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The quantity of naturally processed peptides stably bound by HLA-A*0201 is significantly reduced in the absence of tapasin

Key words:
endogenous peptides; HLA-A*0201; HLA-B*2705; tapasin

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Abstract: Tapasin plays a critical role in promoting peptide binding by major histocompatibility complex (MHC) class I molecules in the endoplasmic reticulum. In its absence, cell surface expression of most allotypes is significantly reduced. Two exceptions are HLA-A*0201 and HLA-B*2705. In this study, the repertoire of peptides bound endogenously by these allotypes in the absence of tapasin was examined and stability of the HLA class I/peptide complexes assessed. Similar quantities of peptides were recovered from B*2705 complexes expressed in the absence and presence of tapasin and the composition of the peptide pools were not radically different. However, the stability of B*2705 molecules expressed at the surface of tapasin-deficient cells was found to be reduced which suggests there are subtle changes to the peptide repertoire. The impact of the absence of tapasin was more dramatic for A*0201. Although equivalent levels of cell surface A*0201 are expressed in the presence and absence of tapasin, very little A*0201 glycoprotein was recovered from tapasin-deficient cells suggesting the complexes readily dissociate. Consistent with reduced stability, A*0201 complexes were found to be rapidly lost from the surface of tapasin-deficient cells. Analysis of the small quantity of endogenously bound peptides recovered from A*0201 expressed in the absence of tapasin revealed a complex mixture typical of A*0201 molecules expressed in normal cells. Therefore these molecules are unable to exploit the alternative supply of TAP-independent A*0201-binding peptides present in the endoplasmic reticulum. Loading of A*0201 with peptides from both TAP-dependent and TAP-independent sources is significantly compromised without tapasin.

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Nascent major histocompatibility complex (MHC) class I molecules bind peptides in the endoplasmic reticulum (ER). Assembly is a tightly regulated process that ensures only those class I complexes containing securely bound peptides are expressed at the cell surface for presentation to CD8+ T lymphocytes. Regulation is achieved by sequential association of the assembling class I molecules with several ER-resident proteins (1). Calnexin and calreticulin promote the more general functions in protein folding but one co-factor called tapasin has a specialised role in the assembly of class I molecules.

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Peptides bound by class I molecules are generated within the cytosol and transported across the ER membrane by the transporter associated with antigen processing (TAP). Tapasin forms a bridge between TAP and peptide-receptive class I molecules in the ER (2). Enforced co-localisation facilitates effective loading with high affinity peptides and release of stable class I/peptide complexes from the ER for transit to the cell surface (3).

In the absence of tapasin, most class I allotypes are poorly expressed at the cell surface (4–6). Two exceptions are HLA-A*0201 and HLA-B*2705 which are both expressed at near normal levels in the tapasin-deficient lymphoblastoid cell line 721.220 (4–6) indicating that certain allotypes are able to bind peptides without associating with the TAP peptide loading complex. In this study, the peptide repertoires bound by A*0201 and B*2705 assembled in the absence of tapasin was examined by direct analysis of the endogenous peptides isolated from these class I molecules and measurement of the stability of these class I/peptide complexes at the cell surface.

Material and methods

Isolation and sequence analysis of peptides bound by HLA class I molecules

Peptides bound by HLA class I molecules in the absence of tapasin were isolated from 721.220/A*0201 cells (kindly provided by Dr. Robert DeMars) and 721.220/B*2705 cells (kindly provided by Dr. Chen Au Peh). Peptides bound by HLA class I molecules in the presence of tapasin were isolated from 721.221/A*0201 and 721.221/B*2705 cells. All cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and penicillin/streptomycin. Cells expressing A*0201 were maintained with 300 μg/ml hygromycin B and cells expressing B*2705 were maintained with 500 μg/ml G418. Peptide isolation and analysis was performed as described previously (7). HLA class I glycoprotein was purified from Nonidet P-40 detergent lysates prepared from 1×10^{10} cells by immunoaffinity purification using monoclonal antibody BB7.2 (8) that recognises all A*0201/peptide complexes equally (9) or monoclonal antibody ME1 (10) that recognises B*2705/peptide complexes expressed in the presence or absence of tapasin equally (11). Bound peptides were eluted by acid denaturation of the class I molecules and recovered by filtration using a 10,000 dalton molecular weight exclusion devise. Peptides were separated by reversed-phase HPLC using a gradient made from 0.06% trifluroacetic acid in water (buffer A) and 0.05% trifluroacetic acid in 80% acetonitrile with water (buffer B) and starting from 5% buffer B at 60 min rising to 80% B at 110 min. Peptide-binding motifs were determined by Edman degradation sequencing of the pool of peptides eluting between 70 and 85 min in the reversed-phase HPLC gradient. The most abundant and best-resolved peptides separated by reversed-phase HPLC were selected for individual sequence analysis by Edman degradation. Searching the GenBank database identified proteins from which the peptides could be derived.

Measurement of the decay of cell surface HLA class I molecules

Aliquots of 3×10^5 tapasin-deficient 721.220/A*0201 or 721.220/B*2705 and tapasin-competent 721.221/A*0201 or 721.220/B*2705 cotransfected and expressing the human tapasin gene were grown in 0.5 ml RPMI 1640 supplemented with 10% FCS and containing 10 μg/ml brefeldin A (Sigma, St. Louis, MO, USA) at 37°C. Cells were harvested at 0, 1, 3, 6 and 20 h post brefeldin A treatment. Surface expression of HLA class I was quantified by indirect immunofluorescent staining using a saturating concentration of the conformation-dependent HLA class I-specific monoclonal antibody

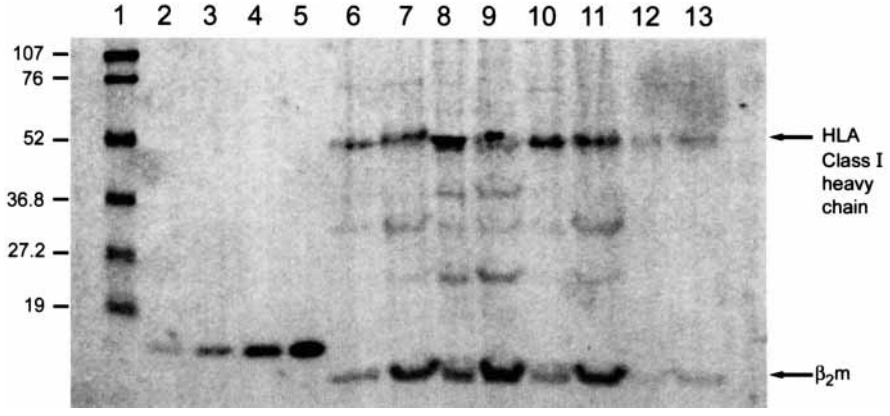

Fig. 1. **Estimation of HLA class I glycoprotein yield from tapasin-competent and tapasin-deficient cells by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining.** Lane 1, molecular weight marker (Kilodaltons). Lanes 2–5, lysozyme at 0.5, 1, 2 and 4 μg. Lanes 6–7, 721.221/B*2705 at 5 and 10 μl from 540 μl total. Lanes 8–9, 721.220/B*2705 at 5 and 10 μl from 550 μl total. Lanes 10–11, 721.221/A*0201 at 5 and 10 μl from 610 μl total. Lanes 12–13, 721.220/A*0201 at 5 and 10 μl from 93 μl total.

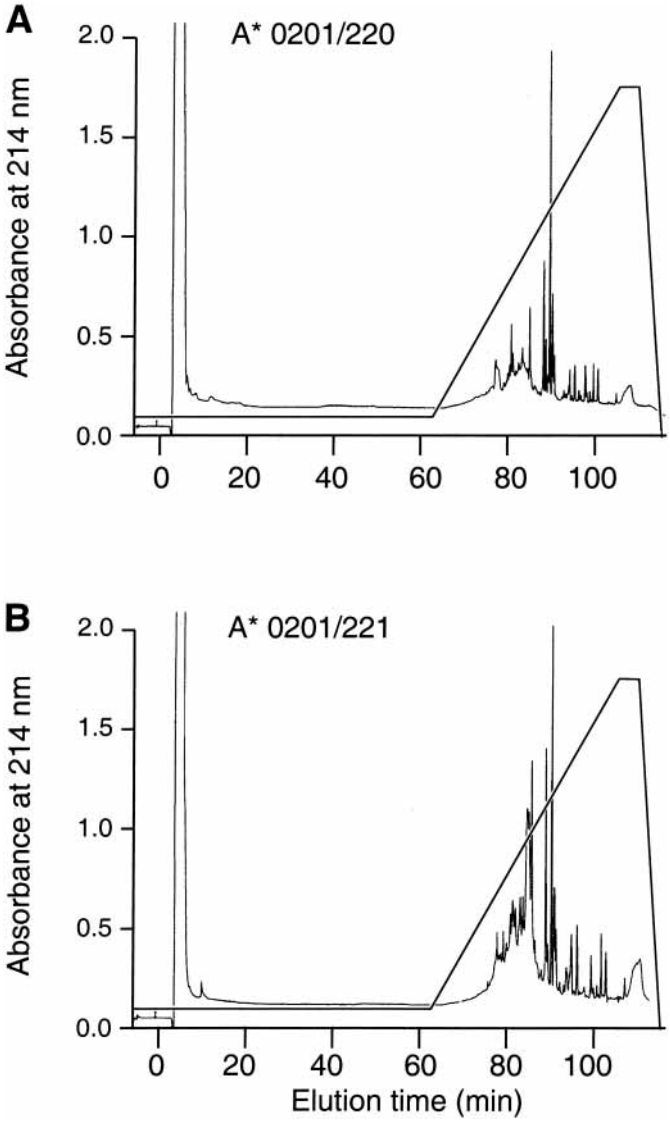

Fig. 2. **Analysis by reversed-phase HPLC of the naturally processed peptides isolated from A*0201 expressed in the absence (panel A) or presence (panel B) of tapasin.** Peptides elute between 70 and 85 min. Material eluting later than 90 min derives from detergent used to prepare the cell lysates.

W6/32 (12) followed by fluorescent-labelled goat anti mouse IgG and analysis by flow cytometry.

Results

Peptides bound by A*0201 were isolated from the tapasin-deficient cell line 721.220/A*0201 and the tapasin-competent partner 721.221/A*0201. Although both cell lines express near identical levels of cell surface A*0201 monitored by immunofluorescent staining and flow cytometry using the monoclonal antibody BB7.2 (data not shown), only a small quantity of A*0201 glycoprotein was recovered from the tapasin-deficient cells by immunoaffinity purification using BB7.2. By SDS-PAGE and Coomassie blue staining and quantification by densitometry based on comparison of β_2-microglobulin (β_2m) to known amounts of a protein standard (lysozyme), only 11 μg of A*0201 glycoprotein was recovered from the tapasin-deficient cells compared to 450 μg from an identical number of tapasin-competent cells (Fig. 1, lanes 10–13). Although the quantity of peptides extracted from A*0201 molecules expressed in the absence of tapasin was much lower than from tapasin-competent cells due to the low yield of class I glycoprotein, those peptides recovered comprise a complex mixture with no high abundance components and is therefore typical of class I peptide repertoires. The ultraviolet (UV) absorbance profiles of the peptides separated by reversed-phase HPLC were very similar for A*0201 from tapasin-deficient and tapasin-competent cells (Fig. 2A, B). Sequence analysis of the pool of A*0201 bound peptides isolated from tapasin-deficient cells yielded a peptide-binding motif comprising leucine at position 2 and an aliphatic hydrocarbon at position 9 (Table 1). This motif is characteristic of A*0201-binding peptides (13–15). Yield for the strong anchor leucine at position 2 for peptides from the tapasin-deficient cells was 42 pmoles and 4093 pmoles from tapasin-competent cells. Peptide yield from tapasin-deficient cells is approximately two-fold lower than predicted from the quantity of A*0201 glycoprotein re-

Peptide-binding motif for HLA-A*0201 expressed in the absence of tapasin

	Peptide position								
	1	2	3	4	5	6	7	8	9
Dominant									
Strong		L	D			I		H	
			Y						
Weak		M		E					L
		Q		H		T			V
				T					F

Residue preferences at each position are grouped into the following categories: dominant; (yield of only one amino acid increases, and the increase is several fold over the previous cycle); strong (amino acids with more than a 2-fold increase over the previous cycle); weak (amino acids with an increase of between 1.5- and 2-fold over the previous cycle). Residues at each position are listed in order of decreasing size of the picomole yield relative to the previous cycle. Because comparison with previous cycles is not possible for cycle 1, any residue preferences at this position are based on percent molar yields of each amino acid with greater than 10% representing enrichment. No significant amino acid signals were detected beyond Edman sequencing cycle 9 indicating that most peptides were nine amino acids or fewer in length. Cysteine was not derivitized and therefore not detectable.

Table 1

A) Peptide-binding motif for HLA-B*2705 expressed in the absence of tapasin

	Peptide position								
	1	2	3	4	5	6	7	8	9
Dominant		R							
Strong	R		Y	E			W		Y
			F	D			F		M
			I	P					F
			M	G					
			N	Q					
			L						
			W						
			H						
			V						
			K						
			D						
			T						
			A						
			Q						

B) Sequences of individual peptides bound endogenously by HLA-B*2705 in the absence of tapasin

Peptide sequence	Homologous protein	Comments
GRLTKHTKF	Homology to rat ribosomal protein L36 36–44	Previously isolated from B*2701 (26), B*2702 (17), B*2704 (27), B*2705 (17) and B*2710 (28)
GRIDKPILK	Ribosomal protein L8 173–181	Previously isolated from B*2705 (16) and B*2710 (28)
GRIGVITNR	40S ribosomal protein S4 189–197	Previously isolated from B*2710 (28)
ARLQTALLV	Homology to rat core histone 188–196	Previously isolated from B*2709 (29) and 8-mer 188–195 isolated from B*2705 (17)
RRFVNVVPTF	40S ribosomal protein S30 114–123	Previously isolated from B*2702 (17)

Table 2

covered. This may result from the A*0201 complexes expressed in the absence of tapasin being more susceptibility to peptide dissociation or proportionately greater losses incurred during isolation and handling of peptides owing to the very small quantity of A*0201 recovered from tapasin-deficient cells.

In contrast to the low recovery of A*0201 glycoprotein, much higher quantities of B*2705 was obtained from tapasin-deficient 721.220/B*2705 cells (330 μg) and a similar amount was obtained from the same number of tapasin-competent 721.221/B*2705 cells (350 μg) (Fig. 1, lanes 6–9). Peptides isolated from B*2705 expressed in the absence of tapasin contained a typical B*2705 peptide-binding motif comprising arginine at position 2 and tyrosine, phenylalanine or methionine at the carboxy-terminal position nine (Table 2A) consistent with previous studies (16, 17). The yield for the dominant anchor arginine at position 2 was 2936 pmoles from tapasin-deficient cells and 3170 pmoles from tapasin-competent cells. Sufficient material was recovered from the tapasin-deficient cells to determine the sequences of five individual peptides from the mixture (Table 2B). These were derived from proteins of cytosolic origin and all have previously been recovered from B*27 molecules expressed by tapasin-competent cells. Therefore the absence of tapasin has less impact on the B*2705 peptide repertoire.

The low yield of A*0201/peptide complexes recovered from tapasin-deficient cells suggests they are unstable and rapidly dissociate. This was examined by measuring the stability of HLA class I/peptide complexes expressed at the surface of tapasin-deficient and tapasin-competent cells. Cells were cultured in the presence of brefeldin A which blocks transport of nascent class I molecules from the ER to the cell surface. The decline of pre-existing HLA class I/peptide complexes at the cell surface was measured at various time points after brefeldin A treatment (Fig. 3). The absence of tapasin had a dramatic effect on the stability of A*0201. A*0201 was rapidly lost from the surface of tapasin-deficient cells. A 25% decrease in the number of molecules at the cell surface was evident after only 100 min compared to 800 min in the presence of tapasin. The decay of B*2705 from the surface of tapasin-deficient cells was slower confirming that B*2705 is more stable than A*0201 in the absence of tapasin. A 25% decrease in the numbers of B*2705 molecules at the surface of tapasin-deficient cells was observed after 160 min. However, the decline in cell surface expression of B*2705 was more rapid on the tapasin-deficient cells compared to the tapasin-competent cells (25% decrease in expression observed after 235 min). Therefore tapasin does enhance the stability of B*2705 molecules.

Discussion

These results show that the repertoire of peptides bound by A*0201 and B*2705 molecules expressed at the cell surface in the absence of tapasin is altered. B*2705 is least affected by the absence of tapasin. The quantity of peptides recovered was consistent with the majority of cell surface B*2705 molecules possessing stably bound peptides. However, the more rapid decay of cell surface B*2705 in the absence of tapasin indicates some changes to the peptide repertoire. A recent detailed analysis of the peptides bound by B*2705 in the absence of tapasin detected subtle differences (18). Some pep-

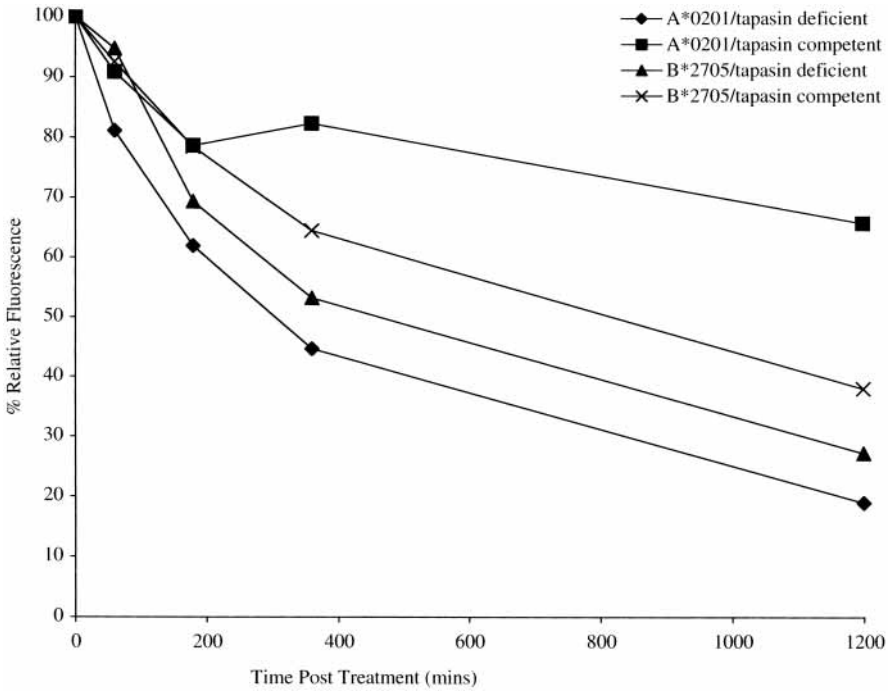

Fig. 3. **Decay of cell surface HLA-A*0201 and B*2705 molecules expressed in tapasin-deficient cells (721.220/A*0201 and 721.220/B*2705) and tapasin-competent cells (721.221/A*0201 and 721.220/B*2705 cotransfected with human tapasin gene).** Cells were treated with brefeldin A, aliquots harvested after 0, 60, 180, 360 and 1200 min and immunofluorescent staining performed with the conformation-dependent HLA class I specific monoclonal antibody W6/32. Results show% relative fluorescence calculated as the percentage of mean channel fluorescence at time 0. Similar rates of decay were observed in two separate experiments.

tides were preferentially or uniquely recovered from either the tapasin-deficient or tapasin-competent cells. However, consistent with our findings, most peptides were not differentially expressed. The significant level of peptide loading achieved by B*2705 in the absence of tapasin may occur because empty peptide receptive B*2705 molecules are inherently more stable than other allotypes (19) which potentially prolongs residence in the ER where they can bind peptides despite not being drawn into intimate contact with TAP by the tapasin bridge. In addition, the abundance of suitable peptides may be relatively high because B*2705 peptides are preferentially transported by TAP (20, 21) and increased peptide concentration in the ER could compensate for loss of tapasin-assisted loading. Alternatively, another mechanism may exist for promoting efficient peptide binding by some allotypes such as B*2705 that operates independent of tapasin-mediated association of HLA class I with TAP (T. Elliott and A. Williams, manuscript submitted).

A*0201 is more dependent on tapasin for efficient peptide loading than B*2705. The majority of A*0201 molecules expressed in the absence of tapasin are unstable. Most A*0201 complexes dissociate during cell lysis in the presence of non-denaturing detergent and loss of A*0201 from the cell surface is very rapid in the absence of tapasin. The instability of these A*0201 complexes indicates that either they are devoid of bound peptides or occupied by low-affinity peptides that readily dissociate. It has been speculated that class I allotypes expressed in the absence of tapasin might be able to utilise an alternative TAP-independent source of peptides (5). Stable A*0201 molecules are expressed at high levels in TAP-deficient cells because the peptide repertoire shifts to a limited number of high abundance species derived from cleaved leader sequences generated in the ER (22, 23). However, our results suggest A*0201 expressed in the absence of tapasin can not exploit this alternative supply of peptides. Although detergent cell lysis conditions and the monoclonal antibody used for purification were the same as those employed to study the peptide repertoire of A*0201 expressed in the absence of TAP (22, 23), the reversed-phase HPLC profile of our peptides did not comprise a few high abundance peaks indicative of high levels of a limited number of peptides. Tapasin is therefore required for efficient loading of TAP-independent peptides. Consistent with this conclusion, the tapasin gene is present in TAP-deficient cells being located just outside the MHC class II region deletion that removes the TAP genes (24) and a study using insect cells expressing various combinations of the molecules involved in peptide loading observed that tapasin promoted stable assembly of A*0201 even in the absence of TAP (25). By promoting the physical association of class I molecules with TAP, tapasin enhances peptide loading when supply is limited as is likely for A*0201 because TAP does not transport A*0201 peptide ligands efficiently (20, 21). Our results also show that tapasin promotes the binding of peptides from both TAP-dependent and TAP-independent sources that produce stable A*0201 complexes.

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