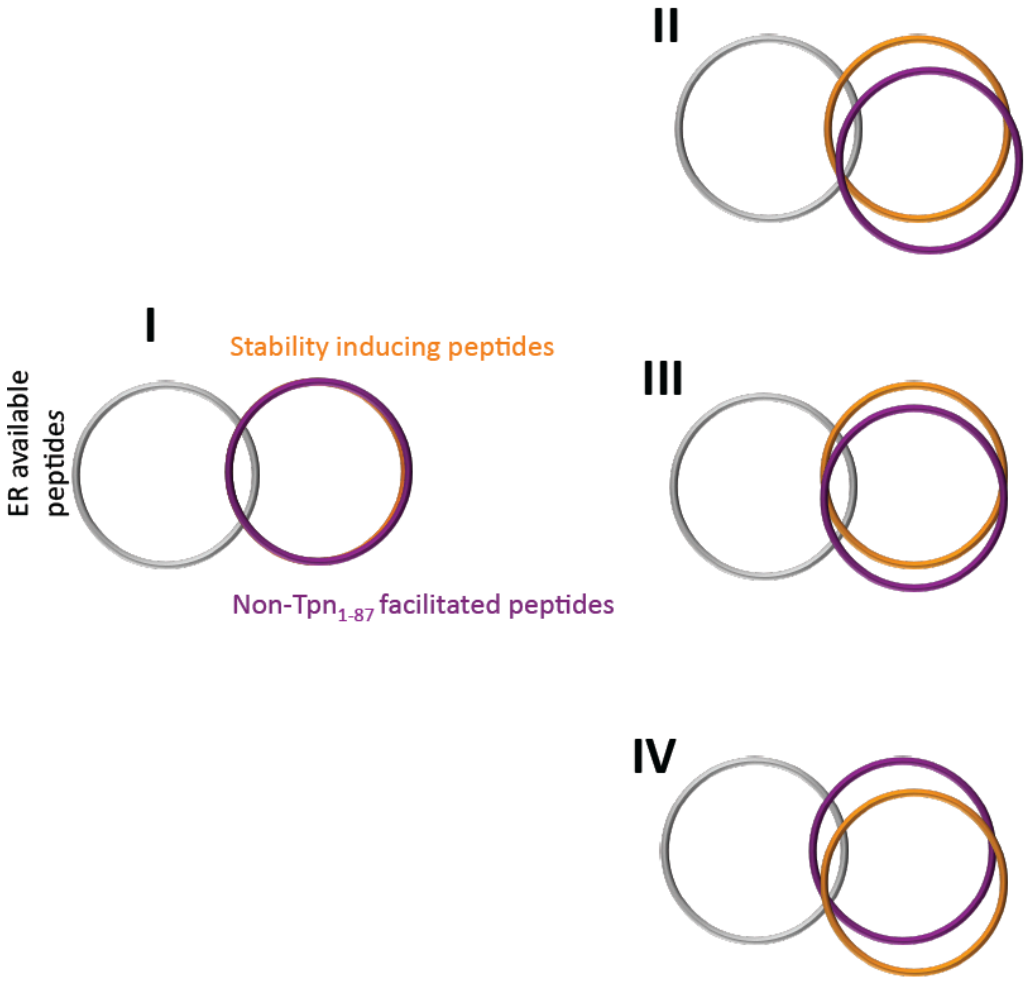

II
I
Stability inducing peptides
ER available peptides
III
Non-Tpn1-87 facilitated peptides
IV

Figure 5.

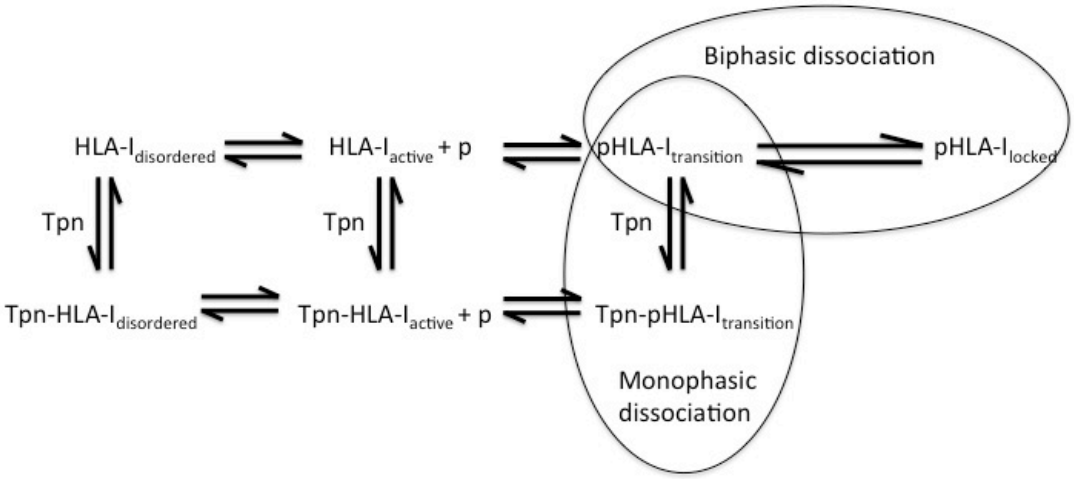

Biphasic dissociation
HLA-I disordered
HLA-I active + p
pHLA-I transition
pHLA-I locked
Tpn
Tpn
Tpn
Tpn-HLA-I disordered
Tpn-HLA-I active + p
Tpn-pHLA-I transition
Monophasic dissociation

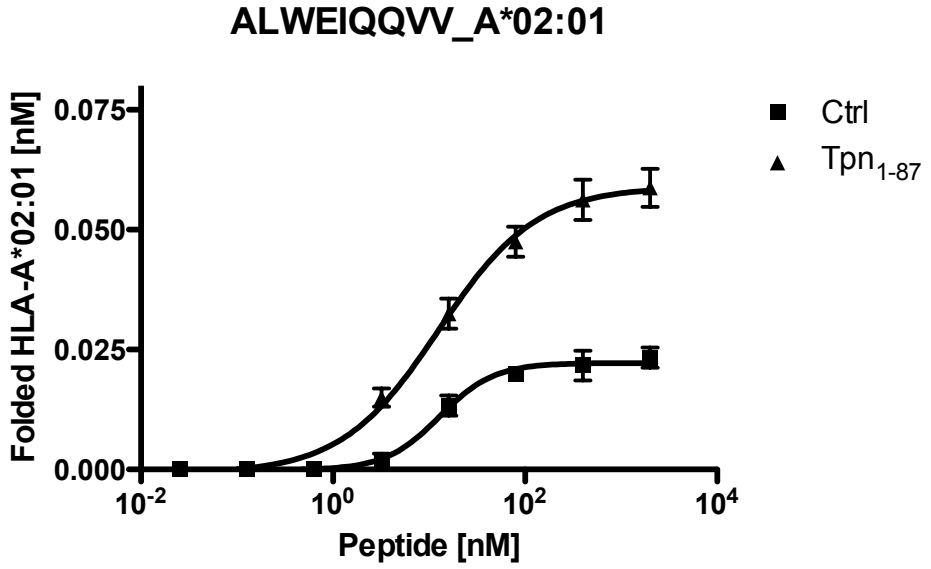
Table 1. In silico predicted affinities of HLA-A*02:01 and HLA-B*08:01 binding peptides.

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 analysed after folding with AlphaScreen assay.

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

Supplementary figure 1.



Tapasin-facilitation of natural HLA-A and -B allomorphs is strongly influenced by peptide length and separates closely related allomorphs

Linda Geironson[1], Camilla Thuring[1], Mikkel Harndahl[2], Michael Rasmussen[2], Søren Buus[2], Gustav Røder[2], Kajsa Paulsson[1*]

[1]Department of Experimental Medical Science, Immunology section, Lund, Sweden

[2]Department of Experimental Immunology, Institute of International Health, Immunology and Microbiology, Copenhagen, Denmark.

*Corresponding author

E-mail: Kajsa_M.Paulsson@med.lu.se

Phone number: +46-46-222 4167

Fax Number: +46-46-222 4218

Address: Immunology, Lund University, BMC D14, 221 84 Lund, Sweden

Key words: HLA-I, tapasin, tapasin-facilitation, peptide length

Abstract

Despite a plethora of peptides inside the cell only a very small fraction ends up presented by HLA-I on the cell surface. The presented peptides have HLA-I allomorph specific motifs and are shown to be restricted in length. So far, detailed length studies have been limited to rather few allomorphs. Peptide-HLA-I complexes of different allomorphs are to different degree qualitatively and quantitatively influenced by tapasin, but again only a small number of HLA-I allomorphs have been studied in this regards. Despite the limited studies both peptide length and tapasin-dependence are of well-established importance for HLA-I peptide presentation, but the relationship between these have not been studied at all. We have here used random peptide libraries, from 7- to 13-mers and studied binding in the presence and absence of a recombinant truncated tapasin. These data show a major difference in how HLA-I allomorphs are affected by tapasin, and significantly extend the spectrum-range of tapasin-dependence for natural allomorphs. Different lengths of peptides generated different amounts of peptide-HLA-I complexes, and the results call for refining the view that optimal length is restricted to 8-10 amino acids of HLA-I presented peptides. Finally, we show that tapasin-facilitation varies for different peptide lengths in an HLA-I allomorph unique fashion.

Introduction

Mature HLA-I molecules present peptides to CD8+T lymphocytes at the cell surface of all nucleated cells. The maturation of the HLA-I molecule is a complex process that takes place in the lumen of the endoplasmic reticulum (ER). During late state maturation, HLA-I is incorporated into the peptide-loading complex (PLC), assuring quality control, optimal peptide binding and subsequent cell surface presentation. In addition to the HLA-I molecule the PLC consists of the peptide transporter TAP, the general chaperone calreticulin, the ERp57 oxidoreductase and the HLA-I maturation dedicated protein tapasin. Tapasin stabilizes and/or promotes formation of TAP heterodimers and enhances peptide binding to the cytosolic part of TAP (1,2). Furthermore, tapasin facilitates peptide binding to HLA-I (3-5), retains and recycles HLA-I molecules loaded with suboptimal peptides (6-9), and has been suggested to edit the peptide repertoire bound to HLA-I (5,10-13).

Tapasin assures that only the small fraction of peptides that satisfy the groove of HLA-I with peptide of right length, stability and sequence (i.e. optimal peptide) can bind in a way that results in tapasin-disengagement, and thus export of a mature peptide-HLA-I (pHLA-I) complex from the PLC to the cell surface (14). Different HLA-I allomorphs display a wide spectrum of variation in their dependency on tapasin for presentation of peptides on the cell surface (15). HLA-B*44:02 (B*44:02) and B*27:05 are well-studied allomorphs in the aspect of tapasin dependence, and have been shown to be on opposite sides in the tapasin-dependence spectrum, with high and low dependence, respectively (12,15-19). Although being less dependent on tapasin for surface expression, a proportion of B*27:05 is still incorporated into the PLC and its surface expression is both quantitatively and qualitatively increased by tapasin (12,15,16,19,20). In contrast, a mutant of the fairly tapasin dependent allomorph A*02:01 with the threonine at position 134 mutated to a lysine, A*02:01-T134K, does not interact with tapasin and is shown to be tapasin-independent (21,22).

Peptides bind to the peptide-binding groove on HLA-I mainly via highly conserved hydrogen bonds between the peptide N-terminal and residues in the A-pocket in the groove, and between the peptide C-terminal and residues in the F-pocket (23). In addition, the peptide binds with its side-chain residues to the other central pockets in the groove. Different HLA-I allomorphs prefer different residues at specific positions in the peptide for binding into the pockets (24,25).

The proteasome, which is the main proteolytic enzyme for HLA-I presented peptides, cleaves proteins into peptides, with a bell-shaped distribution around 11-mer peptides (26). Before translocation across the membrane into the ER by TAP, the peptides are further cleaved by cytosolic amino- and endopeptidases. TAP has a preference for peptides ranging from 8 to 15 amino acids in length but can also transport much longer peptides (27,28). Inside the ER, aminopeptidases further trim the peptides, and the majority of the peptides presented by HLA-I molecules at the cell surface are considered to be 8-10 amino acids in length (29). This is generally thought to be due to the binding preference of the HLA-I molecules. However, it has also been suggested that length is rather controlled by the peptide length availability after antigen processing (30). Indeed it was shown that some HLA-I allomorphs bind very long peptides, 11-25 amino acids, with equal or even greater efficiency than to shorter peptides (30).

We have recently shown that a recombinant protein consisting of only the first 87 amino acids of tapasin (Tpn_{1-87}) has an effect (i.e. increase in B_{max}), termed tapasin-facilitation, that depends on the specific HLA-I allomorph and peptide-sequence (4). Here, we have greatly extended the number of HLA-I allomorphs, and thus this is the first study ever of such a large cohort of HLA-I molecules in terms of tapasin-facilitation. Importantly, the study shows that there is a wide spectrum of tapasin-facilitation for HLA-I allomorphs. We also show a variation in the amount of pHLA-I complexes formed depending on peptide-length, and how tapasin-facilitation affects binding of these peptides from X_{7-13} libraries. Finally, we here for the first time address the effect of peptide length on tapasin-facilitation in a controlled system using peptide libraries.

Results

*Peptides of various lengths bind to HLA-A*02:01 and T134K*

It is known that the preference for certain amino acids at specific positions of the peptides varies for different HLA-I allomorphs, even for allomorphs differing in only one amino acid (17). Peptide binding motifs have been studied with a focus on sequence, with generally less focus on length, resting on the central dogma that HLA-I molecules bind peptides of 8-10 amino acids in length (29). However, this suggested strict length preference is the result of a limited number of studies and the set-ups have suffered from limitations in experimental

procedures. Binding of longer peptides has been shown for several HLA-I allomorphs, and for A*02:01 several CTL epitopes of 11 amino acids in length have been found, and thus the classical length preferences are now challenged (31-33). In this regard, we here set out to study the influence of peptide length on binding to wild type A*02:01 and the recombinant mutant A*02:01-T134K, using X_n peptide libraries with random sequences of 7-13 amino acids in length. Strikingly, the highest amount of peptide-A*02:01 complexes were formed in the presence of peptides longer than 9 amino acids, with 10-, 11-, and 12-mers almost equally well binding (**Fig 1A** and **B**). According to the previously considered length preference the maximum complex-formation for A*02:01 is with 10-mers (29).

Tpn_{1-87} facilitates binding of peptides of different length to HLA-A*02:01 but not to T134K mutant HLA-A*02:01

Using large sets of affinity-paired peptides we have recently shown that tapasin-facilitation in a highly sensitive and specific manner discriminates SYFPEITHI peptides (SYFPEITHI is a database of natural HLA-I ligands, with a high proportion of peptides proved to be immunogenic) from other A*02:01 binding peptides (non-SYFPEITHI peptides) (4). We also found that the tapasin-facilitation varies for different HLA-I allomorphs, e.g. B*44:02 was highly facilitated while A*02:01-T134K was unaffected. Binding of peptides of all studied lengths to A*02:01 was increased in the presence of Tpn_{1-87} (**Fig 2A**). The tapasin-facilitation of peptide binding varied for the different peptide lengths with the highest facilitation seen on X_7 and X_8 peptides, whereas the binding of and X_9-X_{13} peptides were only tapasin-facilitated to a low degree (**Fig 2B**). In line with other studies of A*02:01-T134K both in cellular and biochemical models, the peptide binding to A*02:01-T134K was not influenced by Tpn_{1-87} (**Fig 2A** and **B**) (21,22). For A*02:01 the relatively high complex formation of 9-13-mers and the lower tapasin-facilitation of complex formation with 9-13-mers suggest that there exist relatively few suboptimal peptides in the 9-13-mer libraries, whereas a larger proportion instead indeed are optimal peptides (of course the main bulk of peptides in the libraries are non-binders or very poor-binders). For the 7- and 8-mers instead the situation is reversed with a high facilitation and a lower amount of complex formed. This would then point at an abundance of suboptimal binding peptides while the proportion of peptides inducing full maturation is low.

Tapasin differentially facilitates binding of peptides to a large set of natural HLA-A and –B allomorphs

The exact molecular features in different HLA-I allomorphs dictating the variation in tapasin-dependence are not known. Previous studies of tapasin-dependence have been limited to labour consuming cellular models where only a few HLA-I allomorphs at the time could be studied, and different amounts of HLA-I expression after transfection made the results harder to interpret and compare from one HLA-I allomorph to another (15,20). We have now a recombinant HLA-I peptide binding assay were we can study several HLA-I allomorphs in the presence and absence of $Tpn_{1\text{-}87}$ at the same time under identical conditions.

We have previously observed that $Tpn_{1\text{-}87}$ facilitates folding of A*02:01, B*08:01, B*44:02, B*27:05 to different degree, but not A*02:01-T134K. This is in perfect accordance 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). Here, we have used recombinant versions of additionally 11 allomorphs, and together with A*02:01, B*08:01, B*44:02, B*27:05 and A*02:01-T134K studied the folding of these. Based on the prevailing idea that 8- to 10-mers are the optimal lengths of peptides for binding to HLA-I (29), we studied the tapasin-facilitation of the 16 HLA-I molecules when binding peptides of X_8-X_{10} libraries. The allomorphs were divided into three groups based on their tapasin-facilitation: high tapasin-facilitation (>2.5), intermediate facilitation (2.5>1.5) and low facilitation (<1.5) (**Fig 3** and **4**)(**supp. Fig 2**). The highly tapasin-facilitated group consists of the allomorphs that have an average facilitation >2.5, including B*44:02, B*51:01, B*27:03 and B*08:01. Intermediate average facilitation, i.e. 2.5>1.5, was seen for B*15:01, A*01:01, B*27:05, A*24:02, A*30:01, A*11:01, B*40:01, and A*02:01. The low facilitation group with an average facilitation <1.5 consists of B*18:01, B*35:01, A*02:10 and the mutant A*02:01-T134K. The average degree of tapasin-facilitation (i.e. highest [pHLA-I] in the presence of $Tpn_{1\text{-}87}$ / highest [pHLA-I] in the absence of $Tpn_{1\text{-}87}$) observed when using X_8-X_{10} peptides is shown in figure 3. These data suggest the span of tapasin-dependence to be broader than previously known and still only 15 out of several thousands of HLA-I allomorphs have been studied in this respect.

Binding of X_7-X_{13}-peptide-libraries is differentially facilitated by tapasin in an allomorph dependent manner

To further study a possible difference in the effect of $Tpn_{1\text{-}87}$ on different lengths and not least to study the length preferences in general for these HLA-I allomorphs we then extended the

study to use peptides of lengths, X_7-X_{13} (**Fig 4**). When basing the order of tapasin-facilitation on the results using averages of all studied Xn libraries, the order of the HLA-I allomorphs within the different groups changed to some extent, but no allomorph shifted its position to an extent allowing inclusion into a new group (i.e. no allomorph shifted from high- to low or intermediate to low or vice versa) (**Suppl. Fig 1**).

From these results it is clear that both in the presence and in the absence of $Tpn_{1\text{-}87}$ less tapasin-dependent HLA-I allomorphs form more pHLA-I complexes compared to the highly tapasin-dependent allomorphs (**Fig 4A** and **B**). As expected 7- and 13-mers form least amount of pHLA-I complexes for most allomorphs, while 9-11-mers form the most pHLA-I complexes both in presence and absence of $Tpn_{1\text{-}87}$ (**Fig 4C**). Allomorphs in the low tapasin facilitated group and some of the intermediate facilitated allomorphs, i.e. A*02:01, B*27:05 and A*24:02, are less restricted to the prevailing dogma that optimal peptides for binding are of 8-10 amino acids in length and form a high amount of complexes even with peptides >11 amino acids. 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 for B*27:03 also 10-mers. Moreover, these highly tapasin-facilitated allomorphs form several times less pHLA-I complex in the absence of optimal peptide lengths (**Fig 4C**). Consistently for all studied allomorphs, the binding of 10- and 11-mers was least facilitated by tapasin (**Fig 4D**).

Discussion

Here, we have under strictly controlled conditions and in exactly the same setups determined the tapasin-facilitation of 16 different HLA-I allomorphs. This is the first time a tapasin study of this many HLA-I allomorphs has been performed at one time, and under controlled conditions. The study demonstrates that the tapasin-facilitation of pHLA-I complexes indeed varies over a wide spectrum according to both the HLA-I allomorph and peptide length.

The B*44:02 allomorph has in several studies been shown to be highly tapasin-dependent (15,17,20,34). In this study we have not found a more tapasin-dependent HLA-I allomorph than B*44:02, which allows B*44:02 to keep the position as the most tapasin-dependent allomorph known today. However, on the other side of the tapasin dependency spectrum the

allomorph B*27:05 has been shown to be one of the most tapasin-independent natural allomorph (12,15,16,18,19). Here we have extended beyond the level of independency of B*27:05 with several natural HLA-A and -B allomorphs shown to be more tapasin-independent i.e. B*35:01 and B*18:01 and A*02:10 **(fig. 3 and 4)**. In this study, the non-natural allomorph A*02:01-T134K, known to not interact with tapasin (21), constitutes the extreme of the tapasin-independent side of the spectrum.

The amino acid positions in the HLA-I structure that influence the tapasin-interaction are to some extent known, but still the exact molecular features responsible for a tapasin-dependent or independent phenotype is not characterized (35). It has been hypothesized that the ionic/hydrophobic environment at the bottom of the F-pocket, which is influenced by several amino acids, affects the conformational flexibility of peptide-free HLA-I molecules and is responsible for the degree of tapasin dependence (17,19,36). For some HLA-I allomorphs a single amino acid difference at a specific position in the HLA-I molecule can make a huge difference in tapasin-dependence. The amino acid occupancy at both position 114 and 116 have been shown to be of crucial importance for how dependent different HLA-I molecules are on tapasin for a set of allomorphs (17,20). Garskta et al. have studied the result of different amino acids in position 116, which is the only position that differs between B*44:02 and B*44:05 (17). Replacement of aspartate at position 116 in B*44:02 with histidine (whose shape resembles the one of tyrosine, present in the tapasin independent B*44:05 allele) resulted in a tapasin-independent molecule with stable conformation of the F-pocket region. However, in another study where the TAP interaction of different allomorphs was compared it was concluded that B*44:03, which only differ from B*44:02 at position 156 (aspartate in B*44:02 and leucine in B*44:03) associated very inefficiently with TAP (34). Another highly tapasin-facilitated allomorph, B*27:03, differ from the well studied relatively tapasin independent allomorph B*27:05 only at position 59. This indicates an important role for several specific positions in the peptide-binding groove although this does not exclude that the over-all conformational stability of the HLA-I molecule also may influence the tapasin-dependence.

Comparison of HLA-I expression at the cell surface of B*08:01 transfected tapasin-deficient 721.220 cells and 721.220 tapasin cells has shown that the B*08:01 expression was weakly tapasin dependent (20). In another study using the same cell lines, transfection of B*08 showed a five-fold difference in surface expression with and without tapasin (15). B*08 has also been shown to efficiently associate with TAP (34), which is in accordance with our

results of B*08:01 that show a three-fold increase in the amount of folded B*08:01-peptide complexes in the presence of Tpn_{1-87}.

The surface expression of B*35:01-transfected 721.220 cells has been shown to be >5-fold decreased in the absence of tapasin (20), which contradicts our results showing that B*35:01 has a low tapasin-facilitation. The tapasin dependence was suggested to be due to the presence of aspartate at residue 114 of B*35:01 (15,20). However, in another study it was shown that B*35 allomorphs containing aromatic amino acids at position 116 associated with TAP, while B*35 with serine at position 116, like B*35:01, did not interact with TAP, indicating that it is tapasin independent (12,34). The amino acid at position 134 has a major influence for tapasin dependence of A*02:01, mutation of threonine at position 134 to lysine results in a complete disruption of the tapasin interaction of this allomorph with most peptides (21)(unpublished data). The influence of certain amino acids at specific positions of the HLA-I molecule might explain tapasin-dependency to some degree but is not the only regulating factor for this interaction i.e. the peptide identity is another regulator of the HLA-I-tapasin interaction. We have recently shown that Tpn_{1-87} discriminates the folding of A*02:01 with SYFPEITHI peptides (natural ligands eluted from the cell surface) from non-SYFPEITHI peptides (4). How the tapasin-facilitation relates to peptide length has not been studied previously. Hence, we here have set out to study this parameter using peptide libraries of lengths from 7 to 13 amino acids. In this study we show that more tapasin-facilitated allomorphs were generally more sensitive to the length of the peptides while the allomorphs that were tapasin-facilitated to a low degree were less dependent on the optimal peptide lengths for peptide binding. Under conditions requiring endogenous antigen processing, peptide lengths of 8-10 amino acids have been shown to constitute the bulk of HLA-I bound peptides. However, we here show that longer peptides bind to HLA-I allomorphs to varying extents. We hence propose that it is highly relevant to also consider longer peptides in approaches using synthetic peptides (e.g. peptide-based vaccines) and that it may be time to change our present view of the limitations in the proteome for antigenic peptide candidates.

B*35:01 and A*24:02 have earlier been shown to well accept and bind peptides of different lengths (30). In that study it was suggested that the limitation of some HLA-I to bind peptides longer than 10 amino acids is due to the low availability of longer peptides after antigen processing rather than the preference of the HLA-I molecule per se. B*35:01 and A*24:02 were shown to bind peptides with maintained primary anchor residues but with several central insertions of glycine residues without loosing, and even increasing, their binding to the

allomorphs. The same insertions to peptides binding to A*02:01 and B*08:01 however resulted in reduced binding. This was most evident for peptides binding to the more tapasin sensitive allomorph, B*08:01, resulting in complete loss of peptide binding. The inability of B*08:01 to bind longer peptides, which form looped conformations, has been suggested to be due to its central anchor residue in addition to the B- and the F-pocket (37). However this does not explain the inability of the other tapasin-dependent allomorphs, having anchor residues in the B- and the F-pockets, to interact with longer peptides.

Previously, we have shown that $Tpn_{1\text{-}87}$ discriminates the folding of A*02:01 with SYFPEITHI peptides (natural ligands eluted from the cell surface) from non-SYFPEITHI peptides (4). A tapasin-facilitation <1.5 was determined as the threshold level of facilitation for A*02:01 binding 9-mers, distinguishing SYFPEITHI from non-SYFPEITHI peptides. In this study we demonstrate that specific lengths for several allomorphs, have a tapasin-facilitation value below 1.5. This should however not be interpreted in a way that peptides facilitated less than 1.5 are potential SYFPEITHI peptides. It rather indicates that each allomorph binding to specific peptide lengths have a different tapasin-facilitation threshold value and this may be used to discriminate of SYFPEITHI from non-SYFPEITHI-peptides.

Both in the presence and in the absence of $Tpn_{1\text{-}87}$ there is a clear trend that tapasin-independent allomorphs (i.e. independent is not an absolute independence but rather a lower degree of tapasin dependence) form more pHLA-I complexes compared to the tapasin dependent allomorphs (**Fig. 4A** and **B**). In the absence of tapasin, less tapasin-dependent HLA-I allomorphs exist as a pool of pHLA-I complexes with a higher average stability than tapasin-dependent allomorphs (12). A low degree of tapasin-facilitation in our in vitro binding assay corresponds to a low degree of tapasin-dependence in cellular context (15,38,39) and hence the tapasin-independent allomorphs in our assay fold to a higher degree with peptides regardless of the presence of $Tpn_{1\text{-}87}$. The amount of formed pHLA-I complexes in the ER and the amount subsequently expressed on the cell surface of different HLA-I allomorphs on the cell surface is poorly characterised (34). If the allomorphs proportions expressed in the presence and absence of tapasin would be the subject of more studies this would be an interesting and highly relevant contribution to understand how the effect of tapasin affects antigen presentation. This would be highly relevant for example to analyse during different states when tapasin function is set aside (i.e. when viral immune evasins inhibit the expression of tapasin-dependent allomorphs, or in tumour cells where tapasin is down-regulated).

Figure 5 shows a hypothetical average HLA-I allomorph based on the values from all here studied high-low facilitated HLA-Is (for the values of the average HLA-I allomorphs representing the groups of high, intermediate and low facilitated HLA-I allomorphs, see **supplementary fig 3**). The highest amount of pHLA-I complexes formed with the average HLA-I allomorph, based on normalised values of binding in the absence and in the presence of $Tpn_{1\text{-}87}$, resemble to a great extent and not surprisingly the allomorphs of the intermediary facilitated group. The peptide pool binding to the average HLA-I allomorph in the absence of $Tpn_{1\text{-}87}$ is to a higher proportion suggested to constitute optimal peptides. With $Tpn_{1\text{-}87}$ present both suboptimal peptides and optimal peptides are suggested to bind, but the main increase in pHLA-I complexes is likely to arise from binding of suboptimal peptides. It is important to keep in mind the distinction between suboptimal peptides and poor- or non-binders. Suboptimal peptides are believed to be important during intermediate HLA-I maturation stages and are retained in the ER by tapasin until optimal peptide has been loaded (9). Binding of 8-mers to the average HLA-I allomorph forms less pHLA-I complexes compared to binding of 9-mers, but have slightly higher tapasin-facilitation. This indicates that the X_9-library contains a higher proportion of optimal peptides that bind irrespective of tapasin (**Fig 5**). The facilitation of 10- and 11-mers was lower (**Fig. 4D** and **5B**), and their formation of pHLA-I complexes was higher (5A) than for 12- and 13-mers. This suggests that in the X_{12} and X_{13} libraries there is a smaller proportion peptides able to form suboptimal pHLA-I complexes in addition to a higher proportion peptides able to form pHLA-I complexes in the absence of tapasin with 10- and 11-mers than with 12- and 13-mers (**Fig 5**). Formation of pHLA-I complexes with 7-mers are facilitated to the same high extent as 9-mers and very close to the level of 8-mers, however the amount of complex formed is significantly lower indicating a higher amount of non-binders in the 7-mer library together with a higher amount of optimal peptides in the 8- and 9-mer-libraries.

Based on the peptide-binding motif, both considering length and sequence, we are today able to, with some accuracy, predict what peptides are likely to be immunogenic. Recent advances have shifted focus from affinity, as the key factor in predictions, to stability of pHLA-I complexes (39,40). Even more recent advances suggest that tapasin-facilitation with both high specificity and sensitivity can be used to predict and screen for immunogenic peptides(39). We have here performed a study of tapasin-facilitation on a total of 15 different natural HLA-I allomorphs, the largest in its kind, which shows that the tapasin-facilitation spans over a wide spectrum. This study is also the first attempt to understand the relationship between

tapasin-facilitation and peptide length. In conclusion, these results support findings that peptide sequence including binding motif, but also length, must be evaluated to understand pHLA-I complex formation. We suggest that it is highly justifiable to consider and further study peptide length preferences for HLA-I on its own. Moreover, peptide length is relevant also to elucidate the mechanisms behind tapasin-facilitation that is dictated by both HLA-I allomorph unique features and peptide unique traits, i.e. by the amino acid occupancy at anchor positions and the length of the peptide. The unique peptide specificity of different HLA-I allomorphs but also quality control such as tapasin-facilitation, shapes the peptide-HLA-I profile expressed at the cell surface. These factors are important to investigate to be able to understand the rules for selection of immunogenic peptides and in a translational setting have in mind in the development of candidates for peptide-based vaccines.

Material and methods

Recombinant protein production. HLA-I heavy chains and β_2m were generated as previously described (41,42). Briefly, HLA-I heavy chains encoding the soluble part of the protein were fused at the C-terminal with a histidine affinity (HAT)-tag and a biotinylation signal peptide (BSP). The protein was expressed in inclusion bodies in *E.coli*, extracted and purified. The degree of biotinylation (usually over 95%) was determined by a gel-shift assay. The gene for β_2m was expressed fused with a HAT-tag sequence and FXa cleavage site in the N-terminal. The β_2m was expressed in *E.coli* as inclusion bodies, purified, folded and subjected to FXa protease digestion. $Tpn_{1\text{-}87}$ was expressed and purified as previously described (43). In brief, a construct encoding the first 87 amino acids of tapasin was tagged in the N-terminal with sequences for GrpE and an FXa cleavage site. The construct was expressed in *E.coli* as inclusion bodies, extracted in 8M urea and subsequently purified.

Peptide synthesis. All peptides were synthesised from Schafer-N (Denmark). Briefly, all the synthetic random peptide libraries (7–13 amino acids in length) libraries were synthesized (Applied Biosystems, model 431A), using F-moc (*N*-(9-fluorenyl) methoxycarbonyl) chemistry for the introduction of random degenerate positions (x = 19 L-amino acids, cysteine excluded). The peptides were subsequently purified by RP-HPLC and freeze dried for storage at room temperature.

Peptide – HLA-I folding assay. Peptide-HLA-I folding was monitored in a luminescence oxygen channelling immuno (LOCI) assay (AlphaScreen, Perkin Elmer) as previously described (43). Briefly, biotinylated recombinant HLA-I HCs were diluted in a buffer containing peptide mixes, β_2m and presence or absence of $Tpn_{1\text{-}87}$. The reaction mixtures were incubated at 18°C for 48 hrs to allow pHLA-I complex formation to reach steady state. Peptide-HLA-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-I standard of known concentration.

Data analysis. All data were analysed in GraphPad (Prism 5.0). Tapasin-facilitation was calculated as the highest [pHLA-I] in the presence of $Tpn_{1\text{-}87}$/ the highest [pHLA-I] in the absence of $Tpn_{1\text{-}87}$ (Ctrl). The values for the average HLA-I allomorph were calculated after normalisation of 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 normalisation using the ratio of ((the highest [pHLA-I] $Tpn_{1\text{-}87}$ X_n)/ the highest [pHLA-I] ctrl X_n)/(the highest [pHLA-I] $Tpn_{1\text{-}87}$ of $X_{\text{highest value for any length}}$/the highest [pHLA-I] ctrl of $X_{\text{highest value for any length}}$))/number of studied natural allomorphs. The values representing all studied HLA-I allomorphs were then based on the average of the normalised values calculated for each separate allomorph.

Acknowledgements

We thank the following foundations and councils for financial support: Novo Nordisk foundation, Lundbeck foundation, the Swedish Medical Research Council - grant 2006-6500, the Crafoord foundation, the Royal Physiographic Society in Lund, Thelma Zoegas, Magnus Bergvalls, Österlunds, Groschinskys, Greta & Johan Kocks foundations.

Conflict of interest

The authors have no financial conflict of interest

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Figure legends

Figure 1. Random peptide libraries of 7-13 amino acids in length bind differently to HLA-A*02:01 and HLA-A*02:01T134K. Synthetic random peptide libraries (X) of 7-13 amino acids (X= 19 L-amino acids, cysteine excluded) were analysed for pHLA-I complex formation in a luminescent oxygen channelling immuno assay (LOCI). We here used biotinylated HLA-I allomorphs bound to streptavidin coated donor beads that upon binding of β2m and peptide were detected by the conformation specific W6/32 antibody, pre-conjugated to acceptor beads allowing quantification of folded pHLA-I complexes. Highest amount of [pHLA-I] formed with each peptide length is shown in arbitrary units (AU) after normalisation against the highest amount of [pHLA-I] formed with Xn(highest complex formation). A) $X_{7\text{-}13}$ libraries complex formation with HLA-A*02:01 and B) with HLA-A*02:01-T134K. The experiment shown is representative from duplicate set-ups from several similar experiments.

Figure 2. $Tpn_{1\text{-}87}$ facilitates the binding of X_7-X_{13} peptide libraries to HLA-A*02:01 but not to HLA-A*02:01-T134K. A) A*02:01 and A*02:01-T134K were folded with X_n libraries in the presence and absence of $Tpn_{1\text{-}87}$ and analysed by LOCI (see figure legend 1, and material and methods). Highest amount of [pHLA-I] formed with each peptide length is shown in arbitrary units (AU) after normalisation against the highest amount of [pHLA-I] formed with Xn(highest complex formation). B) From the maximum number of pHLA-I complexes formed ([pHLA-I]) in the presence and absence of $Tpn_{1\text{-}87}$ (i.e.the highest [pHLA-I] in the presence of $Tpn_{1\text{-}87}$/ the highest [pHLA-I] in the absence of $Tpn_{1\text{-}87}$) tapasin-facilitation values are calculated. The experiment shown is representative from duplicate set-ups from several similar experiments.

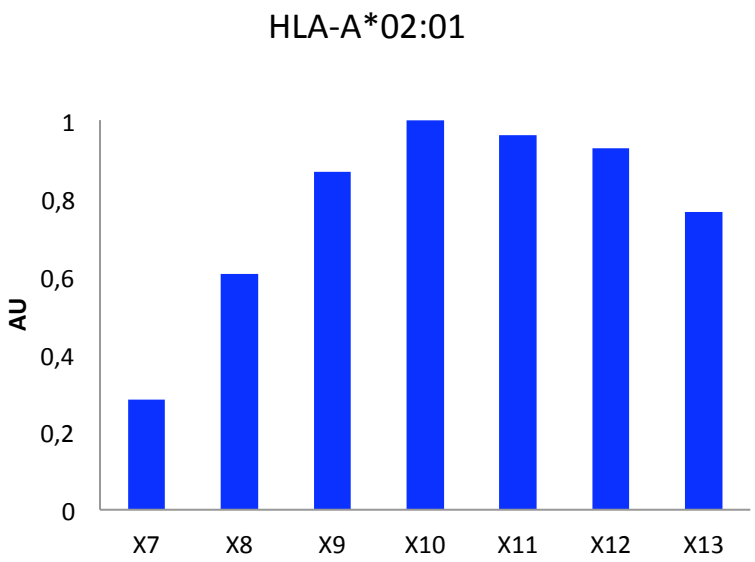
Figure 3. $Tpn_{1\text{-}87}$ facilitates peptide binding of 15 natural HLA-I molecules to a higher degree than the tapasin-inert HLA-A*02:01T134K and separates them over a wide spectrum of tapasin-dependence. Using 16 different recombinant HLA-I molecules and $Tpn_{1\text{-}87}$ in assays with X_n libraries of traditionally considered optimal lengths of peptides for HLA-I molecules, X_8, X_9 and X_{10} complex formation was studied with LOCI assay (see figure legend 1, and material and methods). Based on the different degree of tapasin-facilitation (i.e.the highest [pHLA-I] in the presence of $Tpn_{1\text{-}87}$/ the highest [pHLA-I] in the absence of $Tpn_{1\text{-}87}$) HLA-I allomorphs were divided into three groups: tapasin-influenced to a high degree, tapasin-facilitated to a medium degree and tapasin-influenced to a low degree. Each

point represents the average tapasin-facilitation on X_{8-10} of a unique HLA-I allomorph. The data shown is the average of quadruplicates from duplicate samples run twice in two independent set-ups (data from each set-up in supplementary figure 1).

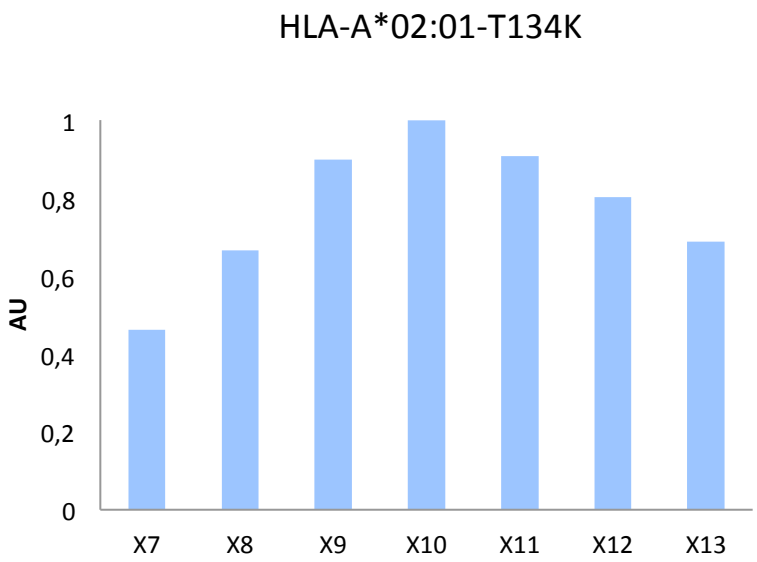
Figure 4. Peptide length influences the degree of tapasin-influence of HLA-A and -B molecules, with partly allomorph specific preferences. Using 16 different recombinant HLA-I molecules, and Tpn_{1-87} in assays with X_7-X_{13} libraries of random peptides, pHLA-I complex formation were studied in the LOCI assay (see figure legend 1, and material and methods). A) Maximum amount of complexes formed (highest [pHLA-I]) for the 16 studied HLA-I molecules. B) Highest [pHLA-I] in the presence of Tpn_{1-87} for 16 studied HLA-I molecules. C) The highest [pHLA-I] values for each peptide length, in the presence and absence of Tpn_{1-87}, for each studied HLA-I allomorph. D) Tpn_{1-87} facilitation of all allomorphs folded with X_{7-13} random peptide libraries. The data shown is the average quadruplicates from duplicate samples run twice in two independent set ups.

Figure 5. The relative average proportions of optimal peptides and suboptimal peptides in X_{7-13} random peptide libraries. The proportion of peptides binding to HLA-I was calculated as an average for all studied HLA-I allomorphs based on highest [pHLA-I] values for each separate allomorph. A) Highest [pHLA-I] for each length +/- Tpn_{1-87} was respectively normalised to the highest value of [pHLA-I] in the presence of Tpn_{1-87} for any length. Values for each length is shown as an average for all 15 studied natural allomorphs. The largest proportion of the peptides in each of the random Xn libraries used, and for each of the HLA-I allomorphs studied, are thought to be suboptimal-binders and suggested to be in vast majority in the Xn library, here illustrated in red. B) The average HLA-I Tpn_{1-87} facilitation for each length was calculated as the normalised ratio of $(([pHLA\text{-}I]Tpn_{1-87}{}^{Xn} / [pHLA\text{-}I]^{Xn})/([pHLA\text{-}I]Tpn_{1-87}{}^{Xhighest} / [pHLA\text{-}I]^{Xhighest}))$/ 15 allomorphs.

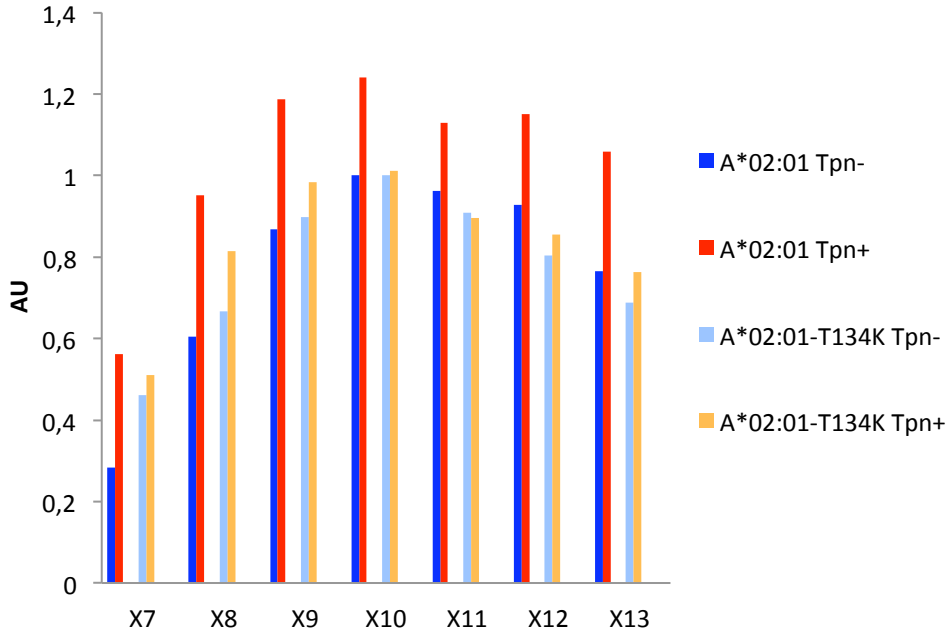
1A.


HLA-A*02:01
AU
1
0,8
0,6
0,4
0,2
0
X7
X8
X9
X10
X11
X12
X13

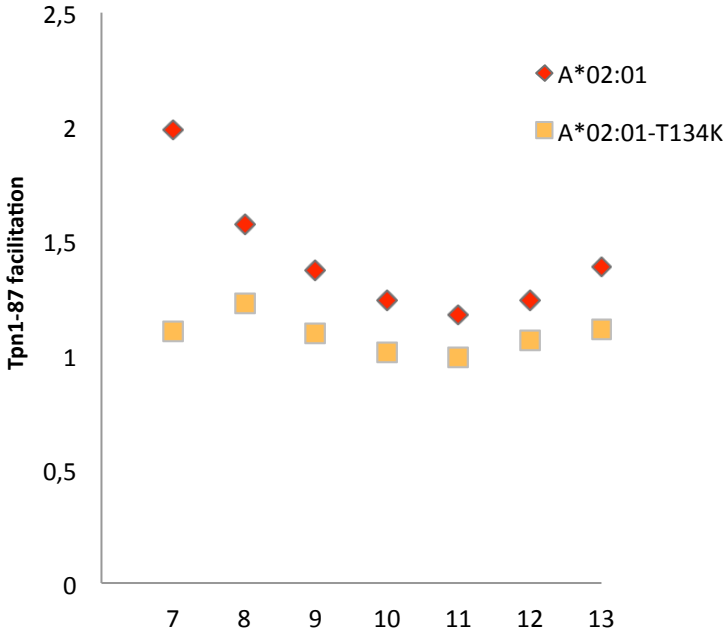
1B.


HLA-A*02:01-T134K
AU
1
0,8
0,6
0,4
0,2
0
X7
X8
X9
X10
X11
X12
X13

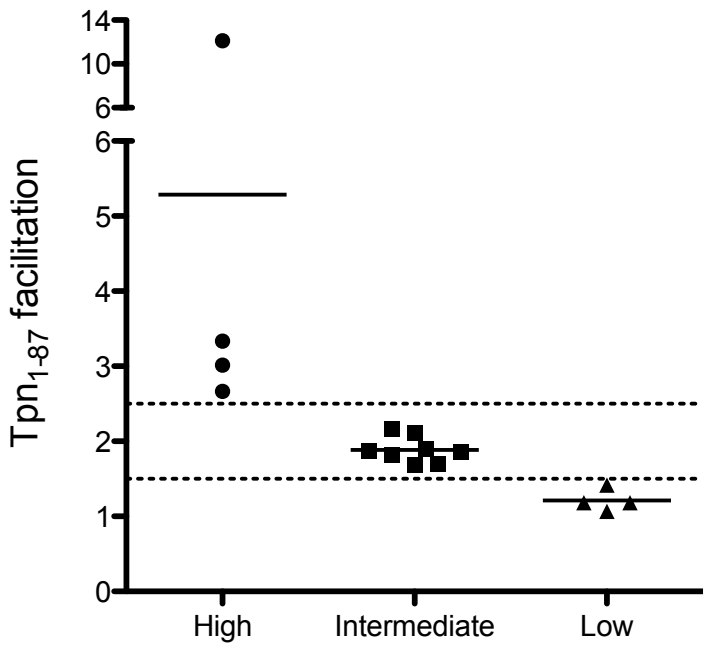
2A.


1,4
1,2
1
0,8
0,6
0,4
0,2
0
AU
X7
X8
X9
X10
X11
X12
X13
A*02:01 Tpn-
A*02:01 Tpn+
A*02:01-T134K Tpn-
A*02:01-T134K Tpn+

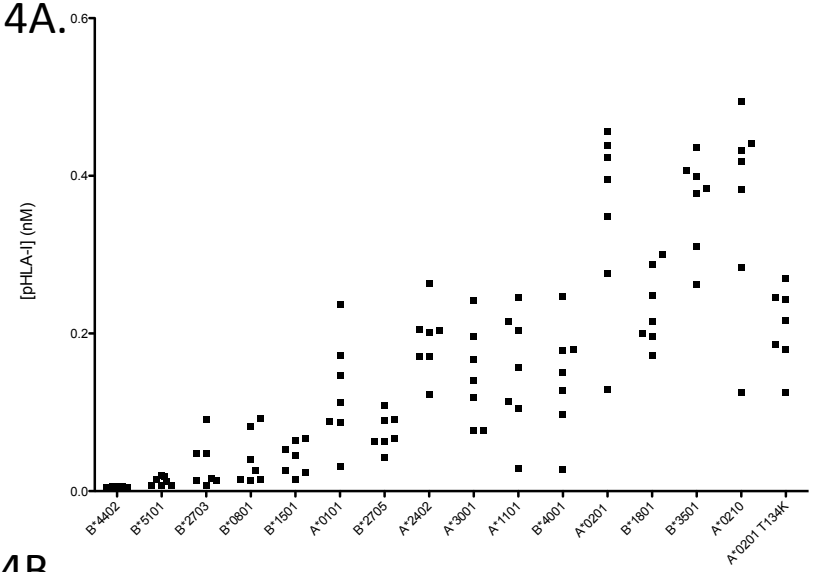
2B.


2,5
2
1,5
1
0,5
0
Tpn1-87 facilitation
7
8
9
10
11
12
13
A*02:01
A*02:01-T134K

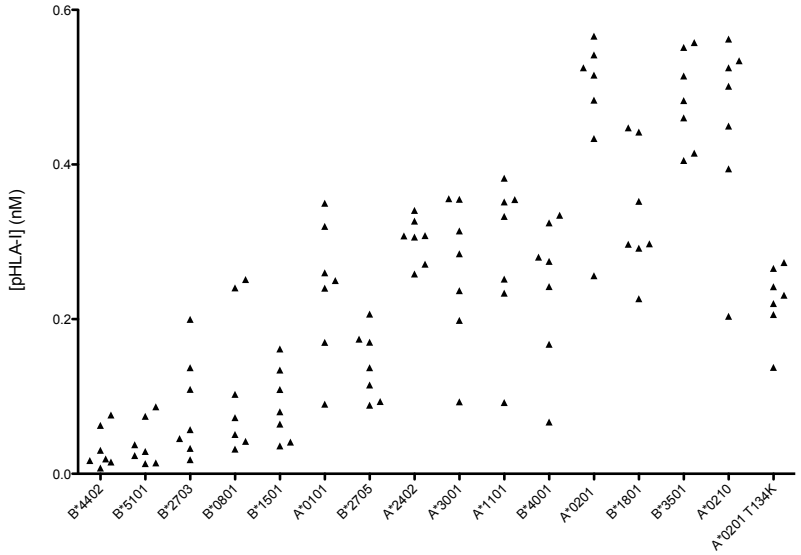
3.



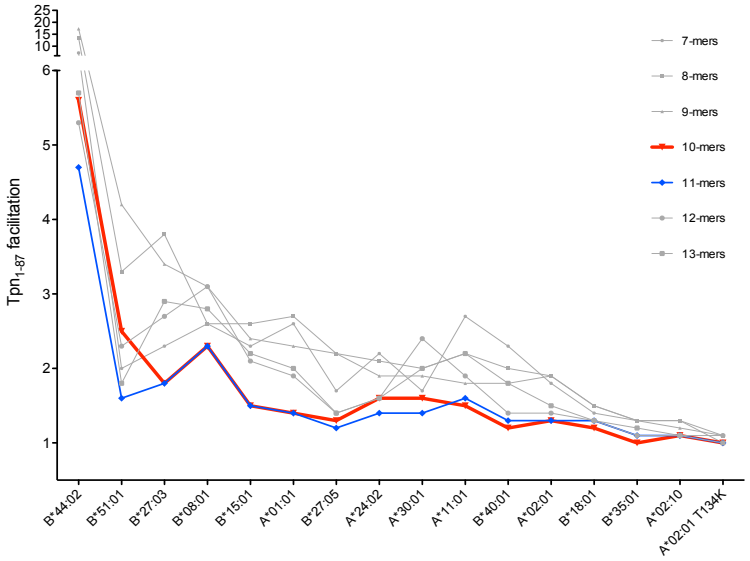
4A.


[pHLA-I] (nM)
0.6
0.4
0.2
0.0
B*4402
B*5101
B*2703
B*0801
B*1501
A*0101
B*2705
A*2402
A*3001
A*1101
B*4001
A*0201
B*1801
B*3501
A*0210
A*0201 T134K

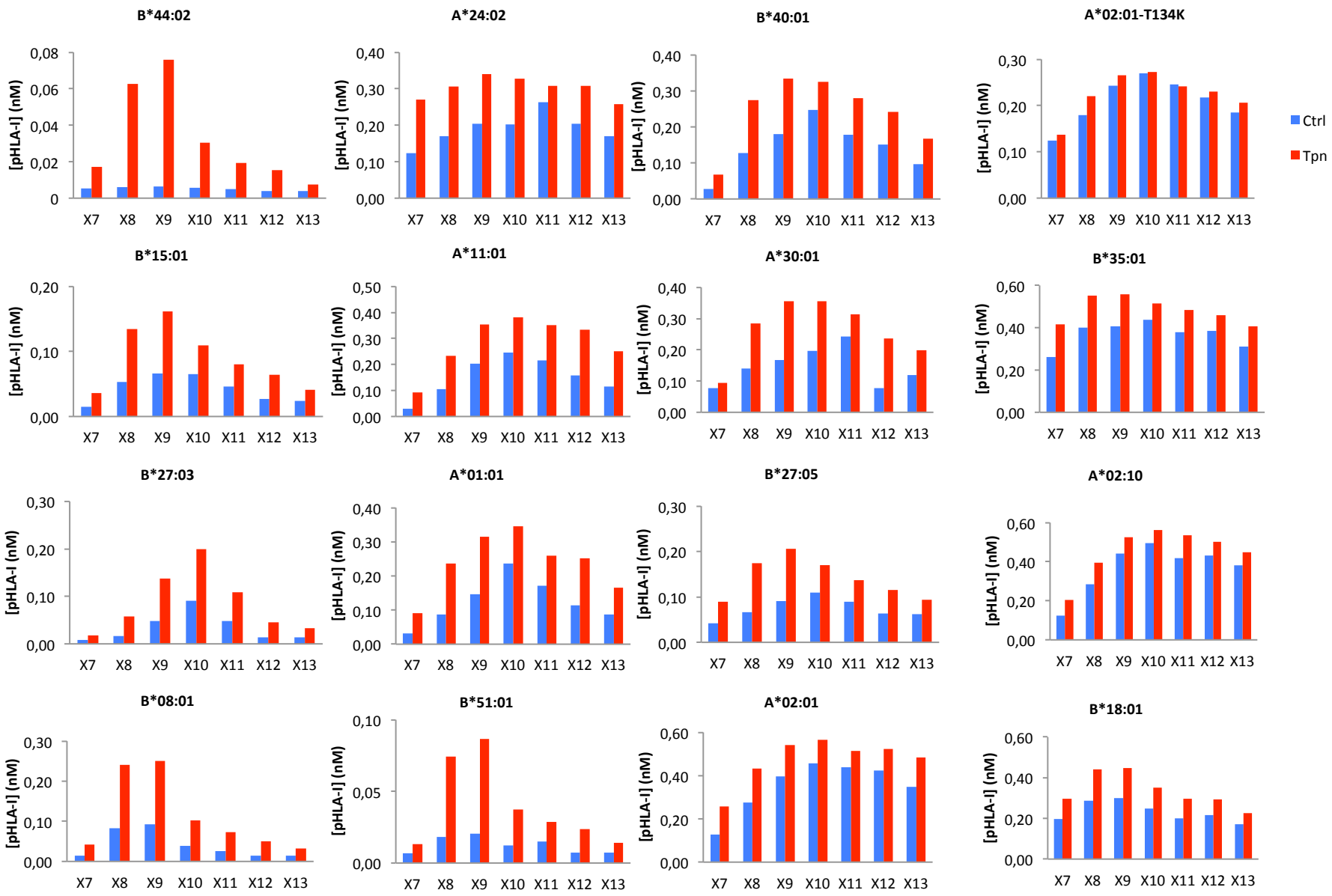
4B.


[pHLA-I] (nM)
0.6
0.4
0.2
0.0
B*4402
B*5101
B*2703
B*0801
B*1501
A*0101
B*2705
A*2402
A*3001
A*1101
B*4001
A*0201
B*1801
B*3501
A*0210
A*0201 T134K

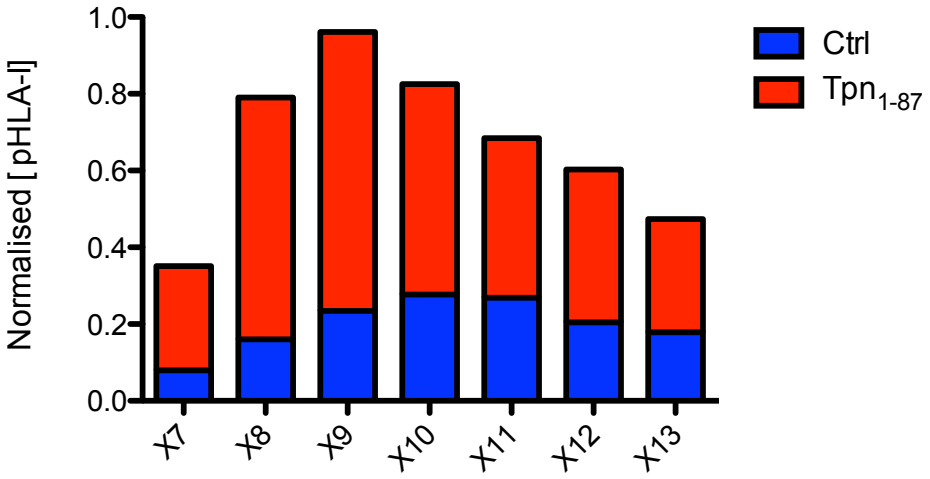
4D.


Tpn1-87 facilitation
25
20
15
10
6
5
4
3
2
1
7-mers
8-mers
9-mers
10-mers
11-mers
12-mers
13-mers
B*44:02
B*51:01
B*27:03
B*08:01
B*15:01
A*01:01
B*27:05
A*24:02
A*30:01
A*11:01
B*40:01
A*02:01
B*18:01
B*35:01
A*02:10
A*02:01 T134K

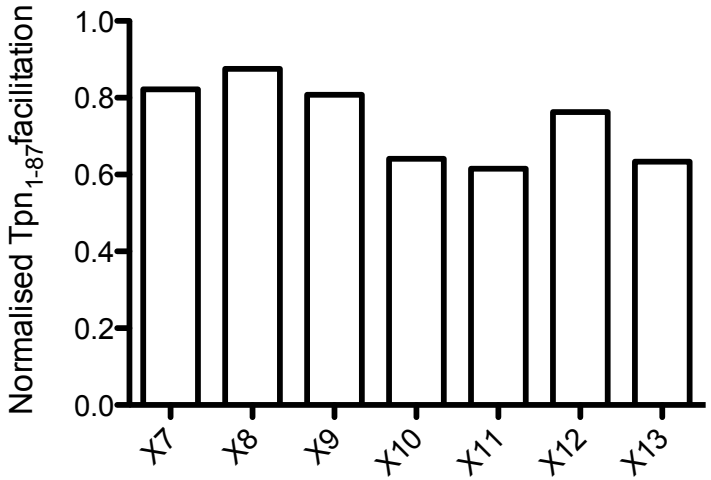
4C.


High
Intermediate
Low
B*44:02
A*24:02
B*40:01
A*02:01-T134K
B*15:01
A*11:01
A*30:01
B*35:01
B*27:03
A*01:01
B*27:05
A*02:10
B*08:01
B*51:01
A*02:01
B*18:01
[pHLA-I] (nM)
X7 X8 X9 X10 X11 X12 X13
Ctrl
Tpn

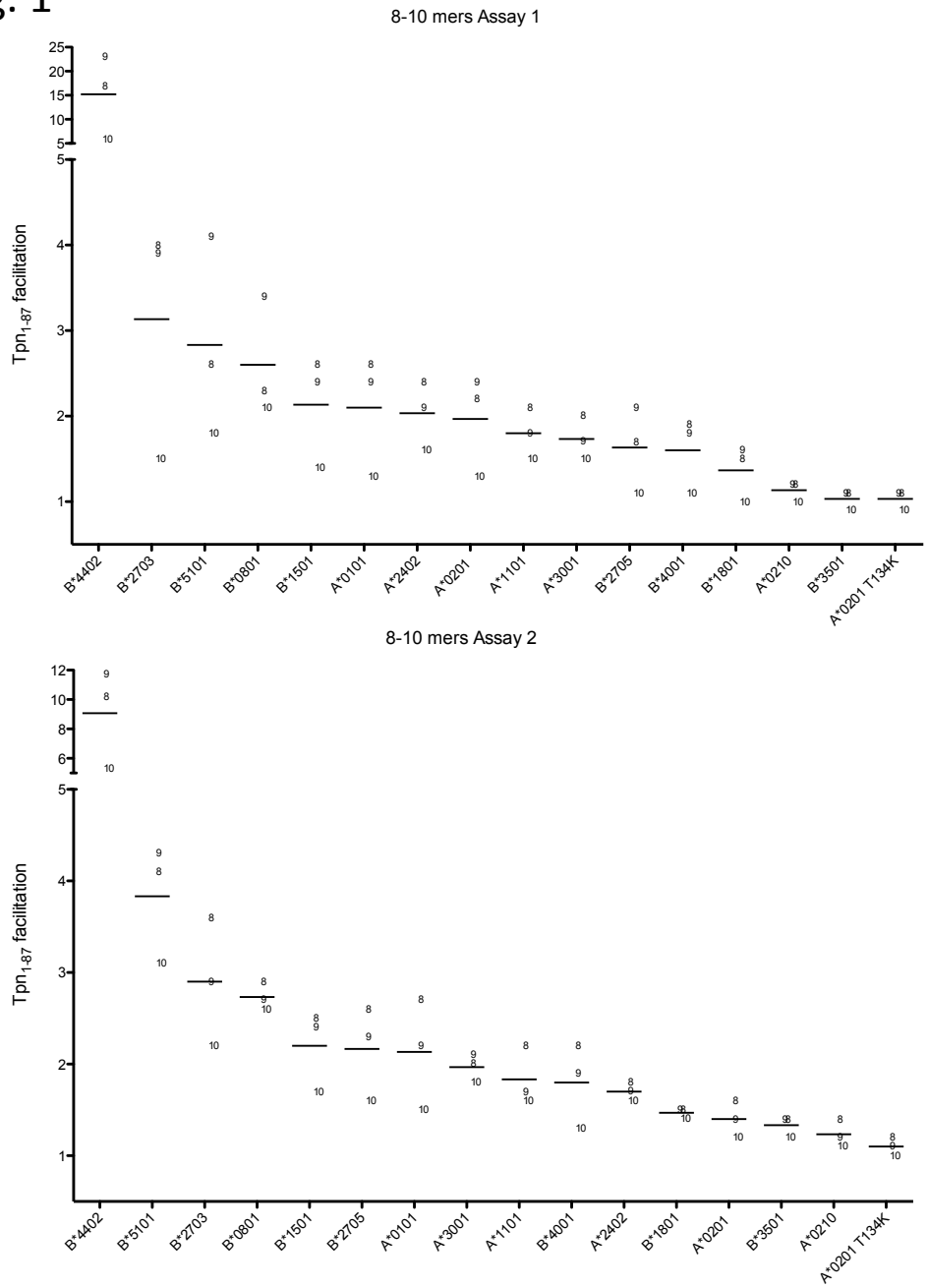
5A.


Normalised [pHLA-I]
1.0
0.8
0.6
0.4
0.2
0.0
X7
X8
X9
X10
X11
X12
X13
Ctrl
Tpn1-87

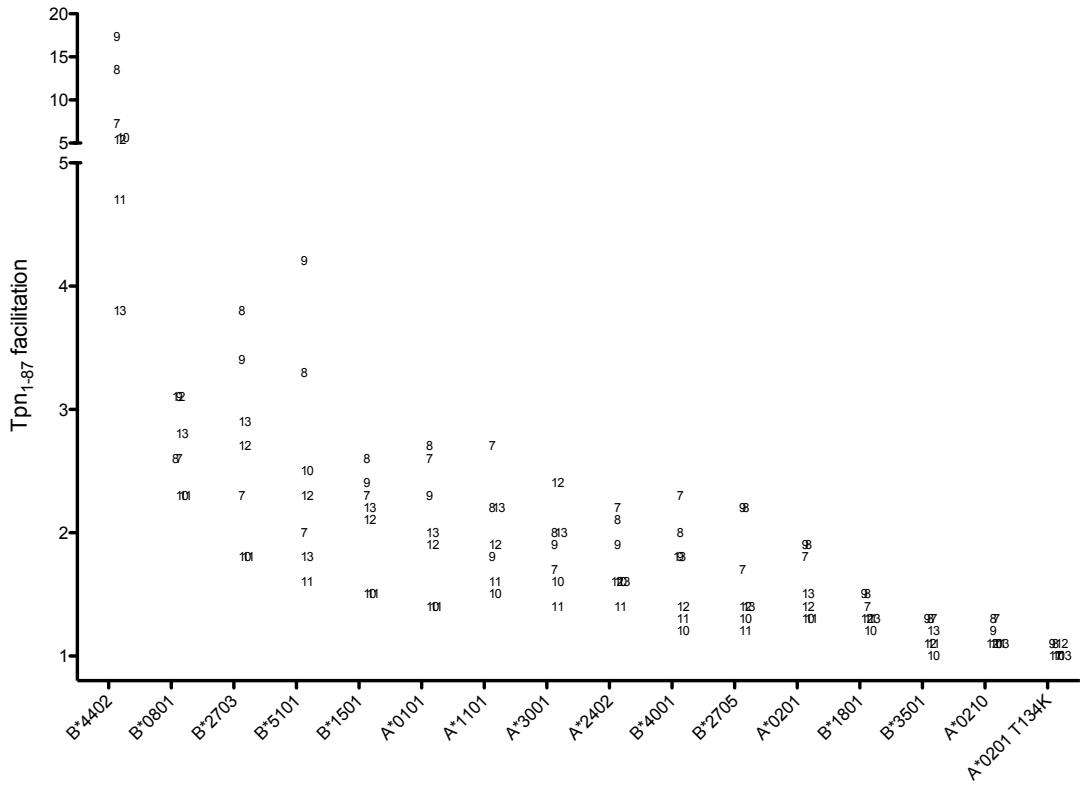
5B.


Normalised Tpn1-87facilitation
1.0
0.8
0.6
0.4
0.2
0.0
X7
X8
X9
X10
X11
X12
X13

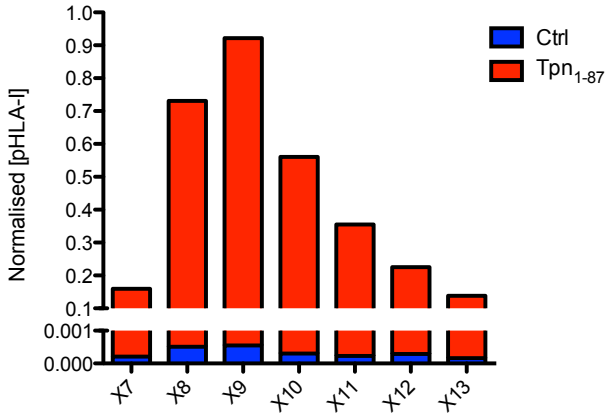
Supp. Fig. 1

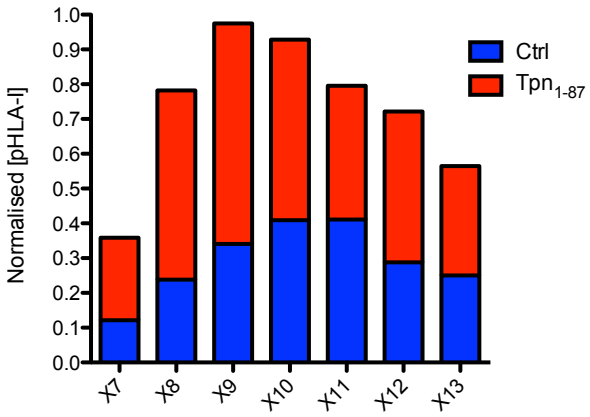

8-10 mers Assay 1
Tpn1-87 facilitation
B*4402
B*2703
B*5101
B*0801
B*1501
A*0101
A*2402
A*0201
A*1101
A*3001
B*2705
B*4001
B*1801
A*0210
B*3501
A*0201 T134K
8-10 mers Assay 2
Tpn1-87 facilitation
B*4402
B*5101
B*2703
B*0801
B*1501
B*2705
A*0101
A*3001
A*1101
B*4001
A*2402
B*1801
A*0201
B*3501
A*0210
A*0201 T134K

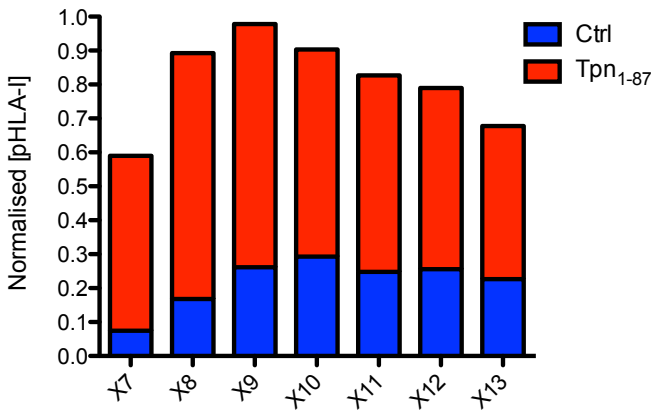
Supp. Fig. 2


Tpn1-87 facilitation
20
15
10
5
5
4
3
2
1
B*4402
B*0801
B*2703
B*5101
B*1501
A*0101
A*1101
A*3001
A*2402
B*4001
B*2705
A*0201
B*1801
B*3501
A*0210
A*0201 T134K

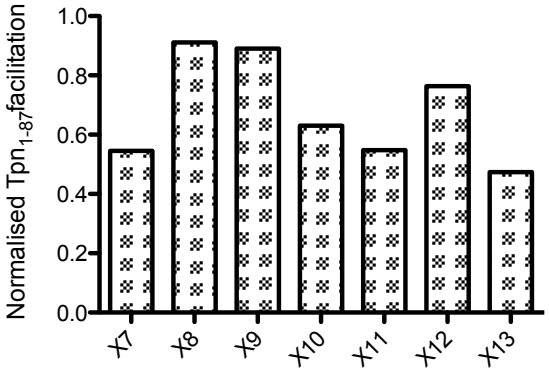
Supp. Fig. 3



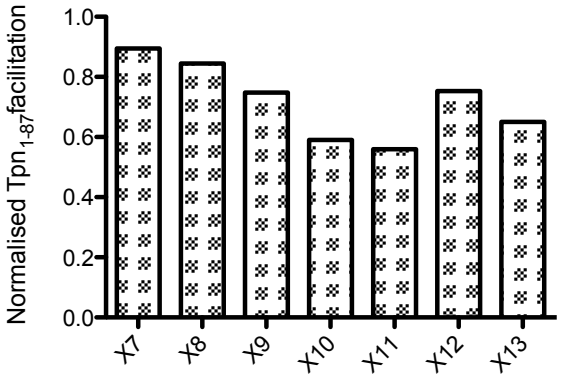




High



Intermediate



Low