TAP- and tapasin-dependent HLA-E surface expression correlates with the binding of an MHC class I leader peptide

Veronique M. Braud*, David S.J. Allan*, Douglas Wilson† and Andrew J. McMichael*

Background: The human major histocompatibility complex (MHC) class Ib molecule HLA-E is transcribed in most tissues but little is known about its localisation within the cell. We have recently shown that HLA-E binds signal-sequence-derived peptides from human MHC class I molecules *in vitro*.

Results: Using a newly characterised antibody recognising HLA-E, we show that HLA-E is expressed at the cell surface. We demonstrate that HLA-E surface expression is correlated with the presence of MHC class I molecules which provide suitable leader sequence peptides capable of binding to HLA-E. Further studies on the interaction of HLA-E with molecules in the endoplasmic reticulum revealed that HLA-E associates with the transporter associated with antigen processing (TAP) and calreticulin, and that HLA-E expression is TAP-dependent and tapasin-dependent. In addition, HLA-E dissociates from TAP upon binding of MHC class I leader sequence peptides.

Conclusion: These experiments establish that surface expression of HLA-E is regulated by the binding of a restricted pool of peptides from the leader sequence of MHC class I molecules. The correlation between HLA-E and MHC class I surface expression might be relevant to the function of HLA-E. Our results also show that, although these HLA-E binding peptides are derived from signal sequences, they may be released back into the cytosol and subsequently translocated by the TAP complex and loaded onto HLA-E molecules.

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Background

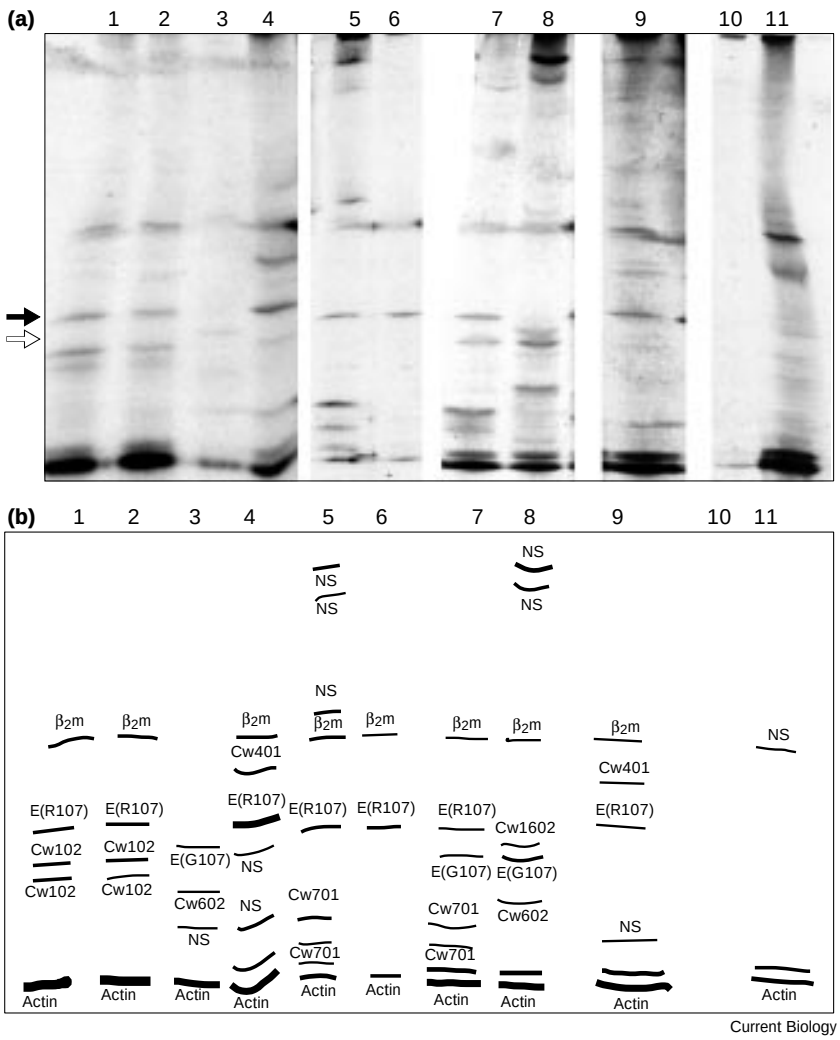
Major histocompatibility complex (MHC) class Ia molecules present peptides derived from cytosolic proteins to $CD8^+$ T cells. Assembly of MHC class I molecules occurs in the endoplasmic reticulum (ER) and requires peptide translocation through the transporter associated with antigen processing, TAP (reviewed in [1]). In human cells, newly synthesised MHC class I heavy chains associate with calnexin which is later displaced by the association of β_2-microglobulin (β_2m) [2–4]. Following dissociation of calnexin, class I–β_2m heterodimers are stably associated with another ER-resident protein, calreticulin [5]. Another molecule, tapasin, which is associated with TAP and with MHC class I–calreticulin complexes, acts as a bridge between them. MHC class I association with TAP facilitates peptide binding and the class I molecules are released and exported to the cell surface upon stable loading of peptide [2,6–8].

Recently, some of the nonclassical MHC class Ib molecules have been shown to bind peptides to fulfil specialised antigen-presentation functions. HLA-G is mainly expressed on human trophoblast and binds a wide range of peptides in a TAP-dependent manner [9,10]. The non-MHC-encoded CD1 may present peptides as well as lipids and glycolipids to T cells [11–14]. The mouse Qa-2 molecule also has the capacity to present a diversity of naturally processed peptides [15]. Murine H-2M3 presents N-formylated peptides to specific T cells, and the presentation requires TAP [16,17]. The mouse Qa-1 molecule binds a very limited pool of peptides derived from MHC class I leader sequences [18,19]. These observations suggest that nonclassical MHC class Ib molecules bind peptides in a similar way to classical MHC class I molecules and that, for most, loading is TAP-dependent.

Some epitopes follow an alternative route to be presented at the cell surface. Signal-sequence-derived peptides are loaded onto HLA-A*0201 and exported to the plasma membrane in the TAP negative cell line T2 [20,21]. This suggests that some leader sequences might be degraded within the ER and the resultant peptides loaded onto newly synthesised class I molecules, independently of TAP. However, presentation of certain epitopes located in signal sequences requires TAP [22,23], and the nonclassical Qa-1 molecule presents leader sequence peptides in a TAP-dependent manner [18].

Recently, we have shown that another human MHC class Ib molecule, HLA-E, is able to bind signal-sequence-derived

Figure 1



(a,b) Characterisation of DT9 reactivity by immunoprecipitations and analysis by one-dimensional isoelectric focusing electrophoresis (1D-IEF). [^{35}S]-methionine and [^{35}S]-cysteine-labelled cell lines: .45 (lane 1), .174 (lane 2), Daudi (lane 3), C1R (lane 4), KM BCL (lane 5), .221 (lane 6), NR BCL (lane 7), JO BCL (lane 8), BM36.1 (lane 9), and mouse L cells transfected with HLA-A*0301 and human β_2m (lane 10) were used for immunoprecipitations with DT9. An immunoprecipitation in .221 cells using the irrelevant antibody K918 is shown in lane 11. Bands were identified according to the HLA typing and compared with immunoprecipitations with the anti-MHC class I antibody W6/32 (data not shown). In (b), nonspecific bands (NS) were seen in samples processed in a similar way, but without addition of any antibodies (data not shown). In (a), the closed arrow indicates the HLA-E*0101 allele which has an arginine at residue 107, and the open arrow the HLA-E*0103 and E-N3 alleles which have a glycine at position 107. HLA typing was as follows: .45 (HLA-A*0201, B*5101, Cw*0102, E*0101); .174 (HLA-A*0201, B*5101, Cw*0102, E*0101); Daudi (HLA-A*0102, A*6601, B*5801, B*5802, Cw*0302, Cw*0602, E*0103, E-N3); C1R (HLA-B*3501, Cw*0401, E*0101); KM BCL (HLA-A*0101, A*68, B*8, Cw*701, E*0101, E*0101); .221 (HLA-E*0101); NR BCL (HLA-A*0101, A*0301, B*8, B*60, Cw*3, Cw*0701, E*0101, E*0103); JO BCL (HLA-A*0201, A*0301, B*5101, B*5701, Cw*1602, Cw*0602, E*0103, E-N3); BM36.1 (HLA-A*0101, B*3501, Cw*4, E*0101, E*0101).

peptides similar to those binding to Qa-1 [18,19,24,25]. The optimum binding peptide is a nonamer, and using alanine and glycine substitutions we established primary anchor residues at positions 2 and 9 and secondary anchor residues at position 7 and possibly 3. Until now, data suggested that HLA-E was largely localised in the ER. Neither mouse cells transfected with HLA-E and human β_2m, nor the LCL721.221 cell line (.221 cells), which only expresses HLA-E and HLA-F, show cell-surface expression of HLA-E [25–27].

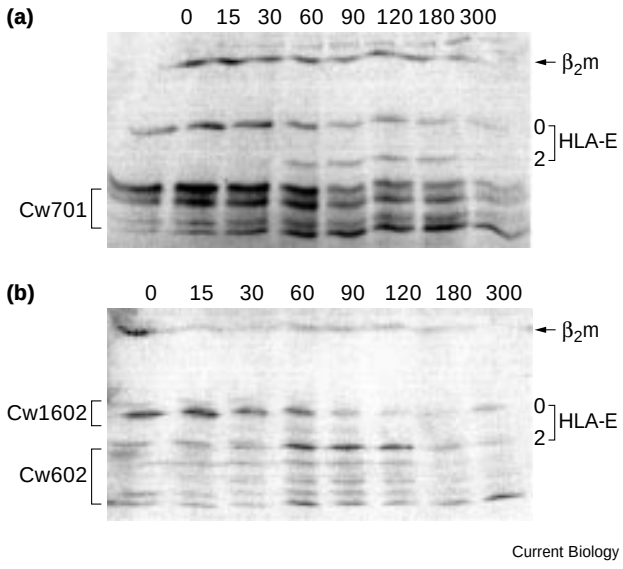
We report here that HLA-E is expressed at a low level on the surface of cells. Its expression is correlated with co-expression of human MHC class I molecules, providing leader sequence peptides capable of binding to HLA-E. Loading of these signal-sequence-derived peptides is TAP and tapasin-dependent and HLA-E assembly appears to be similar to classical MHC class I assembly.

Results

Maturation of HLA-E in lymphoblastoid cells detected by the monoclonal antibody DT9

We recently characterised a mouse monoclonal antibody DT9 that recognises cottontop tamarin MHC class I molecules and cross-reacts with human HLA-E and HLA-C molecules. This antibody detects both HLA-E heavy chains complexed with human β_2m and free HLA-E heavy chains, as shown by immunoprecipitations from B-cell lines and the β_2m-negative cell line Daudi respectively (Figure 1). DT9 recognises the two previously reported HLA-E alleles, which differ by a single amino-acid residue at position 107, HLA-E-arginine-107 and HLA-E-glycine-107 (Figure 1) [28]. Immunoprecipitations from B-cell lines expressing different HLA haplotypes demonstrated that DT9 does not cross-react with HLA-A and HLA-B alleles but does recognise HLA-C molecules (Figures 1,2a,2b and data not shown). The

Figure 2



HLA-E matures in lymphoblastoid cells. Pulse-chase experiments using DT9 were performed in **(a)** KM BCL cells and **(b)** JO BCL cells. Cells were pulsed for 15 min with [^{35}S]-methionine and [^{35}S]-cysteine and chased for the times indicated (in min) along the top of the gels. DT9 immunoprecipitates were analysed by 1D-IEF. The numbers to the right of the gels correspond to the number of sialic acid groups on HLA-E.

cross-reaction with HLA-C was confirmed in .221 cells transfected with HLA-C alleles, in which staining with DT9 was equivalent to that with the anti-MHC class I antibody W6/32 (data not shown).

We used DT9 to study the maturation of HLA-E in B-cell lines by pulse-chase experiments followed by one-dimensional isoelectric focusing electrophoresis (1D-IEF). In homozygous B cell lines, both HLA-E alleles matured to sialated forms after a 1 hour chase, indicating that the molecule had left the ER and reached the Golgi network (Figure 2a,b). The more acidic bands appearing with time were removed by treatment of the precipitate with neuraminidase, confirming that they represented the sialated forms of HLA-E (see Supplementary material). The rate of intracellular trafficking was slow compared with most HLA-A and HLA-B molecules, which are sialated after 15–30 minutes [29,30]. HLA-E matured with a half-time between 60 and 90 minutes. Mature HLA-E molecules disappeared after 3–5 hours, implying that HLA-E turnover is more rapid than that of classical class I molecules.

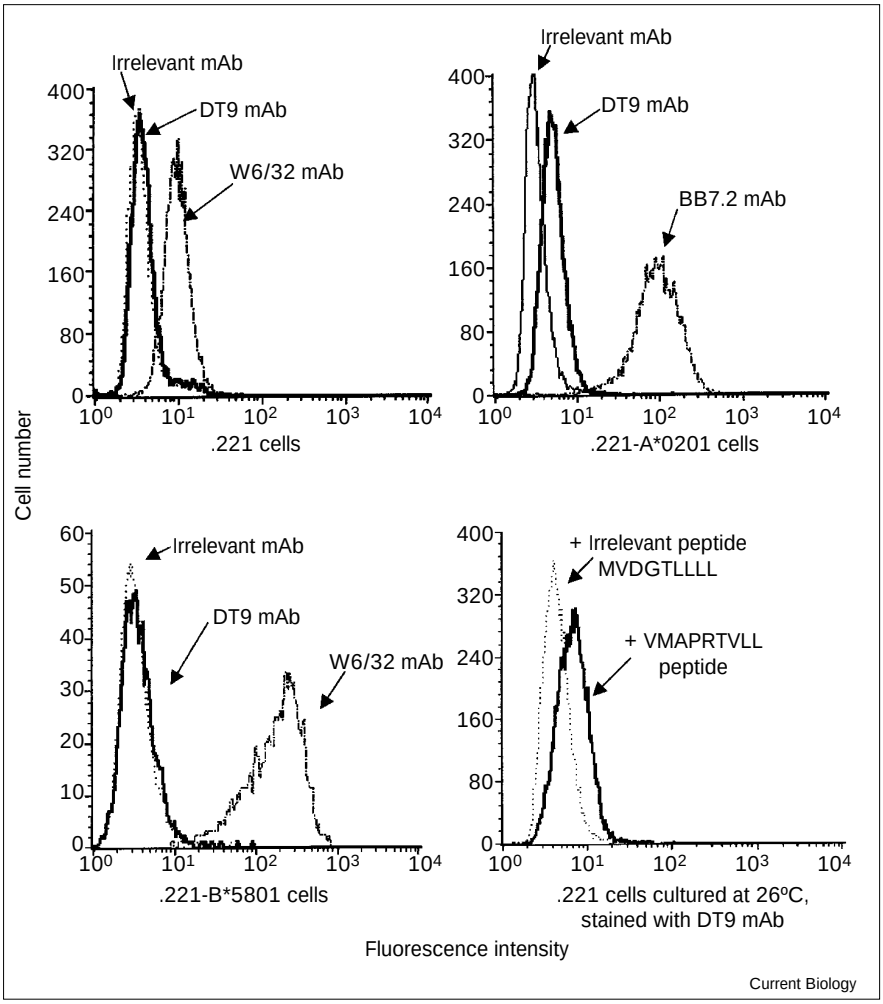
Surface expression of HLA-E correlates with the binding of MHC class I signal-sequence-derived peptides

DT9 was used to detect HLA-E at the cell surface by flow cytometry. As shown in Figure 3, DT9 mAb did not stain HLA-A, HLA-B, HLA-C and HLA-G negative .221 cells, indicating that in these cells HLA-E does not traffic to the plasma membrane. The weak staining by the anti-class I antibody W6/32 was possibly due to surface expression of HLA-F; a sialated form of HLA-F appeared after 90 minutes in a pulse-chase experiment using .221 cells and W6/32 (data not shown). Lack of surface expression of HLA-E in .221 cells was consistent with pulse-chase experiments, which showed a single band representing the nonsialated form of HLA-E retained in the ER (Figure 4a).

We previously reported that HLA-E, like the mouse Qa-1 molecule [18,19,24], binds *in vitro* peptides derived from the leader sequence of some MHC class I molecules [25]. Because their leader sequences are shortened, peptides capable of binding to HLA-E cannot be generated from the endogenous HLA-E and HLA-F in .221 cells [25]. To test whether lack of HLA-E surface expression in .221 cells could be explained by lack of binding of a signal-sequence-derived peptide, we analysed the maturation and expression of HLA-E in .221 cells transfected with HLA-A*0201, whose leader sequence peptide binds to HLA-E [25]. The .221-A*0201 cells, in contrast to .221, were stained by DT9, suggesting that the leader peptide might contribute to HLA-E surface expression (Figure 3). The surface expression was low in comparison with the expression of classical MHC class I molecules. Sialated forms of HLA-E were also detected after 1 hour in a pulse-chase experiment using DT9 in .221-A*0201 cells (Figure 4b). It was confirmed that DT9 can detect HLA-E binding to such leader sequence peptides as staining was observed on .221 cells incubated at 26°C in the presence of the leader-sequence peptide 3–11 from HLA-B*0801 but not with an irrelevant peptide (Figure 3).

To rule out the possibility that HLA-E was binding an HLA-A*0201 peptide distinct from the signal sequence-derived peptide 3–11, we stained several other .221 cells transfected with HLA-A and HLA-B alleles (Figure 3, Table 1). The leader peptide 3–11 from these alleles displays some polymorphism, mainly at positions 2, 7 and 8 in the peptide (Table 1). The corresponding leader peptides were synthesised and tested for binding to HLA-E in the *in vitro* peptide-binding assay previously described ([25]; data not shown). The substitution of the methionine for a threonine at position 2 in the peptide, which is an anchor residue, disrupted peptide binding whereas the polymorphisms at positions 7 and 8 (leucine for valine or phenylalanine) did not. DT9 bound to .221 cells transfected with an HLA allele with a leader peptide capable of binding to HLA-E (Figure 3, Table 1). The only exceptions were HLA-B*1302 and HLA-B*2705, which induced a low level of HLA-E surface expression despite having the inappropriate sequence (Table 1); this is being fully investigated. Among the HLA alleles possessing a leader peptide capable of binding to HLA-E, there was no common amino-acid sequence with an HLA-E-binding

Figure 3



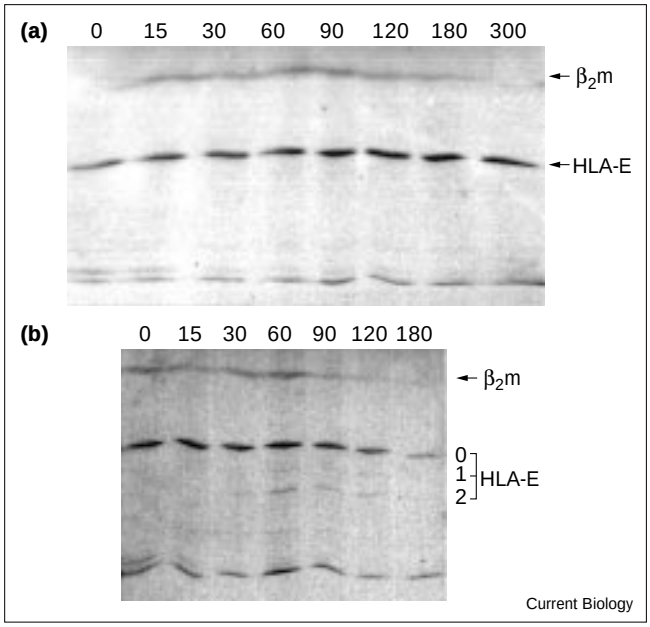
HLA-E is expressed at the cell surface when an MHC class I leader-sequence peptide capable of binding to HLA-E is available. HLA-E cell surface expression was analysed by flow cytometry on .221, .221-A*0201 and .221-B*5801 cells using DT9, W6/32 or BB7.2 monoclonal antibodies (mAb), and K918 as an irrelevant antibody. HLA-E was also stabilised at the cell surface by culturing .221 cells at 26°C in the presence of exogenous added peptides: MVDGTLLLL from HLA-E leader sequence which is not capable of binding to HLA-E *in vitro* and VMAPRTVLL from HLA-B*0801 which can bind to HLA-E.

motif except for the leader sequence peptide 3–11. The correlation therefore demonstrates that HLA-E expression is significantly increased on binding of peptides derived from the leader sequences of some MHC class I molecules and implies that HLA-E binds a restricted pool of peptides. The mouse HLA-E homologue Qa-1 also predominantly binds a single leader sequence peptide from mouse MHC class I molecules [19,24].

HLA-E interaction with ER-resident molecules

We studied HLA-E interactions with ER chaperone molecules and the TAP complex. Immunoprecipitations in mild detergents such as digitonin maintain TAP–class I or calreticulin-class I interactions [2,5]. The results of immunoprecipitations in digitonin with anti-TAP2, and anti-calreticulin antibodies are shown in Figure 5. Whereas no HLA-E–β_2m complexes were recovered in the TAP-negative cell line T2 following an anti-TAP2 immunoprecipitation, HLA-E was associated with the TAP complex in C1R-A2 and .221 cells (Figure 5a). HLA-Cw*0401 and HLA-A*0201 were also associated with TAP in C1R-A2 cells (Figure 5a and data not shown). Thus, the HLA-E–TAP interaction occurred in cells in which MHC leader sequence peptides capable of binding to HLA-E were (C1R-A2) or were not (.221) available. HLA-E does not mature rapidly (Figure 2a,b) and might therefore stay associated with TAP while waiting to be loaded with peptide. Ortmann *et al.* [2] previously failed to detect any MHC class I molecules associated with TAP in digitonin lysates of .221 cells. This difference might be due to an insufficient exposure time, as the amount of HLA-E is lower than classical class I molecules. HLA-E–TAP association was also recently observed in the trophoblast-derived choriocarcinoma cell line JAR [31]. Immunoprecipitations in digitonin with an anti-calreticulin antibody also showed that HLA-E associated with calreticulin in both TAP-negative and TAP-positive cells (Figure 5b). HLA-E therefore seems to behave like classical MHC class I molecules, associating with both TAP and calreticulin.

Figure 4



HLA-E remains in the ER in .221 cells but export to the Golgi can be restored by transfection of HLA-A*0201. Pulse-chase experiments using DT9 for the times indicated (in min) in (a) .221 and (b) .221-A*0201 cells were performed and analysed by 1D-IEF. The numbers to the right of the gel in (b) correspond to the number of sialic acid groups on HLA-E.

Table 1

HLA-E cell-surface expression

HLA class I alleles	Signal sequence-derived peptide (residues 3–11)	.221 transfectants	FACS staining with DT9 mAb
Possessing a leader sequence peptide (residues 3–11) capable of binding to to HLA-E*	VMAPRTLLL	.221-A*0101	+
	VMAPRTLVL	.221-A*0201	+
	VMAPRTLVL	.221-A*0211	+
	VMAPRTLLL	.221-A*0301	+
	VMAPRTLVL	.221-A*2403	+
	VMAPRTLVL	.221-A*2501	+
	VMAPRTLLL	.221-A*3601	+
	VMAPRTVLL	.221-A*0702	+
	VMAPRTVLL	.221-A*0801	+
	VMAPRTLFL	.221-G	+
Not possessing a leader sequence peptide (residues 3–11) capable of binding to HLA-E*	†	.221	–
	VTAPRTLLL	.221-B*1302	±
	VTAPRTVLL	.221-B*1501	–
	VTAPRTLLL	.221-B*2705	±
	VTAPRTVLL	.221-B*3505	–
	VTAPRTVLL	.221-B*4601	–
	VTAPRTVLL	.221-B*5101	–
	VTAPRTLLL	.221-B*5401	–
	VTAPRTLLL	.221-B*5501	–
	VTAPRTVLL	.221B*5801	–

*The binding of synthetic peptides was tested in an *in vitro* peptide-binding assay, as previously described ([25] and data not shown).†.221 cells do not express HLA-A, HLA-B, HLA-C and HLA-G class I molecules but express HLA-E and HLA-F, which have a shorter leader sequence and lack the appropriate peptide capable of binding to HLA-E.

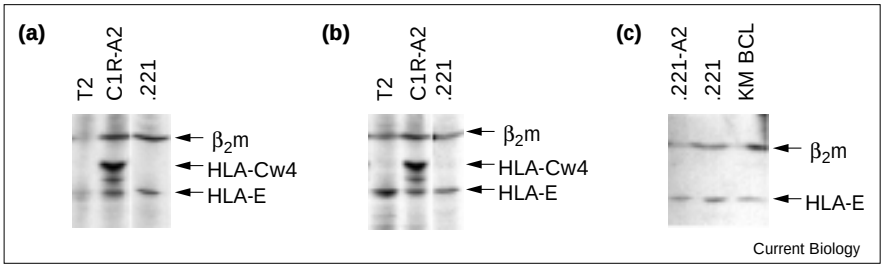
To confirm the above result and verify that in B-cell lines HLA-E was part of the TAP–tapasin–class I–calreticulin complexes described by Sadasivan *et al.* [5], we performed two sequential immunoprecipitations in cells of the KM B-cell line, .221-A*0201 and .221 cells. The first immunoprecipitation was done in digitonin using an anti-TAP2 antibody in order to immunoprecipitate the multicomplex. As the TAP and calreticulin association with MHC class I is not maintained in NP-40 lysis buffer, we destabilised the complex by addition of this detergent and performed a second immunoprecipitation using DT9. HLA-E was recovered from KM BCL, .221-A*0201 or .221, following these sequential immunoprecipitations (Figure 5c). It has previously been shown that an equal amount of class I can be recovered after immunoprecipitation with an anti-TAP antibody followed by an anti-calreticulin or an anti-class I antibody, suggesting that the pool of class I molecules associated with TAP is simultaneously associated with calreticulin [5]. In light of the results shown in Figure 5c, we conclude that HLA-E is also part of this multicomplex.

Interaction with tapasin was also studied using the tapasin-defective cell line .220 transfected with HLA-A*0201 (.220-A*0201) [32,33]. Whereas HLA-A*0201 is detected at the cell surface of .220-A*0201 [32], no HLA-E surface expression was observed (Figure 6), despite its presence in .220-A*0201 cells, detected by immunoprecipitations using W6/32 and DT9 (data not shown). No HLA-E molecules were recovered following TAP coprecipitation in .220-A*0201 (data not shown), consistent with the HLA-B8, -A1 and -G phenotype in .220 described elsewhere [33]. We conclude that HLA-E assembly and maturation requires tapasin, acting as a bridge between TAP and HLA-E–calreticulin complexes. HLA-E therefore interacts with TAP, calreticulin and tapasin, like classical MHC class I molecules, although it binds signal-sequence-derived peptides.

Binding of signal-sequence-derived peptides to HLA-E is TAP-dependent

We assessed the contribution of the TAP complex to the loading of peptides onto HLA-E. Using DT9, we stained three TAP-deficient cell lines in which TAP1 and TAP2 are either missing (T2 [34] and .174 [35]) or not functional because of a frameshift mutation in one of the subunits (BM36.1 [36]). A negligible level of HLA-E expression was observed on the surface of the cells (Figure 7a and data not shown). Furthermore, the sialated forms of HLA-E were not detected in a pulse-chase experiment in T2-A3 cells (data not shown). In contrast, when BM36.1 infected by a recombinant vaccinia virus encoding both human TAP1 and TAP2 subunits was metabolically labelled for 6 hours, sialated HLA-E appeared (Figure 7b).

Figure 5

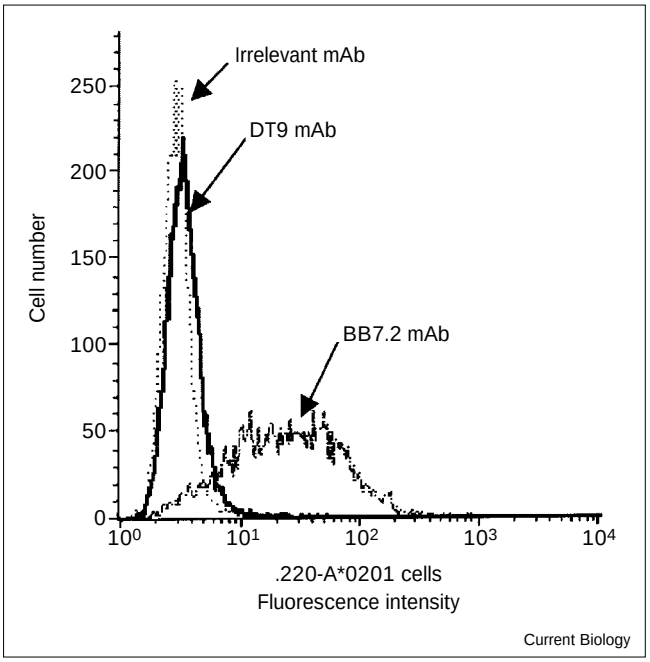


HLA-E associates with TAP and calreticulin. (a) TAP coprecipitation in digitonin lysate using a rabbit anti-TAP2 serum was performed in T2, C1R-A2 and .221 cells. Immunoprecipitates were analysed by 1D-IEF. (b) Calreticulin coprecipitation in digitonin lysate using a rabbit anti-calreticulin serum was also performed in T2, C1R-A2 and .221 cells and analysed by 1D-IEF. (c) Molecules associated with TAP were first immunoprecipitated in digitonin lysate using a rabbit anti-TAP2 serum from .221-A*0201, .221 and KM BCL cells. TAP-associated molecules were then eluted in NP- 40 lysis buffer and a second immunoprecipitation using DT9 was performed. Samples were analysed by 1D-IEF.

Together, these results demonstrate that HLA-E expression is TAP-dependent.

We then investigated whether HLA-E, like classical MHC class I molecules, dissociates from TAP upon binding of peptides translocated from the cytosol: .221 cells were metabolically labelled and permeabilised with streptolysin O and after incubation with or without leader peptides, TAP was precipitated in digitonin [2]. The rate of dissociation of HLA-E from TAP was enhanced by the introduction into the cytosol of the leader sequence peptide from HLA-A*0201 which binds to HLA-E (Figure 8, lane 2), compared to no peptide (lane 1) or the HLA-E leader peptide (lane 3), which does not bind to HLA-E [25]. Thus, leader-sequence peptides from MHC class I molecules can be translocated by the TAP complex, which is also important for loading onto HLA-E. These results imply that, although located in leader sequences, these peptides must reach the cytosol and then be translocated back into the ER by the TAP complex to be loaded onto HLA-E.

Figure 6



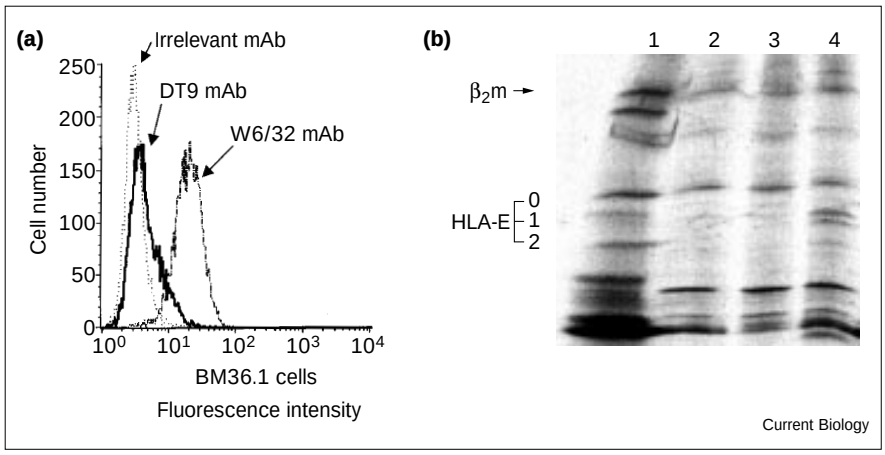
HLA-E expression is tapasin-dependent. Flow cytometry staining of .220-A*0201 cells using DT9 and BB7.2 monoclonal antibodies (mAb) and K918 as an irrelevant antibody.

Discussion

We used an antibody that recognises HLA-E and HLA-C molecules to demonstrate that HLA-E is stably expressed at the surface of human cells that also express certain MHC class I molecules. HLA-E surface expression correlates with binding of signal sequence-derived peptides from MHC class I molecules. HLA-E therefore binds a restricted pool of peptides, in a similar fashion to the mouse MHC class Ib Qa-1 molecule, which predominantly binds a single peptide derived from the signal sequence of H-2D and H-2L class I molecules [18,19]. Mouse Qa-1 and HLA-E might have a similar and conserved function; both have limited polymorphism, a broad tissue distribution, a low cell-surface expression and a slow maturation rate [37]. They also share some unique substitutions in the binding groove, especially a serine replacing a tryptophan at position 147, which disrupts the highly conserved hydrogen bond to the main-chain carbonyl oxygen of P8 of the peptide found in other MHC class I molecules [25,38]. This might explain the restricted pool of peptides, and the kinetics of HLA-E turnover observed in pulse-chase experiments (Figure 2a,b).

We also showed that HLA-E expression is TAP-dependent and tapasin-dependent and that HLA-E associates with calreticulin, tapasin and TAP. HLA-E assembly therefore seems to be similar to MHC class I assembly. This is consistent with HLA-E sharing many of the characteristics of classical MHC class I molecules. First, the percentage of amino-acid sequence homology between HLA-E and HLA-A, HLA-B or HLA-C molecules varies from 50 to 90%, depending on the exon [39]. Second,

Figure 7



HLA-E expression is TAP-dependent. (a) FACS analysis of the TAP-negative cell line BM36.1. Cells were stained with DT9 and W6/32 monclonal antibodies (mAb), and K918 as an irrelevant antibody. (b) CO BCL cells (lane 1); BM36.1 untreated (lane 2), infected with wild-type vaccinia virus (lane 3), or infected with a vaccinia virus encoding the two human subunits of TAP (lane 4) were metabolically labelled for 6 h and immunoprecipitations were carried out using DT9 and analysed by 1D-IEF. The numbers to the left of the gel in (b) correspond to the number of sialic acid groups on HLA-E.

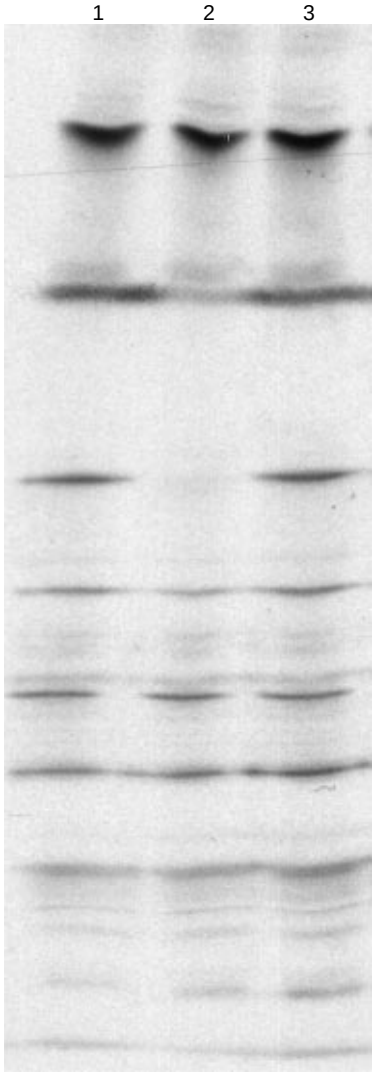
HLA-E has a very similar structure to its classical MHC counterparts (C. O'Callaghan *et al.*, personal communication). Third, HLA-E heavy chain associates with β_2m and binds peptides [25,27,40]. Fourth, HLA-E tissue distribution matches classical MHC class I tissue distribution [41].

It is surprising that the loading of the signal-sequence-derived peptide requires the presence of TAP, but similar observations have been made for the mouse Qa-1 molecule [18]. TAP-dependent HLA-E expression could result from TAP acting as a chaperone in the ER. If leader sequences are generated in the ER, HLA-E might load peptides only when associated with TAP, without requiring peptide translocation. This is, however, unlikely, because HLA-E is not expressed at the cell surface of BM36.1, which has a normal TAP1 subunit to interact with class I, and a different cytosolic carboxy-terminal domain in TAP2. Despite this nonfunctional TAP2, the complex still assembles and can be immunoprecipitated with an anti-TAP1 antibody [36]. It is therefore likely that HLA-E associates normally with TAP in BM36.1, making a simple non-transporting role for TAP quite unlikely.

The full-length leader sequences of MHC class I molecules, or a peptide generated from them, might recycle back to the cytosol. Studies on the processing of signal sequences suggest that some signal-sequence peptides are released into the cytosol. The processing of the preprolactin signal sequence in eukaryotic cells results in the production of two fragments, the amino-terminal peptide being released into the cytosol [42]. Peptides derived from MHC class I signal sequences might follow this pathway, particularly as the peptide binding to HLA-E is at the amino terminus of the signal sequence. One other leader sequence peptide known to be presented in a TAP-dependent manner is also located at the amino terminus [23]. In addition, other MHC class I leader sequence peptides have been eluted from MHC class I or class II molecules, all located at the amino terminus of the signal sequence, close to the peptide binding to HLA-E [43,44]. This suggests that HLA class I signal sequences might all be processed in a similar way. Further investigations are in process to confirm these hypotheses. Another possibility is that the full-length signal sequence from MHC class I molecules, once released into the ER, cannot be degraded by ER resident proteases (reviewed in [1]). Long peptides or misfolded proteins can recycle back into the cytosol to be further degraded by the proteasome to generate nonamer peptides, which are then translocated back into the ER through the TAP complex [45–48]. Signal sequences from class I molecules could follow this route.

It is intriguing that the two nonclassical class I molecules Qa-1 and HLA-E bind and present signal-sequence-derived peptides at the surface of cells expressing MHC class I molecules. It is interesting that HLA-E expression is controlled not only by MHC class Ia molecules, but also by HLA-G [41,49]. It is likely that HLA-E trafficks to the cell surface of trophoblast cells because HLA-G leader peptide 3–11 can bind to HLA-E and HLA-E is expressed on .221-G cells (Table 1) [25]. HLA-E might therefore have a function that correlates with the expression of other MHC class I molecules. One possibility might be that HLA-E interacts with a receptor on natural killer (NK) cells in order to protect cells expressing MHC class I from NK cell-mediated lysis. Alternatively, HLA-E might interact with $CD8^+$ T cells, as the residues involved in CD8 interaction are conserved in HLA-E [50]. HLA-E, like Qa-1, might therefore be involved in alloreactive responses and in immune regulation by $CD8^+$ T cells of $CD4^+$ T cells expressing a specific V_β T-cell receptor [37,51,52]. The leader-sequence peptides from MHC class I could also be displaced by specific peptides during bacterial or viral infections and HLA-E might present these peptides to $\alpha\beta$ or $\gamma\delta$ T cells.

Figure 8



HLA-E dissociates from TAP on binding of MHC leader-sequence peptide. Metabolically labelled .221 cells were permeabilized using streptolysin O and incubated for 10 min at 37°C, with no peptide (lane 1), VMAPRTLVL peptide (lane 2), or MVDGTLLLL peptide (lane 3) to allow transport through the TAP transporter. The VMAPRTLVL peptide from the leader sequence of HLA-A*0201, is capable of binding to HLA-E *in vitro*, whereas the MVDGTLLLL peptide from HLA-E leader sequence is not. Cells were then lysed in digitonin. TAP and molecules associated with TAP were immunoprecipitated using a rabbit anti-TAP2 serum. Precipitates were analysed by 1D-lEF.

Conclusions

Here, we demonstrate for the first time that HLA-E is stably expressed at the cell surface. HLA-E surface expression correlates with the expression of those MHC class I molecules which possess a peptide in their leader sequence capable of binding to HLA-E. Like classical MHC class I molecules, HLA-E is associated with calreticulin, tapasin and TAP in the ER and HLA-E surface expression is TAP-dependent and tapasin-dependent. These findings might be relevant to protection from NK cell-mediated lysis.

Materials and methods

Cell lines and HLA class I typing

The mutant cell lines LCL721.221 (.221), .221-A*0201 and .220-A*0201 were kindly provided by R.DeMars (University of Wisconsin, Madison). All the other .221 cells transfected with various HLA alleles were generously given by P. Parham (Stanford University, Stanford, California). Mouse L cells transfected with HLA-A*0301 and human β_2m were provided by F. Lemonnier (Pasteur Institute, Paris). The HLA-A, -B, and -C typing was performed on DNA by PCR utilising sequence-specific primers (PCR–SSP), as described elsewhere [53,54]. The HLA-E DNA typing was kindly performed by R. Steffensen (Aalborg Hospital, Aalborg, Denmark), by PCR utilising sequence-specific oligonucleotides (PCR–SSO), according to the 12th International Histocompatibility Workshop protocol.

Antibodies, peptides and vaccinia viruses

DT9 was generated by immunisation of mice with purified tamarin MHC class I molecules (using anti-class I antibody BB7.7), and screened by flow cytometry on tamarin B-cell lines, tamarin T-cell lines and human B-cell lines. W6/32 detects a conformational epitope on human MHC class I molecules. BB7.2 sees a specific determinant on HLA-A2 and A69 molecules loaded with peptides. K918 monoclonal antibody, which recognises H-2K^d molecules was used in all the experiments as an irrelevant antibody, and was kindly provided by A. Townsend (Institute of Molecular Medicine, Oxford). A polyclonal rabbit anti-TAP2 serum kindly provided by J. Neefjes (The Netherlands Cancer Institute, Amsterdam) was used for the coprecipitations in digitonin lysates. The polyclonal rabbit anti-calreticulin serum was purchased from Affinity Bioreagents. Peptides from MHC class I leader sequences were synthesised on a SMPS-350 automated peptide synthesiser (Zinsser Analytical) by conventional Fmoc chemistry, precipitated with ether, lyophilised, and subjected to HPLC to ascertain purity (> 95%). The vaccinia virus encoding human TAP1 and TAP2 subunits (hTAP1 +2-Vac) was kindly provided by J. Yewdell (National Institute of Allergy and Infectious Diseases). BM36.1 cells were infected with 5 plaque forming units (PFU) per cell, with wild-type vaccinia virus (WT-Vac) or hTAP1+2-Vac for 90 min at 37°C during the metabolic labelling.

Flow cytometry

HLA-E and HLA class I expressions were monitored by flow cytometry using the panel of antibodies described above and a fluorescein-conjugated goat anti-mouse IgG F(ab')$_2$. Cells were analysed on a FACScan (Becton Dickinson). For the stabilisation of HLA-E at the cell surface, .221 cells were cultured overnight at 26°C in the presence of 50 μM peptides.

Pulse-chase experiments

Pulse-chase experiments were performed as previously described [30]. Cells were pulse-labelled for 15 min with 100 μCi [^{35}S]-methionine + [^{35}S]-cysteine (Amersham) and chased for the indicated times. A standard immunoprecipitation of HLA-E was performed using DT9. Samples were analysed by 1D-lEF, followed by autoradiography.

Coprecipitation in digitonin lysates

Metabolically labelled cells were lysed in digitonin lysis buffer, as previously described [7] A standard immunoprecipitation was then performed by addition of the anti-TAP2 or anti-calreticulin polyclonal rabbit sera (10 μl and 5 μl respectively). For the sequential immunoprecipitations, after immunoprecipitation by the anti-TAP2 antibody in digitonin, dried beads binding the multicomplex calreticulin–class I–tapasin–TAP were resuspended in NP-40 lysis buffer. Supernatants containing the molecules dissociated from TAP were subjected to a second immunoprecipitation with DT9.

Dissociation of MHC class I molecules from TAP

Metabolically labelled cells were washed in transport buffer (5 mM Hepes pH 7.3, 130 mM KCl, 10 mM NaCl, 1 mM $CaCl_2$, 2 mM EGTA, 2 mM $MgCl_2$) and permeabilized by addition of 2.5 IU ml^{-1} streptolysin O (Wellcome Diagnostic) for 4 min at 37°C. Peptides and ATP were added at a final concentration of 100 μM and 10 mM respectively, and cells were incubated for another 10 min at 37°C, to allow translocation through the TAP complex. Transport was stopped by addition of 10 mM EDTA and 0.02% NaN_3. A standard TAP coprecipitation in digitonin was then performed as described above.

Supplementary material

Figure S1 showing identification of the sialated forms of HLA-E and Figure S2 demonstrating HLA-E cell-surface expression when an MHC class I leader-sequence peptide capable of binding to HLA-E is available are published with this paper on the internet.

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