HLA-E: Strong Association with β_2-Microglobulin and Surface Expression in the Absence of HLA Class I Signal Sequence-Derived Peptides[1]

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The nonclassical class I HLA-E molecule folds in the presence of peptide ligands donated by the signal sequences of permissive class I HLA alleles, with the aid of TAP and tapasin. To identify HLA-E-specific Abs, four monoclonals of the previously described MEM series were screened by isoelectric focusing (IEF) blot and immunoprecipitation/IEF on >30 single-allele class I transfectants and HLA-homozygous B lymphoid cells coexpressing HLA-E and HLA-A, -B, -C, -F, or -G. Despite their HLA-E-restricted reactivity patterns (MEM-E/02 in IEF blot; MEM-E/07 and MEM-E/08 in immunoprecipitation), all of the MEM Abs unexpectedly reacted with β_2-microglobulin (β_2m)-free and denatured (but not β_2m-associated and folded) HLA-E H chains. Remarkably, other HLA-E-restricted Abs were also reactive with free H chains. Immunodepletion, in vitro assembly, flow cytometry, and three distinct surface-labeling methods, including a modified (conformation-independent) biotin-labeling assay, revealed the coexistence of HLA-E conformers with unusual and drastically antithetic features. MEM-reactive conformers were thermally unstable and poorly surface expressed, as expected, whereas β_2m-associated conformers were either unstable and weakly reactive with the prototypic conformational Ab W6/32, or exceptionally stable and strongly reactive with Abs to β_2m even in cells lacking permissive alleles (721.221), TAP (T2), or tapasin (721.220). Noncanonical, immature (endoglycosidase H-sensitive) HLA-E glycoforms were surface expressed in these cells, whereas mature glycoforms were exclusively expressed (and at much lower levels) in cells carrying permissive alleles. Thus, HLA-E is a good, and not a poor, β_2m assembler, and TAP/tapasin-assisted ligand donation is only one, and possibly not even the major, pathway leading to its stabilization and surface expression. ***The Journal of Immunology,*** **2008, 181: 5442–5450.**

The expression of the nonclassical HLA-E molecule on the cell surface requires the stabilization of the heterodimer formed by the HLA-E H chain (42 kDa) with its light (12-kDa) β_2-microglobulin (β_2m)[3] subunit (1–3). There are two nonsynonymous HLA-E alleles (4): HLA-E*0101 (HLA-E^{107R}) and HLA-E*0103 (HLA-E^{107G}), less and more abundant on the cell surface, respectively (5). In several experimental systems, the stabilization of HLA-E^{107R} depends on the binding of short peptide ligands (nonamers) cleaved from the signal sequences of other classical (HLA-A, -B, -C) and nonclassical (HLA-G) class I H chains, often referred to as permissive alleles, and by the coexpression of two endoplasmic reticulum resident proteins and members of the so-called peptide-loading complex, e.g., TAP and tapasin (1–3, 6–9). The former transports the precursors of HLA-E ligands across the endoplasmic reticulum membrane; the latter provides crucial chaperoning/peptide-loading functions.

HLA-E molecules loaded with signal sequence-derived peptides engage the inhibitory immune receptor NKG2A, and protect target cells from NK lysis (1–3, 7). Thus, HLA-E monitors for integrity a large section of, and several functions crucial for, class I Ag processing, loading, and presentation. Accordingly, the crystallography of HLA-E is consistent with structural constraints on binding throughout the entire length of the peptide, e.g., tight association with a defined set of ligands (10).

These data illustrate the role of HLA-E as a dedicated acceptor of signal sequence-derived peptides. However, HLA-E may also trigger NK-CTLs, a more recently described subset of CD8 effectors with memory phenotype that directly recognize and lyse target cells via (oligo)-clonally rearranged TCRs (2, 11). In addition, several biochemical observations listed below (numbered 1–7) argue against selective assembly and suggest instead multiple mechanisms of stabilization, possibly mediated by alternative classes of ligands.

For instance: 1) HLA-E has been reported to have a low affinity for β_2m and several signal sequence-derived peptide ligands (5), as such being similar to its murine homologue Qa-1 (12); 2) pool sequencing of peptides eluted from HLA-E H chain-β_2m assemblies upon refolding in vitro around a recombinant random peptide library did not support a clear preference for motifs from the signal peptides of class I H chains with methionine at the P2 anchor position, but rather suggested a generic preference for hydrophobic amino acids at many positions (13); 3) several HLA-E ligands from microbial and self proteins have been identified with alternative (not always hydrophobic) residues at anchor and nonanchor

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[3] Abbreviations used in this paper: β_2m, β_2-microglobulin; Endo H, endoglycosidase H; IEF, isoelectric focusing.

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positions (8, 14–21). In addition, 4) widely used Abs to HLA-E (e.g., 3D12 and DT9) did not detectably bind to the surface of HLA-A, -B, -C-negative 721.221 (shortly, 221) cells grown at physiological temperature (3, 6, 7), notwithstanding these cells have long been known to react with Abs to framework H chain epitopes and β_2m (see, for instance, Ref. 22); and 5) in line with this observation, a weak surface binding of Abs to HLA-E could be elicited by cold treatment, cold treatment followed by incubation in the presence of permissive ligands, and transfection with permissive alleles of 221 cells, and yet these minor changes in surface HLA-E expression resulted in major functional effects on NK cytotoxicity and/or the binding of soluble rNKG2A (3, 5, 7, 9, 23, 24). Moreover, 6) the 3D12 and DT9 Abs did not detect HLA-E on the surface of TAP-defective and tapasin-defective human mutants (3, 6), but the requirement for TAP was less stringent for Qa-1 expressed in TAP-defective murine cells (25), for HLA-E expressed in human cells in which TAP is present, but functionally inhibited in its ability to translocate peptides (26), and for HLA-E assembled in the presence of the viral, UL40-derived peptide mimic of class I signal sequences (14, 15). Finally, 7) different authors have used different Abs and methods to detect surface HLA-E H chains, and at least some of these Abs were not specific for HLA-E and/or poorly characterized.

Among these, DT9 binds HLA-E, but cross-reacts with HLA-C (6). V16 has undergone limited testing on murine cells transfected with various HLA-A, -B, -C H chains and human β_2m (27). The 3D12, 4D12, and 7G3 Abs (3, 28) have been reported to be HLA-E specific, but to our knowledge no detailed immunochemical characterization has ever been published. Abs of the MEM series (MEM-E/02, MEM-E/06, MEM-E/07, and MEM-E/08) were described more recently. MEM-E/02 was tested on a very limited number of class I alleles. It reacted with denatured HLA-E H chains in Western blotting, but did not react with native, surface-expressed HLA-E in flow cytometry (29). Conversely, MEM-E/06 was shown not to work in Western blotting, but to work in flow cytometry on murine cells cotransfected with human β_2m, although three human class I alleles (HLA-A3, -A11, and -B7) of eight did cross-react (29). Finally, two additional MEM Abs (MEM-E/07 and MEM-E/08) were claimed to be substantially more restricted than MEM-E/06 in a preliminary workshop report by our group (30, 31), but quite an extensive list of alleles cross-reacting with MEM-E/07 (HLA-B7, -B8, -B27, -B44) and MEM-E/08 (HLA-A24, -B7, -B27, -B51, -B54, and -Cw7) has subsequently been published (32).

In summary, one may wonder why HLA-E should assemble in the presence of signal sequence-derived ligands for which it may have little affinity. In addition, one may question the role of the many known HLA-E ligands in regulating HLA-E expression/function, the contribution of TAP and tapasin to overall HLA-E assembly, and, lastly, the fine specificity of the different Abs used to detect HLA-E. In the present study, we have extensively characterized the four MEM Abs by flow cytometry and immunochemical methods, using exclusively human cells, either single class I allele transfectants in the HLA-A-, HLA-B-, HLA-C-negative 221 cell line, or EBV-immortalized cell lines expressing a representative panel of common HLA-A, HLA-B, and HLA-C alleles. At variance with previous studies (29, 32–35), and quite surprisingly, several Abs (including the MEM Abs) did not appear to efficiently detect surface HLA-E by flow cytometry. Using alternative Abs, we found that HLA-E exists under the species of several distinct, partially overlapping conformers, some of which were detected on the cell surface in the absence of canonical HLA-E ligands. Our results explain why some of the available Abs with nominal HLA-E specificity fail to efficiently bind to the surface of HLA-E-expressing cells, provide insight into the posttranslational regulation of HLA-E, clarify some open issues of HLA-E expression in specific cells, and raise the possibility that different HLA-E conformers/glycoforms might have different functional roles.

Materials and Methods

Cell lines

The 221 cell line and its transfectants 221.AEH (3), 221.G1 (36), 221.A*0201, 221.B*0702, 221.B*1501, 221.B*2705, and 221.Cw*0102 (37, 38) were obtained through either the courtesy of different investigators (see *Acknowledgments*), or the collaborative efforts of the HLA-G and HLA-E workshops. A low-resolution (one- or two-digit) allele denomination (i.e., 221.A2) will be used hereafter. EBV-immortalized B cell lines (distributed through the International Histocompatibility Workshops), and their known (37) HLA-A, -B, -C typing (provided in parentheses) are as follows: BM15 (A1; B49; Cw7); BM92 (A25; B51; Cw1), BSM (A2; B62; Cw9), CALOGERO (A2; B61; Cw2), CJO (A11; B35; Cw4), FPF (A2; B35; Cw4), JVM (A2; B18; Cw5), JY (A2; B7; Cw7), LBF (A30; B13; Cw6), LG2 (A2; B27; Cw1), LKT3 (A24; B54; Cw1), MGAR (A26; B8; Cw7), RM12 (A3; B62; Cw3), RM13 (A2; B18; Cw7), WT46 (A32; B44; Cw5), and WT51 (A23; B65; Cw8). Molt 4 (A1, 25; B18, 57; Cw6, 12) and FO-1-β_2m (A25; B8; Cw7) are a T lymphoblastoid cell line and a β_2m melanoma transfectant (39), respectively. TAP-defective.174 × CEM.T2 (T2) are derived from parental T1 cells (40). Tapasin-defective 721.220 (220) cells are partially isogenic with 221 cells (41). Mouse NIH3T3 cells were from the American Type Culture Collection (http://www.lgcpromochem-atcc.com/).

Nucleic acid biochemistry

Genomic DNAs were HLA-E genotyped by PCR-SSP, using the forward sequence specific primers 2E-382-A-F and 2E-382-B-F, and the reverse universal primer 382-ALL-as, as described (4), and by direct sequencing of a 501-bp amplimer obtained with the forward primer GGACACCGCAC AGATTTTCCG and the reverse primer 382-ALL-as.

Protein biochemistry

The following mouse mAbs were used: MEM-E/02, MEM-E/06, MEM-E/07, and MEM-E/08 (29, 31, 32), all from EXBIO; 3D12 and 4D12 (33), BB7.1 and W6/32 (42), HC10 and HCA2 (43), and Namb-1 and L31 (37, 38). A rabbit polyclonal to ERp57 and all the immunochemical methods are described in previous publications of our group (37–39). For blotting and isoelectric focusing (IEF) blotting, cells were solubilized by the nonionic detergent Nonidet P-40. Cellular proteins (100 μg/lane) were resolved by either SDS-PAGE or IEF slab gels, and electroblotted to nitrocellulose filters. Class I HLA molecules were revealed by staining filters with the indicated Abs, followed by ECL (Amersham Biosciences). For immunoprecipitation studies, cells were metabolically labeled with [^{35}S]methionine (9 MBq/ml) for 2 h. Soluble Nonidet P-40 extracts were incubated for 2 h at 4°C with protein A-Sepharose beads (20 μl) preloaded with rabbit anti-murine Ig and mAbs (10 μg). Washed beads were eluted at 100°C (3 min) in Laemmli buffer, or at 56°C (15 min) in the IEF loading buffer containing urea (8 M). Immunoprecipitates were electrophoresed, and gels were dried and autoradiographed. The synthetic peptide VMAPRTVLL was purchased from Sigma Genosys. In vitro assembly was performed, as described (38), by incubating soluble cell extracts at the indicated temperatures, in the presence or absence of known HLA-E ligands, for the indicated times, before immunoprecipitation.

Surface-labeling studies

Live cells were incubated with either ^{125}I in the presence of iodobeads, or sulfo-*N*-hydroxysuccinimide-biotin, as instructed by the manufacturer (Pierce). Nonidet P-40 extraction, immunoprecipitation, elution, and electrophoresis were as above. Where indicated, washed beads were incubated in the presence of neuraminidase, as described (37, 38). Following electrophoresis, gels were either dried and autoradiographed (^{125}I labeling) or transferred to nitrocellulose filters (biotin labeling), and stained with streptavidin-peroxidase, followed by chemiluminescence detection. In an alternative, conformation-independent protocol, all the biotin-labeled proteins were solubilized by Nonidet P-40. Streptavidin-conjugated agarose beads (50 μl; Pierce) were incubated (30 min) with soluble extracts (1 mg), washed, and either digested with endoglycosidase H (Endo H) (38) or mock incubated. Biotin-labeled proteins were resolved by SDS-PAGE,

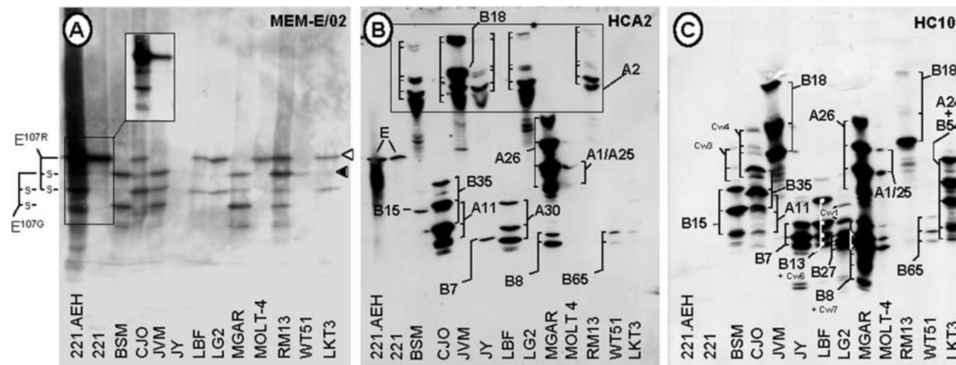

FIGURE 1. IEF blot analysis of the reactivity of MEM-E/02. Nonidet P-40 cell extracts were run on an IEF slab (acidic end, *bottom*) under reducing conditions, electroblotted onto replicate nitrocellulose filters, and stained/stripped with MEM-E/02 (*A*), HCA2 (*B*), and HC10 (*C*), as indicated. IEF positions of HLA-E*0101, HLA-E*0103 (open and closed arrowheads in *A*, respectively), and HLA-A, -B, -C alleles are indicated. HLA-A, -B, -C typing is noted in *Materials and Methods*. Sialylated (s) HLA-E bands are indicated *left* of *A* according to results shown in Fig. S1.[4] The *inset* in *A* shows an underexposed area of the filter. MEM-E/02 staining of HLA-E H chains was incompletely stripped from an area in *B*, as indicated. Irreversible staining in immunoblotting is often observed with this Ab, possibly due to a high binding affinity.

electroblotted to nitrocellulose filters, and identified by staining with specific Abs and chemiluminescence. For flow cytometry, cells were stained on ice at a predetermined, optimal Ab concentration (10 μg/ml). Isotype-matched control Abs were included in all of the experiments. Primary Ab binding was revealed by incubation with FITC-labeled rabbit anti-murine Ig (Cappel), and specifically bound fluorescence was immediately analyzed without fixation by a FACScan flow cytometer (BD Biosciences).

Results

Characterization of Abs to HLA-E

The 221 cells transfected with single HLA class I alleles, and the HLA-A, -B, -C typed cells listed in *Materials and Methods* were selected as a convenient source of Nonidet P-40-soluble class I H chains (both classical and nonclassical), with known IEF migration and sialylation patterns (6, 37). Such a panel samples more than 90, 60, and 95%, respectively, of the serologically defined HLA-A, -B, and -C alleles expressed in Caucasoids and representative of the frequent substitutions in class I primary sequences.

To characterize the allelic specificity of MEM-E/02, previously shown (29) to exclusively work in Western blotting, Nonidet P-40 extracts were denatured by 8 M urea and IEF blotted onto replicate filters with MEM-E/02 and two control Abs (HCA2 and HC10) to denatured HLA-A, -B, -C H chains (Fig. 1, *A–C*, respectively). MEM-E/02 detected much higher HLA-E levels in 221.AEH transfectants as compared with parental 221 cells, as expected (3), and identified from one to four IEF bands in the other cell lines (Fig. 1*A*). Essentially the same bands were detected on filter-blotted W6/32 immunoprecipitates from the same extracts (compare Fig. 1*A* with representative results in supplemental Fig. S1),[4] demonstrating that all the MEM-E/02-reactive IEF components are class I polypeptides. Fully consistent with the known dimorphism of HLA-E (4), the most basic bands from cell lines genotyped as HLA-E*0101 (221.AEH, 221, CJO, LBF, LG2, and Molt 4) and HLA-E*0103 (BSM, JVM, MGAR, WT51, and JY) migrated to the expected (6) IEF positions of HLA-E^{107R} and HLA-E^{107G}, respectively (Fig. 1*A*: a longer exposure of the filter was required in the JY lane; also see Fig. S1).[4] As also expected, HLA-E-heterozygous RM-13 and LKT-3 (presumably from the progenies of nonconsanguineous marriages) displayed both the HLA-E^{107R} and HLA-E^{107G} IEF bands (Figs. 1*A* and S1).[4] Neuraminidase digestion of W6/32 immunoprecipitates from representative cell lines (Fig. S1)[4] identified additional charge heterogeneity due to sialic acid addition, although one-sialic acid species were poorly sensitive to neuraminidase, as described (6, 37). In contrast, HLA-E sialylation was poor and undetectable in 221.AEH and 221 cells, respectively (Fig. S1[4] and Fig. 1*A*, *inset*).

Remarkably, none of the IEF bands detected in Fig. 1*A* overlapped with HLA-A, -B, and -C bands in Fig. 1, *B* and *C*. Similarly, no overlapping with HLA-C bands was detected by probing other filters with the HLA-C-restricted L31 Ab (data not shown). Finally, MEM-E/02 did not react with either HLA-F, expressed (6) by parental 221 cells, or the HLA-G1 isoform, expressed in 221.G1 transfectants (Table I, and see below).

Next, we characterized MEM-E/06, claimed to bind native (surface-expressed) H chains (29), as well as MEM-E/07 and MEM-E/08, submitted to an International HLA-G and HLA-E Workshop as Abs with specificities similar to that of MEM-E/06 (30). To this end, detergent-soluble class I H chains were immunoprecipitated from metabolically labeled cells with these three Abs and, as a control, with W6/32 to conformed HLA-A, -B, -C, -E, and -F molecules (6, 42). Representative immunoprecipitation/IEF gels and a complete synopsis of the results are shown in Fig. 2 and Table I, respectively. MEM-E/06 identified HLA-E and cross-reacted not only with A11 (Fig. 2, *lane 3*), A3, and B7 (Table I), as reported (29), but also with most other HLA-A, -B, and -C H chains (*lanes 8*, *13*, and *18*, and Table I), including alleles (A26, B27, Cw3, and Cw7) previously described (29) to be unreactive when expressed in murine transfectants. In contrast, and quite interestingly, MEM-E/07 and MEM-E/08 cross-reacted more weakly and with a more limited number of alleles than MEM-E/06 (lanes *4*, *5*, *9*, *10*, *14*, *15*, *19*, and *20*, see dots; also see Table I).

Altogether, we conclude that MEM-E/02 binds with remarkable specificity (in Western blotting) a linear epitope carried by both nonsynonymous HLA-E alleles, and none of the tested HLA-A, -B, -C, -F, or -G H chains, and that MEM-E/07 and MEM-E/08 are remarkably restricted for Nonidet P-40-soluble HLA-E in immunoprecipitation.

However, careful inspection of MEM-E/06, MEM-E/07, and MEM-E/08 immunoprecipitates, including those depicted in Fig. 2, revealed that β_2m is present only when cross-reacting HLA-A, -B, -C bands are also present (particularly evident in *lanes 8*, *13*, and *18*), whereas little or no β_2m is detectable when HLA-E is the only or major H chain band (*lanes 3–5*, *14*, *15*, *19*, and *20*). These results are quite puzzling and suggest that the MEM Abs recognize HLA-E H chains poorly associated with β_2m, β_2m being mostly

[4] The online version of this article contains supplemental material.

Table I. *Reactivity of the MEM Abs with class I HLA alleles other than HLA-E*

Alleles	MEM-E0/2	MEM-E0/6	MEM-E0/7	MEM-E0/8
A1	−[a]	−	−	−
A2	−	−	−	−
A3	−	+	−	−
A11	−	+	−	−
A23	−	++	++	+
A24	−	++	−	+
A25	−	−	−	−
A26	−	+	−	−
A30	−	−	−	−
A32	−	−	−	−
B7	−	++	+	−
B8	−	++	+	+
B13	−	++	+	−
B15	−	++	−	−
B18	−	++	−	−
B27	−	++	−	−
B35	−	−	−	−
B44	−	++	+	−
B49	−	+	−	−
B51	−	−	−	−
B54	−	++	−	+
B61	−	++	−	−
B62	−	++	−	−
B65	−	++	+	++
Cw1	−	−	+	−
Cw2	−	−	−	−
Cw3	−	++	−	−
Cw4	−	−	−	−
Cw5	−	+	−	−
Cw6	−	++	−	−
Cw7	−	+	−	−
Cw8	−	NT[b]	NT	NT
G1	−	−	−	−
F	−	+	−	−

[a] −, No detectable IEF bands comigrating with alleles other than HLA-E in the immunoprecipitate. +, The IEF bands of the cross-reacting alleles were less intense than, or as intense as, the HLA-E bands in the same immunoprecipitate. ++, The IEF bands of the cross-reacting alleles were more intense than the HLA-E bands in the same immunoprecipitate.
[b] Not tested.

coimmunoprecipitated with HLA-A, -B, and -C. To address this issue under controlled conditions, immunoprecipitates from parental 221 cells (only expressing HLA-E and HLA-F) were compared with immunoprecipitates from 221.B7 transfectants (coexpressing HLA-B7, a permissive allele that cross-reacts with MEM-E/06).

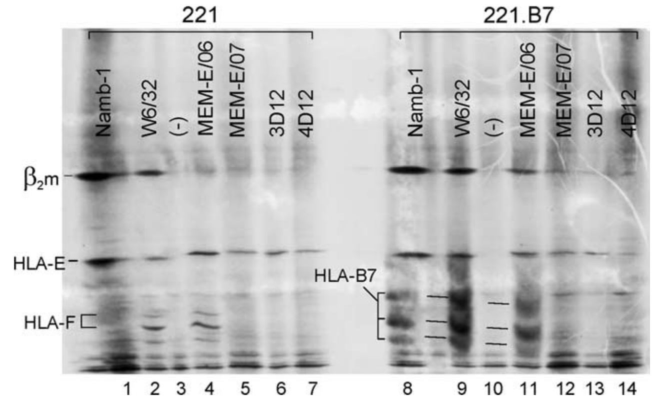

FIGURE 3. β_2m-free and β_2m-associated HLA-E molecules synthesized by 221 cells and 221.B7 transfectants. The indicated cell lines were metabolically labeled, lysed, immunoprecipitated, and electrophoresed on an IEF slab, as described in the legend to Fig. 2.

Several Abs to HLA-E bind H chains free of, or poorly associated with, β_2m

Nonidet P-40 extracts of metabolically labeled 221 and 221.B7 cells were immunoprecipitated by MEM-E/06, MEM-E/07, 3D12, and 4D12 (33) to HLA-E, Namb-1 to β_2m, and W6/32 to β_2m-associated class I H chains. Upon IEF analysis, HLA-E was detected in all of the immunoprecipitates from parental 221 cells, whereas β_2m was clearly detected only in Namb-1 and W6/32 immunoprecipitates (Fig. 3, *lanes 1–7*). It can be concluded that several Abs with nominal HLA-E specificity immunoprecipitate H chains poorly associated with β_2m. Accordingly, coexpression of the permissive HLA-B7 allele in 221.B7 cells enhanced conformed HLA-E H chains reactive with W6/32 (compare *lanes 2* and *9*), most likely as a result of ligand donation, but minimally (if at all) affected HLA-E and β_2m levels in MEM-E/07, 3D12, and 4D12 immunoprecipitates (*lanes 12–14* as compared with *lanes 5–7*). As with EBV-immortalized B cells, β_2m became clearly detectable in the MEM immunoprecipitates only upon cross-reactivity, e.g., in MEM-E/06 immunoprecipitates containing three HLA-B7 bands (*lane 11*). In separate experiments, MEM-E/02 and MEM-E/08 coprecipitated extremely low levels of β_2m, e.g., they behaved like MEM-E/06 and MEM-E/07, whereas BBM-1 (another Ab to β_2m) behaved like Namb-1 (data not shown, and see below). Finally, HLA-F was reactive with W6/32 (*lane 2*), as described (44), and

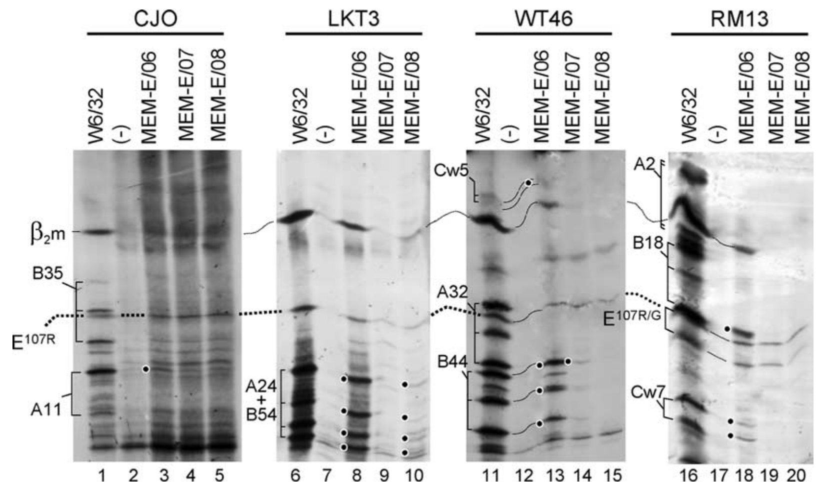

FIGURE 2. IEF analysis of the reactivity of MEM-E/06, MEM-E/07, and MEM-E/08. The indicated cell lines were metabolically labeled for 2 h with [^{35}S]methionine (9 MBq/ml) and lysed by Nonidet P-40 on ice. Lysates were immunoprecipitated at 4°C with either the indicated Abs, or an irrelevant Ab (−), and immunoprecipitates were run on an IEF slab gel under reducing conditions. Dots indicate some evident HLA-A, -B, -C bands present in the MEM lanes (A11 in *lane 3*; A24 and B54 in *lanes 8* and *10*; B44 in *lanes 13* and *14*; and B18 and Cw7 in *lane 18*). In agreement with Fig. 1, RM13 cells display two major HLA-E bands.

with MEM-E/06 (*lane 4*), but not with Namb-1 (*lane 1*, and see below).

To provide conclusive evidence that the β_2m component immunoprecipitated by the MEM Abs is mainly associated with HLA-B7 (and not HLA-E), HLA-B7 and β_2m molecules in Nonidet P-40 extracts of 221.B7 were immunodepleted by BB7.1, an allele-specific Ab to conformed HLA-B7 H chains (42). As shown in a representative experiment of three that were performed (Fig. S2),[4] BB7.1 depleted HLA-B7 and β_2m, but not HLA-E, in MEM-E/06 immunoprecipitates, conclusively showing the preferential association of β_2m with MEM-reactive HLA-B7.

On this basis, we sought to determine whether HLA-E-restricted Abs behave like Abs to β_2m-free HLA-A, -B, -C H chains (reviewed by us in Ref. 38), e.g., whether they bind linear epitopes that become accessible following denaturation. Western blotting experiments were performed in parallel with the same staining concentrations of MEM-E/02, MEM-E/06, MEM-E/07, MEM-E/08, 3D12, and 4D12 on replicate filters (Fig. S3).[4] All of the tested Abs to HLA-E bound denatured HLA-E H chains, although to a different extent.

Altogether, from the results in Figs. 2, 3, S2, and S3,[4] we conclude the following: 1) several Abs with nominal HLA-E specificity, including the widely used MEM and 3D12 Abs, preferentially (and unexpectedly) react with HLA-E H chains poorly associated with β_2m, β_2m being mainly associated with the MEM cross-reacting HLA-A, -B, -C alleles; 2) MEM-E/06 (and possibly the other MEM Abs) recognize unusual epitopes carried by β_2m-free HLA-E and β_2m-associated HLA-A, -B, -C H chains; 3) the free H chain reactivity of these Abs is slightly, if at all, affected by the presence of the known ligand donor HLA-B7, which is instead capable of enhancing the W6/32 reactivity of HLA-E; 4) the MEM and 3D12 epitopes fulfill the definition of linear, because they are hidden and accessible on native (β_2m-associated) and denatured (β_2m-free) HLA-E H chains, respectively; and 5) poor association with β_2m is not a shared feature of all the HLA-E H chains, because some of them are detectably β_2m associated in the same nonionic detergent extracts, as assessed by two distinct Abs to β_2m and an Ab (W6/32) to folded class I H chains.

Coexistence of HLA-E conformers displaying strong and weak association with β_2m

The unexpectedly poor reactivity of conformed HLA-E molecules with Abs to HLA-E prompted us to focus on β_2m-associated HLA-E H chains reactive with W6/32 and Namb-1. To this end, five cell lines and transfectants were selected in which HLA-E is expected to assemble either under favorable conditions, as in FO-1-β_2m transfectants overexpressing β_2m (39) and 221 transfectants (221.G1 and 221.Cw1) capable of providing HLA-E with canonical ligands (6), or under unfavorable conditions, as in 221 cells lacking canonical ligand donors and in peptide loading-defective 220 cells (6, 41). Surprisingly, despite such a wide spectrum of assembly conditions, IEF analysis of W6/32 and Namb-1 immunoprecipitates from cells metabolically labeled at similar sp. act. consistently detected a strong and preferential Namb-1 reactivity of HLA-E complexes in all of the cells. In contrast, five of the coexpressed H chains (HLA-F, HLA-A25, HLA-B8, HLA-Cw7, and HLA-Cw1) were preferentially reactive with W6/32, and one (HLA-G1) was similarly reactive with the two Abs (Fig. S4).[4]

Such an elective coimmunoprecipitation of HLA-E H chains with β_2m implies tight heterodimeric association (possibly tighter than in the case of other classical and nonclassical class I H chains tested in parallel), e.g., high (and not low) affinity for β_2m, whereas failure to acquire the prototypic, framework (42) class I epitope W6/32 upon assembly suggests the formation of nonconventionally folded (W6/32$^{+/-}$) HLA-E H chain conformers. These features of Namb-1-reactive and W6/32-reactive H chains are distinctive of HLA-E and are observed regardless of cell lineage, number and levels of coexpressed class I alleles, available amounts of β_2m and HLA-E ligands, and peptide-loading sufficiency.

This prompted us to another experiment in which we assessed the distribution of the Namb-1 and W6/32 epitopes on the HLA-E molecular pool by reciprocal immunodepletion. In three preliminary experiments (data not shown), we were unable to completely immunodeplete W6/32-reacting H chains from 221 soluble extracts using excess amounts of W6/32. To investigate this issue, we performed four rounds of immunodepletion using immunoadsorbents loaded with W6/32 and Namb-1, and monitored the recovery of HLA-E H chains and β_2m at successive depletion rounds. We found (Fig. S5)[4] that Namb-1 efficiently depletes HLA-E H chains and β_2m within two depletion rounds, whereas W6/32 depletions are largely unsuccessful even following four consecutive rounds. Poor coimmunoprecipitation of HLA-E-associated β_2m by W6/32 was also observed.

The easiest interpretation of these data (Figs. S4 and S5)[4] is that Namb-1 binds with high-affinity HLA-E H chains with a high affinity for β_2m, whereas W6/32 binds with low-affinity HLA-E H chains with a low affinity for β_2m, e.g., Abs to β_2m bind conformers at the tightly assembled end of a spectrum of HLA-E conformations/assemblies. To further investigate this issue, MEM-reactive, W6/32-reactive, and Namb-1-reactive HLA-E H chains were compared by in vitro assembly.

Distinct conformations of HLA-E H chains detected by in vitro assembly

It has been firmly established from experiments of in vitro assembly (45) that newly synthesized class I H chain-β_2m complexes are thermally stable in nonionic detergent extracts at 4°C, become unstable at 37°C, but can be stabilized at this temperature upon the addition of specific peptide ligands. However, several attempts to stabilize at 37°C the MEM-reactive and W6/32-reactive HLA-E molecules contained in soluble extracts from 221 cells by the addition of the known HLA-E ligand VMAPRTVLL were unsuccessful (data not shown). After excluding trivial causes, we repeated the same experiment not only at 37°C, but also at 4°C, and tested the stability of all of the three HLA-E conformers at this low temperature. This experiment (Fig. 4), although confirming that W6/32-reactive HLA-E H chains are refractory to peptide-mediated stabilization at 37°C (compare *lanes 4* and *5*), revealed that, quite unusually, they are also unstable at 4°C, to the extent that their ligand was required to achieve some stabilization even at this low temperature (compare *lanes 2* and *3*). In contrast, the free H chains reactive with MEM-E/08 could be detected, although at very low levels, only in the absence of peptide (slightly better at 4°C than at 37°C; *lanes 7* and *9*), peptide addition preventing in both cases their appearance (*lanes 6* and *8*).

Most surprisingly, Namb-1-reactive HLA-E H chains exhibited a peculiar, sharply different behavior. They were extremely abundant and marginally sensitive to either temperature or ligand addition (*lanes 10–13*). The similar effect of low temperature and the opposite effects of peptide addition on W6/32 conformers and MEM-E/08 conformers, in combination with the results in Figs. S3–S5,[4] provide conclusive evidence of the existence of a large pool of unstable HLA-E conformers that includes a sizeable fraction of W6/32-reactive H chains prone to spontaneous dissociation. In addition, in vitro assembly lends strong support to the existence of Namb-1^{+}/W6/32^{-} HLA-E H chains with unique features.

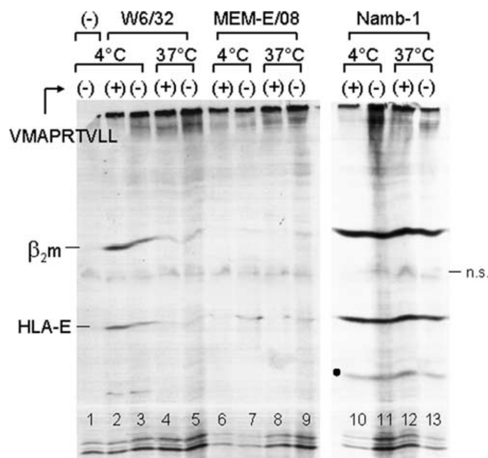

FIGURE 4. Thermal stability and in vitro assembly with specific peptides of three different HLA-E conformers. The 221 cells were metabolically labeled for 30 min with [^{35}S]methionine (18 MBq/ml). Lysates (prepared on ice) were divided in two aliquots that were incubated for 3 h at 4°C and 37°C, respectively, in the absence or presence of a specific HLA-E ligand (VMAPRTVLL, 50 μM), as indicated. Class I molecules were immunoprecipitated at 4°C with the indicated Abs, and resolved on an IEF slab, as above. A nonspecific (n.s.) band and an unknown component (dot) are indicated.

Biochemical features of surface-expressed HLA-E H chains

Next, we tested by flow cytometry the binding of the MEM Abs to the surface of parental 221 cells and 221 cells transfected with representative class I alleles, either nonpermissive or permissive (Fig. S6).[4] All of the cells were either negative or barely reactive, except 221.AEH, which overexpresses a chimeric HLA-E H chain capable of *cis*-ligand donation. Two distinct methods of vectorial cell surface labeling with ^{125}I and biotin (Fig. S7)[4] confirmed the results of flow cytometry. MEM-E/06 and MEM-E/08 detected surface HLA-E H chains on 221.AEH, but not 221, 221.B7, 221.G1, and FO-1-β_2m cells, and even when surface expressed, as in 221.AEH, MEM-reactive H chains were largely free of β_2m, whereas β_2m-associated HLA-E H chains were clearly detectable by W6/32 and Namb-1 on the surface of all of the tested cells.

It can be concluded that β_2m-free, MEM-reactive HLA-E H chains accumulate on the cell surface in clearly detectable amounts only upon overexpression of a hybrid HLA-E H chain in 221.AEH cells, whereas conformed HLA-E H chains associated with β_2m, although unreactive with the MEM Abs, are present on the surface of cell lines of different origin, including parental 221 cells (in the absence of permissive alleles), its transfectants, and a melanoma cell line.

Unfortunately, despite extensive attempts to improve the quality of the IEF patterns, the ^{125}I labeling and biotin-labeling approaches suffered from numerous nonspecific bands (see Fig. S7)[4] that may preclude the interpretation of complex IEF patterns. In view of this, and because the detection of HLA-E on the cell surface appears to critically depend on the Abs used, we resorted to a modified, low-background biotin-labeling assay for the conformation-independent detection of surface HLA-E (see *Materials and Methods*), and used this approach to assess surface HLA-E levels of parental 221 cells, their transfectants, as well as T1 and the peptide loading-defective T2 and 220 cell lines (Fig. 5). Within the accuracy limits of the assay (that is semiquantitative), it was readily apparent that 221.AEH expressed the highest HLA-E levels, much higher than those detected in the parental 221 cells (*lane 51* compared with *lane 47*), as expected. In contrast, no major differences in surface HLA-E expression were detected among 221 cells (lacking HLA-A, -B, -C expression) and other transfectants, regardless of whether expressing permissive (221.B7, 221.G1) or nonpermissive (221.B27 and 221.B15) alleles (compare *lanes 1*, *3*, and *5*, as well as *lanes 47* and *49*; the nonpermissive allele B15 is not shown). Much lower, and similar, HLA-E levels were instead detected in T1 and the peptide loading-defective T2 and 220 cells (*lanes 41*, *43*, and *45*).

Even in the absence of major quantitative differences, qualitative differences were readily apparent in surface HLA-E glycoforms. A canonical (Endo H-insensitive), mature glycoform was exclusively expressed (and at low levels) in 221 cells transfected with permissive alleles (*lanes 4*, *6*, and *52*), whereas an unusual

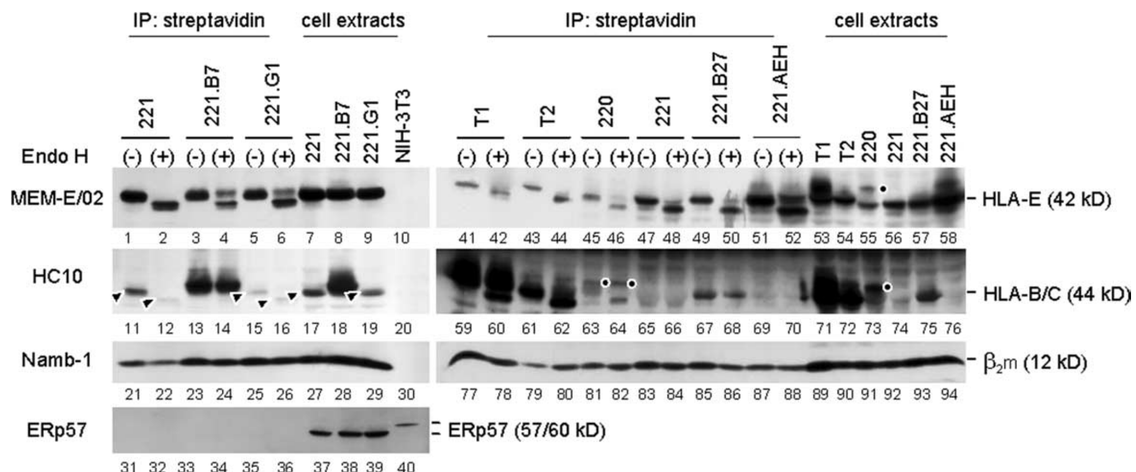

FIGURE 5. Surface HLA-E expression in 221 cells, their transfectants, and isogenic derivatives. The indicated cells were surface labeled with biotin and solubilized by Nonidet P-40. Equal amounts of soluble extracts (1 mg) containing biotin-labeled proteins were purified on agarose beads conjugated to streptavidin, eluted, run on a SDS-PAGE slab, and electroblotted to nitrocellulose filters. Biotin-labeled class I H and L chains were identified by sequentially staining/stripping the filters with the indicated Abs. All samples were prepared in duplicate, and one aliquot was digested with Endo H. Nonidet P-40 extracts (100 μg) of biotin-labeled cells were run side by side. Arrowheads mark residual staining by MEM-E/02 that could not be removed by stripping. Several experiments are shown, each of which includes 221 cells as an internal control to normalize for day-to-day variability. Dots mark a nonspecific component in 220 cells.

(Endo H-sensitive), immature glycoform was the more abundant surface-expressed HLA-E species in permissive transfectants (*lanes 2*, *4*, *6*, and *52*), and the only species in nonpermissive 221 transfectants, T1, T2, and 220 cells (*lanes 2*, *42*, *44*, *46*, *48*, and *50*). The widespread presence of immature HLA-E glycoforms at the cell surface (although possibly at levels lower than those of canonical HLA-A, -B, -C glycoforms, as estimated in Fig. S7)[4] was surprising, but their Endo H insensitivity was in agreement with the neuraminidase insensitivity of the major, surface-expressed, basic IEF HLA-E*0101 band in 221 cells and transfectants (see Figs. S1 and S7).[4] Consistent with surface-specific biotin labeling, endocellular ERp57 was not accessible to biotin conjugation (Fig. 5, *lanes 31–36*), and HC10-reactive H chains recovered from the same soluble extracts were entirely insensitive to Endo H digestion (*lanes 13*, *14*, *67*, and *68*), although quite surprisingly parental T1, and their derivative T2 cells displayed some surface glycoforms (classical H chains as well) of the immature type (*lanes 60* and *62*).

In summary, the presence of canonical HLA-E ligands and an intact peptide-loading machinery are both necessary (although not sufficient, as demonstrated by the T1 exception) for the expression of mature HLA-E glycoforms, but neither is necessary for the expression of immature HLA-E glycoforms.

Discussion

We have assessed the reactivity of the four MEM Abs on a representative panel of classical (HLA-A, -B, -C) and nonclassical (HLA-F and HLA-G) molecules. The Western blotting-grade (29) MEM-E/02 Ab has now been characterized in detail, and found to be HLA-E specific on denatured H chains. The other MEM Abs (MEM-E/06, MEM-E/07, and MEM-E/08), previously thought to bind folded HLA-E H chains, were tested in this study by immunoprecipitation from nonionic detergent cell lysates. MEM-E/06 cross-reacted with HLA-A, -B, -C even more extensively than previously appreciated by flow cytometry on murine transfectants (29). Thus, the specificity of MEM-E/06 differs between different assays (cell surface vs soluble extracts) and/or across species (mouse transfectants vs human cells). As to MEM-E/07 and MEM-E/08, we are aware of only one report stating their cross-reactivity patterns (32). However, no technical details were provided in this report, and the primary data were not shown, making it impossible to compare the range and extent of the described cross-reactions with those documented in the present study. From our data, it appears that MEM-E/07 and MEM-E/08, being much more restricted than MEM-E/06 and DT9 (6, 29), are of interest to screen for the presence of HLA-E polypeptides in cells coexpressing a defined range of class I alleles.

Most unexpectedly, and in stark contrast with previous studies (29, 32), we found that none of the MEM Abs is suitable for the detection of surface-expressed, conformed HLA-E molecules, because all of them are Western blotting grade and bind HLA-E H chains that are associated with β_2m poorly (if at all), and are poorly expressed on the cell surface. Interestingly, this free H chain specificity is shared by a group of Abs that includes the widely used 3D12 Ab. Even the prototypic conformational Ab W6/32 binds with low affinity a poorly β_2m-associated H chain pool. And yet, far from being a poor assembler, as previously reported (5), HLA-E is instead tightly associated with β_2m, even more tightly than other classical and nonclassical H chains tested in parallel, as shown by its preferential reactivity with Abs to β_2m. Thus, the hallmark of HLA-E is the unprecedented (in other class I molecules) coexistence of H chain conformers with drastically antithetic features (very poor and very good association with β_2m). This and other findings reported in this study reconcile previous conflicting observations and suggest alternative, partly unexpected, assembly mechanisms of HLA-E.

Abs to HLA-E are biased to recognize unfolded H chains

The observation that MEM-E/06 and 3D12 work in Western blotting in our hands (Fig. S3),[4] but not in the hands of others (3, 28, 29), was unexpected, but is not entirely surprising when considered in the context of reports showing that 3D12 and DT9 bind both β_2m-free and β_2m-associated H chains in nonionic detergent extracts, and that at least two Abs to HLA-E (7G3 and 4D12) also work in Western blotting (3, 6, 28). Taking into account the present results, at least eight Abs to HLA-E (the four MEM Abs and four additional Abs produced by different groups) have been shown to react with unfolded and/or denatured H chains free of β_2m. Then, regardless of discrepancies and possible differences in Western blotting protocols among different research groups, a likely interpretation is that several investigators have generated Abs to free H chains because these are inevitable, abundant dissociation byproducts in the immunogens prepared in different laboratories.

This interpretation is supported by in vitro assembly experiments. In these experiments, a canonical HLA-E ligand increased and decreased the W6/32-reactive and MEM-reactive H chain pools, respectively (Fig. 4), indicating that the former is loosely associated with β_2m and prone to spontaneous dissociation, but can be protected from melting through forced in vitro assembly in conditions of ligand excess. Thus, although the two H chains have distinct peptide receptivity and affinity for β_2m, they form a somewhat common reservoir of unstable folding intermediates existing at equilibrium, and this is a likely source of free, unfolded H chains in HLA-E preparations used for immunization. Consistent with this idea, W6/32 has been shown to react with isolated and partially denatured class I H chains (46), and to prevent melting of HLA-E^{107R} H chains when added to soluble cell extracts immediately after cell lysis (1). Also consistent with the idea of a common pool of unfolded (or partially folded) HLA-E molecules, the MEM Abs (particularly MEM-E/06) detected some β_2m-associated H chains, although this can be clearly demonstrated only in the case of H chains encoded by the classical class I loci (Figs. 2, 3, and S2).[4] In this respect, restoration of NK-mediated lysis by Abs such as 3D12 (23, 24) may be explained by their ability to identify a partial conformation on class I H chains as they melt, e.g., to catch and block partially folded HLA-E conformers.

HLA-E conformers and mechanisms of HLA-E assembly

Although a substantial conformer overlap and widely different Ab-binding affinities do not allow to sort H chains with homogeneous features by purely serological means, the experiments in Figs. 2–4, S2, S4, and S5[4] suggest a wide range of HLA-E H chain-β_2m interactions, from very weak (MEM$^+$/W6/32$^-$/Namb-1$^-$ conformers) to very strong (MEM$^-$/W6/32$^-$/Namb-1$^+$ conformers).

At one end of the H chain-β_2m association spectrum, MEM-reactive conformers display several features highly reminiscent of β_2m-free HLA-A, -B, -C H chains, recently reviewed by us (38). Free HLA-E H chains: 1) carry bona fide linear epitopes displayed by denatured HLA-E polypeptides (in Western and IEF blotting), but normally hidden in the three-dimensional structure of conformed, β_2m-associated H chains (Figs. 2, 3, S2, and S3);[4] 2) are poorly expressed on the cell surface (Figs. S6 and S7);[4] 3) steadily decrease in the course of pulse-chase experiments (our unpublished results); 4) are unstable at physiological temperature (Fig. 4); and 5) are substantially unable to bind peptide ligands (Fig. 4).

In contrast, W6/32-reactive and Namb-1-reactive conformers drastically differ from β_2m-associated HLA-A, -B, -C H chains.

W6/32 conformers, on the one hand, are recognized (weakly) by an Ab that binds (strongly) HLA-A, -B, -C (Figs. S4 and S5),[4] and require both hypothermia and peptide to be partially stabilized (Fig. 4), whereas either treatment is sufficient to completely stabilize the W6/32-reactive pool of classical class I H chains (see, for instance, Ref. 38). Namb-1 conformers, in contrast, are recognized (very strongly) by Abs to β_2m that also bind (but more weakly) other classical and nonclassical (HLA-F and -G) H chains (Fig. S4).[4] They achieve full thermal stability and become essentially peptide unreceptive within 30 min from synthesis (Fig. 4), e.g., at stages in which classical class I molecules are instead free of, and fully receptive to, specific peptide ligands (see, for instance, Ref. 38).

Ulbrecht et al. (5) found that HLA-E^{107R} has a low affinity for human β_2m when coexpressed in cells from species other than human, including murine X63 myeloma and insect cells. Possibly, the use of Abs to framework class I epitopes, including W6/32, and/or transfection in nonhuman cells prevented the efficient detection of tight HLA-E:β_2m assemblies in this and other previous studies.

HLA-E: tight association with β_2m and surface expression in the absence of canonical ligands, TAP, and functional tapasin

Although some Abs to framework class I epitopes and β_2m bind to the surface of 221 cells (22), there is no conclusive identification, to our knowledge, of the reactive class I molecules as HLA-E, HLA-F, or both. For instance, whether or not HLA-F is surface expressed is open to discussion (44, 47). In contrast, 3D12 and/or DT9 did not significantly bind to the surface of 221 cells (3, 6, 23, 24), TAP-defective.134 and BM36.1 cells, and tapasin-defective 220.A2 cells (3, 6). Altogether, these results do not formally exclude surface expression of HLA-E in these cells, but the consensus view of HLA-E function implies that these surface HLA-E molecules, if any, are unstable, being assembled in the absence of their permissive ligands.

Strikingly, taking advantage of Abs to β_2m and three surface-labeling methods (including a conformation-independent cell surface biotin-labeling assay), we detected HLA-E at the cell surface not only in peptide loading-sufficient cells, e.g., 221 transfectants coexpressing HLA-E and permissive alleles, but also in 221 transfectants, T2 and 220 cells lacking canonical ligands, TAP, and functional tapasin, respectively (Figs. 5 and S7).[4] All of the tested cell lines expressed an immature (Endo H-sensitive) HLA-E glycoform, whereas a mature (Endo H-insensitive) glycoform was exclusively expressed, and at low levels, in 221 cells transfected with permissive alleles. Interestingly, this restricted cell surface distribution of the mature glycoform recapitulates the surface binding of 3D12 and DT9 (3, 6).

These results demonstrate that surface expression in the absence of TAP and tapasin is not a special feature of a limited number of HLA-E molecules nonconventionally loaded with alternative peptides (14, 15, 26), but a widely used, although noncanonical, assembly and expression route of HLA-E that operates in parallel with (and independently of) the canonical signal sequence-dependent route. Many alternative HLA-E ligands have indeed been described (8, 14–21) that may promote the noncanonical route. A nonexclusive possibility is that HLA-E might undergo ligand-independent assembly and expression, as proposed for Qa-1 (12).

Incidentally, the present study provides evidence for immature classical class I H chains on the surface of peptide loading-sufficient and peptide loading-defective T1 and T2 cells, respectively. Thus, HLA-E takes advantage of both canonical and alternative routes, whereas classical class I molecules routinely adopt the former, but may resort to the latter in special cases (e.g., T1) or when the former is damaged (T2 and 220). The surface expression of classical and nonclassical class I H chains bearing immature glycans has been reported by us in the case of HLA-C H chains in the absence of β_2m (39), and by others in the case of HLA-F (47).

In summary, stable HLA-E expression at the cell surface is possible in several conditions that are incompatible with physiological donation, transport, and loading of canonical peptide ligands. Whatever the ligand populations, specific assembly mechanisms, and transport routes, peptide loading with signal sequence-derived peptides is not the only, and probably not even the dominant, mechanism of stabilization and expression of HLA-E. These findings may help to reconcile conflicting findings, listed above and in the Introduction as points 1–7, provide a comprehensive model of HLA-E stabilization/expression, and predict the existence of a range of HLA-E ligands and assembly mechanisms, some of which have been addressed in this study.

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Disclosures

M.S. is a current employee of EXBIO, the company commercializing the MEM Abs.

References

1. Braud, V. M., E. Y. Jones, and A. McMichael. 1997. The human major histocompatibility complex class Ib molecule HLA-E binds signal sequence-derived peptides with primary anchor residues at positions 2 and 9. *Eur. J. Immunol.* 27: 1164–1169.
2. Braud, V. M., D. S. J. Allan, C. A. O'Callaghan, K. Söderström, A. D'Andrea, G. S. Ogg, S. Lazetic, N. T. Young, J. I. Bell, J. H. Phillips, et al. 1998. HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. *Nature* 391: 795–799.
3. Lee, N., D. R. Goodlett, A. Ishitani, H. Marquardt, and D. E. Geraghty. 1998. HLA-E surface expression depends on binding of TAP-dependent peptides derived from certain HLA class I signal sequences. *J. Immunol.* 160: 4951–4960.
4. Grimsley, C., A. Kawasaki, C. Gassner, N. Sageshima, Y. Nose, K. Hatake, D. E. Geraghty, and A. Ishitani. 2002. Definitive high resolution typing of HLA-E allelic polymorphisms: identifying potential errors in existing allele data. *Tissue Antigens* 60: 206–212.
5. Ulbrecht, M., A. Couturier, S. Martinozzi, M. Pla, R. Srivastava, P. A. Peterson, and E. H. Weiss. 1999. Cell surface expression of HLA-E: interaction with human β_2m and allelic differences. *Eur. J. Immunol.* 29: 537–547.
6. Braud, V. M., D. S. J. Allan, D. Wilson, and A. J. McMichael. 1998. TAP- and tapasin-dependent HLA-E surface expression correlates with the binding of an MHC class I leader peptide. *Curr. Biol.* 8: 1–10.
7. Borrego, F., M. Ulbrecht, E. H. Weiss, J. E. Coligan, and A. G. Brooks. 1998. Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis. *J. Exp. Med.* 187: 813–818.
8. Ulbrecht, M., S. Modrow, R. Srivastava, P. A. Peterson, and E. H. Weiss. 1998. Interaction of HLA-E with peptides and the peptide transporter in vitro: implications for its function in antigen presentation. *J. Immunol.* 160: 4375–4385.
9. Brooks, A. G., F. Borrego, P. E. Posch, A. Patamawenu, C. J. Scorzelli, M. Ulbrecht, E. H. Weiss, and J. E. Coligan. 1999. Specific recognition of HLA-E, but not classical, HLA class I molecules by soluble CD94/NKG2A and NK cells. *J. Immunol.* 162: 305–313.
10. O'Callaghan, C. A., J. Tormo, B. E. Willcox, V. M. Braud, B. K. Jakobsen, D. I. Stuart, A. J. McMichael, J. I. Bell, and E. Y. Jones. 1998. Structural features impose tight peptide binding specificity in the nonclassical MHC molecule HLA-E. *Mol. Cell* 1: 531–541.
11. Moretta, L., C. Romagnani, G. Pietra, A. Moretta, and M. C. Mingari. 2003. NK-CTLs, a novel HLA-E-restricted T-cell subset. *Trends Immunol.* 24: 136–143.
12. Kambayashi, T., J. Kraft-Leavy, J. Dauner, B. Sullivan, O. Laur, and P. Jensen. 2004. The nonclassical MHC class I molecule Qa-1 forms unstable peptide complexes. *J. Immunol.* 172: 1661–1669.

13. Stevens, J., E. Joly, J. Trowsdale, and G. W. Butcher. 2001. Peptide binding characteristics of the non-classical class Ib MHC molecule HLA-E assessed by a recombinant random peptide approach. *BMC Immunol.* 2: 5.
14. Ulbrecht, M., S. Martinozzi, M. Grzeschik, H. Hengel, J. W. Ellwart, M. Pla, and E. H. Weiss. 2000. The human cytomegalovirus *UL40* gene product contains a ligand for HLA-E and prevents NK cell-mediated lysis. *J. Immunol.* 164: 5019–5022.
15. Tomasec, P., V. M. Braud, C. Rickards, M. B. Powell, B. P. McSharry, S. Gadola, V. Cerundolo, L. K. Borysiewicz, A. J. McMichael, and G. W. G. Wilkinson. 2000. Surface expression of HLA-E, an inhibitor of natural killer cells, enhanced by human cytomegalovirus gpUL40. *Science* 287: 1031–1033.
16. Michaelsson, J., C. Teixeira de Matos, A. Achour, L. L. Lanier, K. Kärre, and K. Soderstrom. 2005. A signal peptide derived from hsp60 binds HLA-E and interferes with CD94/NKG2A recognition. *J. Exp. Med.* 196: 1403–1414.
17. Li, J., I. Goldstein, E. Glickman-Nir, H. Jiang, and L. Chess. 2001. Induction of TCR Vβ-specific $CD8^+$ CTLs by TCR Vβ-derived peptides bound to HLA-E. *J. Immunol.* 167: 3800–3808.
18. Heinzel, A., J. Grotzke, R. Lines, D. Lewinsohn, A. McNabb, D. Streblow, V. Braud, H. Grieser, and J. Belisle. 2002. HLA-E-dependent presentation of Mtb-derived antigen to human $CD8^+$ T cells. *J. Exp. Med.* 196: 1473–1481.
19. Wooden, S., S. Kalb, R. Cotter, and M. Soloski. 2005. HLA-E binds a peptide derived from the ATP-binding cassette transporter multidrug resistance-associated protein 7 and inhibits NK cell-mediated lysis. *J. Immunol.* 175: 1383–1387.
20. Salerno-Goncalves, R., M. Fernandez-Vina, D. Lewinsohn, and M. Sztein. 2004. Identification of a human HLA-E-restricted $CD8^+$ T cell subset in volunteers immunized with *Salmonella enterica* serovar Typhi strain Ty21a typhoid vaccine. *J. Immunol.* 173: 5852–5862.
21. Nattermann, J., H. D. Nischalke, V. Hofmeister, G. Ahlenstiel, H. Zimmermann, L. Leifeld, E. H. Weiss, T. Sauerbruch, and U. Spengler. 2005. The HLA-A2 restricted T cell epitope HCV core 35–44 stabilizes HLA-E expression and inhibits cytolysis mediated by natural killer cells. *Am. J. Pathol.* 166: 443–453.
22. Pietra, G., C. Romagnani, M. Falco, M. Vitale, R. Castriconi, D. Pende, E. Millo, S. Anfossi, R. Biassoni, L. Moretta, and M. C. Mingari. 2002. The analysis of the natural killer-like activity of human cytolytic T lymphocytes revealed HLA-E as a novel target for TCR α/β-mediated recognition. *Eur. J. Immunol.* 31: 3687–3693.
23. Lee, N., M. Llano, M. Carretero, A. Ishitani, F. Navarro, M. Lopez-Botet, and D. Geraghty. 1998. HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc. Natl. Acad. Sci. USA* 95: 5199–5204.
24. Llano, M., N. Lee, F. Navarro, P. García, J. P. Albar, D. E. Geraghty, and M. López-Botet. 1998. HLA-E-bound peptides influence recognition by inhibitory and triggering CD94/NKG2 receptors: preferential response to an HLA-G-derived nonamer. *Eur. J. Immunol.* 28: 2854–2863.
25. Robinson, P. J., P. J. Travers, A. Stackpoole, L. Flaherty, and H. Djaballah. 1998. Maturation of $Qa\text{-}1^b$ class I molecules requires β_2-microglobulin but is TAP independent. *J. Immunol.* 160: 3217–3224.
26. Ulbrecht, M., V. Hofmeister, G. Yuksekdag, J. Ellwart, H. Hengel, F. Momburg, S. Martinozzi, M. Reboul, M. Pla, and E. Weiss. 2003. HCMV glycoprotein US6 mediated inhibition of TAP does not affect HLA-E dependent protection of K-562 cells from NK cell lysis. *Hum. Immunol.* 64: 231–237.
27. Pacasova, R., S. Martinozzi, H. Boulouis, Y. Szpak, M. Ulbrecht, F. Sigaux, E. Weiss, and M. Pla. 1999. Cell surface detection of HLA-E gene products with a specific monoclonal antibody. *J. Reprod. Immunol.* 43: 195–201.
28. Ishitani, A., N. Sageshima, N. Lee, N. Dorofeeva, K. Hatake, H. Marquardt, and D. Geraghty. 2003. Protein expression and peptide binding suggest unique and interacting functional roles for HLA-E, F, and G in maternal-placental immune recognition. *J. Immunol.* 171: 1376–1384.
29. Menier, C., B. Saez, V. Horejsi, S. Martinozzi, I. Krawice-Radanne, S. Bruel, C. Le Danff, M. Reboul, I. Hilgert, M. Rabreau, et al. 2003. Characterization of monoclonal antibodies recognizing HLA-G or HLA-E: new tools to analyze the expression of nonclassical HLA class I molecules. *Hum. Immunol.* 64: 315–326.
30. LeMaoult, J., M. Le Discorde, N. Rouas-Freiss, P. Moreau, C. Menier, J. McCluskey, and E. D. Carosella. 2003. Biology and functions of human leukocyte antigen-G in health and sickness. *Tissue Antigens* 62: 273–284.
31. Sibilio, L., A. Martayan, R. Fraioli, E. Lo Monaco, E. Melucci, M. Suchànek, and P. Giacomini. 2003. Biochemical characterization of monoclonal antibodies to HLA-E and HLA-G. *Tissue Antigens* 62: 356.
32. Coupel, S., A. Moreau, M. Hamidou, V. Horejsi, J. P. Soulillou, and B. Charreau. 2007. Expression and release of soluble HLA-E is an immunoregulatory feature of endothelial cell activation. *Blood* 109: 2806–2814.
33. Marin, R., F. Ruiz-Cabello, S. Pedrinaci, R. Mendez, P. Jimenez, D. E. Geraghty, and F. Garrido. 2003. Analysis of HLA-E expression in human tumors. *Immunogenetics* 54: 767–775.
34. Palmisano, G. L., E. Contardi, A. Morabito, V. Gargaglione, G. B. Ferrara, and M. P. Pistillo. 2005. HLA-E surface expression is independent of the availability of HLA class I signal sequence-derived peptides in human tumor cell lines. *Hum. Immunol.* 66: 1–12.
35. Derré, L., M. Corvaisier, B. Charreau, A. Moreau, E. Godefroy, A. Moreau-Aubry, F. Jotereau, and N. Gervois. 2006. Expression and release of HLA-E by melanoma cells and melanocytes: potential impact on the response of cytotoxic effector cells. *J. Immunol.* 177: 3100–3107.
36. Lee, N., A. R. Malacko, A. Ishitani, M. C. Chen, J. Bajorath, H. Marquardt, and D. E. Geraghty. 1995. The membrane-bound and soluble forms of HLA-G bind identical sets of endogenous peptides but differ with respect to TAP association. *Immunity* 3: 591–600.
37. Setini, A., A. Beretta, C. De Santis, R. Meneveri, A. Martayan, M. C. Mazzilli, E. Appella, A. G. Siccardi, P. G. Natali, and P. Giacomini. 1996. Distinctive features of the α1 domain α helix of HLA-C heavy chains free of β_2-microglobulin. *Hum. Immunol.* 46: 69–81.
38. Sibilio, L., A. Martayan, A. Setini, R. Fraioli, D. Fruci, J. Shabanowitz, D. F. Hunt, and P. Giacomini. 2005. Impaired assembly results in the accumulation of multiple HLA-C heavy chain folding intermediates. *J. Immunol.* 175: 6651–6658.
39. Martayan, A., R. Fraioli, E. Giorda, A. Setini, G. Ciccarelli, L. Delfino, G. B. Ferrara, and P. Giacomini. 1999. Biosynthesis of HLA-C heavy chains in melanoma cells with multiple defects in the expression of HLA-A, -B, -C molecules. *Br. J. Cancer* 80: 639–649.
40. Steinle, A., and D. J. Schendel. 1994. HLA class I alleles of LCL 721 and 174 × CEM.T2 (T2). *Tissue Antigens* 44: 268–270.
41. Greenwood, R., Y. Shimizu, G. S. Sekhon, and R. DeMars. 1994. Novel allele-specific, post-translational reduction in HLA class I surface expression in a mutant human B cell line. *J. Immunol.* 153: 5525–5536.
42. Brodsky, F. M., P. Parham, C. J. Barnstable, M. J. Crumpton, and W. F. Bodmer. 1979. Monoclonal antibodies for analysis of the HLA system. *Immunol. Rev.* 47: 3–61.
43. Stam, N. J., H. Spits, and H. L. Ploegh. 1986. Monoclonal antibodies raised against denatured HLA-B locus heavy chains permit biochemical characterization of certain HLA-C products. *J. Immunol.* 137: 2299–2306.
44. Wainwright, S. D., P. A. Biro, and C. H. Holmes. 2000. HLA-F is a predominantly empty, intracellular, TAP-associated MHC class Ib protein with a restricted expression pattern. *J. Immunol.* 164: 319–328.
45. Townsend, A., T. Elliott, V. Cerundolo, L. Foster, B. Barber, and A. Tse. 1990. Assembly of MHC class I molecules analyzed in vitro. *Cell* 62: 285–295.
46. Tran, T. M., P. Ivanyi, I. Hilgert, T. Brdicka, M. Pla, B. Breur, M. Flieger, E. Ivaskova, and V. Horejsi. 2001. The epitope recognized by pan-HLA class I-reactive monoclonal antibody W6/32 and its relationship to unusual stability of the HLA-B27/β_2m complex. *Immunogenetics* 53: 440–446.
47. Lee, N., and D. Geraghty. 2003. HLA-F surface expression on B cell and monocyte cell lines is partially independent from tapasin and completely independent from TAP. *J. Immunol.* 171: 5264–5271.