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Analysis of HLA-E Peptide-Binding Specificity and Contact Residues in Bound Peptide Required for Recognition by CD94/NKG2

Joseph D. Miller,* Dominique A. Weber,‡ Chris Ibegbu,* Jan Pohl,† John D. Altman,* and Peter E. Jensen2‡

The MHC class Ib molecule HLA-E is the primary ligand for CD94/NKG2A-inhibitory receptors expressed on NK cells, and there is also evidence for TCR-mediated recognition of this molecule. HLA-E preferentially assembles with a homologous set of peptides derived from the leader sequence of class Ia molecules, but its capacity to bind and present other peptides remains to be fully explored. The peptide-binding motif of HLA-E was investigated by folding HLA-E in vitro in the presence of peptide libraries derived from a nonameric leader peptide sequence randomized at individual anchor positions. A high degree of selectivity was observed at four of five total anchor positions, with preference for amino acids present in HLA-E-binding peptides from class Ia leader sequences. Selectivity was also observed at the nonanchor P5 position, with preference for positively charged amino acids, suggesting that electrostatic interactions involving the P5 side chain may facilitate assembly of HLA-E peptide complexes. The observed HLA-E peptide-binding motif was strikingly similar to that previously identified for the murine class Ib molecule, Qa-1. Experiments with HLA-E tetramers bearing peptides substituted at nonanchor positions demonstrated that P5 and P8 are primary contact residues for interaction with CD94/NKG2 receptors. A conservative replacement of Arg for Lys at P5 completely abrogated binding to CD94/NKG2. Despite conservation of peptide-binding specificity in HLA-E and Qa-1, cross-species tetramer-staining experiments demonstrated that the interaction surfaces on CD94/NKG2 and the class Ib ligands have diverged between primates and rodents. The Journal of Immunology, 2003, 171: 1369–1375.

Human histocompatibility leukocyte Ag-E is nonclassical MHC class I molecule with limited polymorphism that is expressed in a wide range of tissues, but at lower levels than MHC class Ia molecules (1–4). This class Ib protein predominantly binds and displays a closely related set of nonameric peptides derived from the signal sequences of class Ia molecules, in contrast to class Ia molecules, which present a highly diverse repertoire of peptide ligands (5–7). The N-terminal fragment of the class Ia leader sequence appears to be released into the cytoplasm of cells through a mechanism involving cleavage by signal peptidase and the endoplasmic reticulum membrane-imbehded protease, signal peptide peptidase (8). Thus, assembly of HLA-E with the signal peptide-derived ligands is strictly dependent on TAP function for peptide delivery into the endoplasmic reticulum lumen (9).

Recent studies have demonstrated that HLA-E is the primary, and perhaps exclusive, ligand for heterodimeric CD94/NKG2 receptors expressed on NK cells and a subset of T cells (6, 7, 10). CD94 can pair with several different isoforms of NKG2 to form receptors with the potential to either inhibit (NKG2A) or promote (NKG2C) cellular activation (11–13). The inhibitory receptor CD94/NKG2A is the dominant isoform expressed on NK cells, and a number of studies have demonstrated that NK cytotoxicity can be inhibited by interaction of this receptor with HLA-E on target cells (6, 7, 10, 14). This inhibitory receptor therefore provides a mechanism for recognition of missing self, in which self in this case is defined by expression of HLA-E (15, 16). Several studies have provided evidence that recognition by CD94/NKG2 is sensitive to the sequence of peptide bound to HLA-E; thus, peptide replacement in HLA-E provides a potential mechanism to sensitize cells for NK killing (10, 14, 17–19). In addition, it has been demonstrated that human CMV has the potential to use a decoy for CD94/NKG2, by providing an appropriate HLA-E peptide ligand that can be loaded through a TAP-independent pathway (20, 21). The CD94/NKG2-class Ib recognition system appears to have an ancient origin in evolution, because a homologous system is present in rodents. Murine CD94/NKG2 receptors recognize the mouse class Ib molecule Qa-1, which is not a clear ortholog of HLA-E based on overall amino acid sequence comparison (22–24). Nevertheless, Qa-1 selectively binds class Ia leader sequence-derived peptides with sequences similar to the known HLA-E-binding peptides (25, 26).

In addition to serving as a ligand for CD94/NKG2, there is evidence for TCR-mediated recognition of HLA-E (27–32). Thus, HLA-E may also function as a restricting element for conventional or regulatory CD8⁺ T cells. The crystal structure of HLA-E bound to a class Ia leader sequence-derived peptide provided a structural explanation for the restricted peptide-loading specificity of HLA-E (33). It is possible that the intracellular loading pathway and the abundance of class Ia leader peptides also act to limit the diversity of peptides presented by HLA-E under physiological conditions. Very little information is currently available on the extent to which HLA-E is capable of binding and presenting peptides derived from...
sources other than class Ia leader sequences (14, 34). In the present study, we addressed this issue by analyzing the peptide-binding specificity of HLA-E using an approach based on in vitro folding of HLA-E complexes in the presence of limited peptide libraries selectively randomized at specific anchor positions. In addition, we demonstrate that lysine substitutions at P5 or P8 in HLA-E-bound peptide prevent binding to CD94/NKG2, suggesting that these positions serve as receptor contact residues.

Materials and Methods

Peptides

Peptides used in binding assays and for HLA-E tetramers were synthesized by 9-fluorenylmethyloxycarbonyl (F-moc) chemistry peptide on a Ranin Symphony peptide synthesizer. Biotinylation of Qa-1 determinant modifier (Qdm) peptides and assembly of randomized peptide pools were performed, as previously described (26).

Purification of HLA-E and Qa-1b inclusion bodies

To increase HLA-E expression in BL21 DE3 cells, the GC content in the 5’ region of HLA-E was reduced without changing the amino acid content. Using the following 5’ GC primer, GCAGATTCTTCATGGGCTCAGCTCATTCAATGATTTCCACACTTCCGTGTCACGTCCAGGTCGTTGGACACCGTTTCACTCTTGTCGCTAC, the soluble region of HLA-E*0103 was subcloned into a pET23a vector (Novagen, Madison, WI) and fused to a 3’ biotinyl signal peptide to form pHLA-E Delux (35). Removal of 5’ GC content resulted in a 100-fold increase in protein expression (data not shown). Inclusion bodies were prepared, as previously described (35). Briefly, BL21 DE3 bacteria transformed with pHLA-E Delux were grown in Luria-Bertani medium at 37°C until the cultures reached an OD of 0.6 absorbance units at 600 nm. At this point, isopropyl β-D-thiogalactoside was added to a 1 mM final concentration and the cultures were incubated for additional 4 h at 37°C. Harvested bacteria was lysed using a 550 sonic dismembrator (Fisher, Pittsburgh, PA), and inclusion bodies were prepared by washing bacterial lysates with a Triton X-100 solution. Purified inclusion bodies were solubilized in 8 M urea and stored at −80°C until further use. Qa-1b inclusion bodies were purified similarly.

Folding Qa-1b and HLA-E and the formation of MHC tetramers

HLA-E and Qa-1b were folded in vitro, as previously described (35). Briefly, MHC H chain, β2m–microglobulin (β2m), and peptide were injected into t-arginine folding buffer (200 mM t-arginine, 100 mM Tris, pH 8.3, 2 mM EDTA, 5 mM reduced glutathione, and 0.5 mM oxidized glutathione) and incubated at 10°C for 2 days. The concentrated folding reaction was purified by size exclusion chromatography using a S300 column (Pharmacia, Peapack, NJ). Monomers to be used as tetramers were biotinylated overnight using BIR A enzyme. All complexes were further purified by anion exchange using a monoQ column (Pharmacia). Biotinylated MHC monomers were labeled using PE-conjugated streptavidin (Molecular Probes, Portland, OR).

Peptide-binding immunoassay

Peptide-binding assays were performed, as previously described (36). In brief, folded MHC molecules (30 nM) were incubated overnight at room temperature with biotin-Qdm4C (0.2 μM) and dilutions of competitor peptide in pH 5.5 binding buffer (0.01% Nonidet P-40, 10 nM citrate-phosphate buffer, and protease inhibitor cocktail (Roche, Nutley, NJ)). MHC complexes were pH neutralized and captured in anti-β2m mAb (ImmunoTech, Westbrook, ME)–coated wells. The amount of biotin-Qdm4C bound to MHC was determined by adding europium-labeled streptavidin (Wallac Oy, acquired by PE Life Sciences, Boston, MA) to each sample well, measuring the fluorescence intensity at 615 nm using a 1230 ARCS time-resolved fluorometer (LKB Wallac, Boston, MA).

Sequencing of peptides eluted from HLA-E

Elution of peptides from folded HLA-E was performed, as previously described (26). Briefly, HLA-E was folded in vitro, as described above, using peptide pools randomized at a single position. Peptides were eluted from purified MHC molecules under acidic conditions (0.1% trifluoroacetic acid, 10% CH3CN), purified from proteins using a 5-kDa Ultrafree-15 centrifugal filter device (Millipore, Bedford, MA), and concentrated using a speed vac (Labconco, Kansas City, MO). Samples were sequenced by Edman degradation using a Procise cLC peptide sequencer (PE Biosystems, Foster City, CA) (37). HPLC purification and mass spectrometry were performed, as previously described (26).

Staining primary cells with HLA-E tetramers

PBMC were isolated from Macaca mulatta and human peripheral blood using lymphocyte separation medium (Cellgro, Herndon, VA). Murine splenocytes were isolated from C57BL/6 mice by RBC lysis purification (Sigma-Aldrich, St. Louis, MO). All staining Abs were purchased from BD Pharmingen (San Diego, CA), unless noted. Human and nonhuman pri- mate samples were stained at room temperature with HLA-E tetramers and surface-staining mAbs in PBS containing 1% BSA. Human surface mAbs included anti-CD3 FITC, anti-CD8 PerCP, and anti-CD56 APC. Nonhuman plaque surface stains included anti-CD16 FITC, anti-CD8 PerCP, and anti-CD3 APC. Murine samples were stained at 4°C using anti-DX-5 FITC, PE-labeled MHC tetramers, anti-CD8 PerCP, and anti-CD3 APC in PBS containing 1% BSA. All samples were fixed in PBS containing 1% paraformaldehyde and analyzed by flow cytometry using a FACScan with Luminex technology (BD Biosciences, San Jose, CA) and FlowJo data analysis software (Treestar, San Carlos, CA).

Results

The peptide-binding specificity of HLA-E has previously been investigated using assays based on biochemical stabilization of HLA-E in cell lysates (5, 6) and stabilization of cell surface molecules measured by flow cytometry (7, 10, 34, 38). We developed a peptide-binding assay using soluble rHLA-E and a biotin-labeled variant of the HLA-A2 leader peptide (VMAPRTTLV). The Pro at position 4 in the peptide was replaced with Cys, allowing selective biotin labeling through a thiol group in a position predicted to be solvent exposed in the HLA-E-peptide complex (33). Biotin-peptide-HLA-E complexes were quantified using a europium-streptavidin fluorescence immunoassay (Fig. 1). Peptide binding was observed to be saturable and inhibited by excess unlabeled HLA-A2 leader peptide.

In competition-binding studies, the mouse leader peptide Qdm (AMAPRTLLL) was observed to bind HLA-E with affinity similar to that of the HLA-A2 leader (Fig. 1C). Previous reports showing that Qdm binds to HLA-E with somewhat lower affinity than the HLA-A2 leader peptide (5, 19) might reflect differences in the

FIGURE 1. Peptide binding to rHLA-E. A, Various concentrations of purified rHLA-E were incubated for 18 h with 2 μM biotin-A2 P4C peptide (sequence: VMAARPTTLV). B, rHLA-E (50 nM) was incubated for 18 h with various concentrations of biotin-A2 P4C peptide. C–E, rHLA-E (50 nM) was incubated with 0.2 μM biotin-A2 P4C in the presence of various concentrations of the indicated unlabeled competitor peptides. HLA-E-bound biotin peptide was measured using a europium-streptavidin fluorescence immunoassay, as described in Materials and Methods. Data represent fluorescence counts per second. The mouse leader peptide Qdm (AMAPRTTLV) was observed to bind HLA-E with full affinity. HLA-E-A2 leader peptide (VMAPRTTLV). Binding affinity was markedly reduced in Qdm peptides with Lys substitutions at P2, P6, P7, or P9.
assays used: HLA-E stabilization as opposed to measurement of peptide exchange in the current study. Lysine-substituted peptides based on the Qdm sequence were used to evaluate anchor positions. Substitutions at positions 2, 6, 7, or 9 markedly reduced binding affinity, with IC₅₀ values 2–3 logs greater than wild-type Qdm (Fig. 1D). Lysine substitutions at positions 1, 3, 4, 5, or 8 produced relatively small effects in competition-binding experiments (Fig. 1, D and E). These results confirm and extend a previous study in which each residue of the human HLA-B*0801 leader peptide (VMAPRTVLL) was replaced with alanine and tested for the capacity to stabilize HLA-E in cell lysates (5). The latter study demonstrated primary anchor residues located at positions 2 and 9, with secondary anchor residues at position 7 and possibly position 3. In contrast to our findings, the P6 position was not identified as an anchor, presumably because alanine, but not lysine, is tolerated at this position. The combined results are consistent with the HLA-E crystal structure, which demonstrated deep pockets accommodating the side chains of P2, P7, and P9 and shallow pockets accommodating P3 and P6 (33).

An approach based on in vitro folding of HLA-E with various peptide libraries was used to characterize the peptide-binding motif of this protein. Several attempts were made to fold HLA-E with a fully random nonamer peptide library. Whereas high yields were obtained in folding reactions with the mouse leader peptide Qdm, little or no folded HLA-E was obtained with high concentrations (100 μM) of the fully random peptide library (Fig. 2). The concentration of peptides in the library with an appropriate peptide-binding motif was apparently too low to support efficient refolding. Therefore, to determine the amino acid preferences at each anchor position, we refolded HLA-E H chain and β₂m with separate libraries of the Qdm peptide (AMAPRTVLL) randomized selectively at P2, P3, P6, P7, or P9. Randomized peptide libraries displayed an equal representation of each naturally occurring amino acid with the exception of cysteine, which was omitted from the peptide synthesis to prevent cross-linking. Refolded HLA-E-peptide complexes were isolated by size exclusion chromatography (Fig. 2) and further purified by anion exchange chromatography. Peptides from purified HLA-E complexes were eluted under acidic conditions and subjected to mass spectrometry and Edman degradation to determine amino acid preferences and percentages at each anchor position, respectively (Table I).

Folded HLA-E complexes strongly preferred Met and Leu at P2 and P9, respectively, consistent with the assignment of these positions as dominant anchors (5). Indeed, the greatest selectivity was observed with the peptide pool that was randomized at P9, with 82% of bound peptide having Leu at this position. Peptides eluted from HLA-E refolded with the P2-randomized Qdm peptide library showed a strong preference for methionine (66%) and leucine (25%). A preference for Ala (52%) was observed at the P6 anchor position. Thr (39%) was also well represented; this amino acid is present at P6 in all HLA-E-binding human leader sequences. The naturally occurring residues at P7, Leu, and Val were clearly dominant in HLA-E folded in vitro with the library randomized at this position. Chemically diverse amino acids at P3 were selected by HLA-E, indicating that preference at this position may be less constrained. Ala, the residue found at this position in human leader peptides, represented only 15% of HLA-E-bound peptide. The peptide with Lys at this position bound preferentially (36%), consistent with competition-binding data showing that Lys substitution at this position had a relatively small effect on binding affinity (Fig. 1D).

The crystal structure of HLA-E complexed with a nonamer peptide (VMAPRTVLL) demonstrates two conformations for the P5 Arg side chain: completely solvent exposed or bound to the negatively charged Glu residue on the α2 helix (33). To determine whether interactions between the P5 side chain and the α2 helix confer a preference for specific amino acids at P5, we refolded HLA-E with a Qdm peptide library randomized at P5. Peptides eluted from HLA-E folded with P5-randomized peptides showed a preference for positively charged amino acids Arg (43%) and Lys (19%) (Table I), suggesting that a salt bridge formed between these residues and the negatively charged Glu residue of HLA-E confers selectivity for positively charged amino acids at P5 (33). Overall, our findings demonstrate a remarkable preference for the
Amino acid residues naturally present in MHC class Ia leader peptides (shown in bold in Table I) at the positions analyzed, indicating that these leader peptides have optimal sequences for binding HLA-E and suggesting that peptide-binding specificity is relatively constrained as compared with class Ia molecules. Nevertheless, it is clear that peptides with alternative amino acids at various positions can bind HLA-E.

Because P1, P4, P5, and P8 do not serve as anchor residues, we sought to determine whether these positions function as contact residues for interaction with CD94/NKG2 receptors. HLA-E tetramers were generated with the HLA-A2 leader peptide (VMAPRTLVL) containing lysine substitutions at P1 (A1K), P4 (P4K), P5 (R5K), or P8 (V8K). By means of biochemical analysis, each of these HLA-E tetramers folded equivalently (data not shown). Saturating tetramer-staining conditions were determined by titrating each HLA-E tetramer (data not shown). HLA-E/A1K and HLA-E/P4K tetramers bound a similar percentage of CD56+/CD3− lymphocytes. B. Fresh PBMC were stained with HLA-E tetramers folded with multiple HLA leader peptides that varied at the P8 contact residue: VMAPRTLFL (LFL), VMAPRTLIL (LIL), VMAPRTLVL (LVL), and VMAPRTVLL (VLL). Histograms are gated on CD56+ , CD3− lymphocytes.

The peptide-binding specificity of HLA-E, as determined in this study by analysis of peptide selectivity during in vitro folding, is remarkably similar to that previously determined for Qa-1 using a similar experimental approach (26) (Table I). Given the shared specificity for MHC class Ia leader sequence-derived peptides, we wondered whether the interaction surface with CD94/NKG2 receptors might also be conserved between the primate and mouse proteins. Tetrameric HLA-E and Qa-1 proteins containing the HLA-A2 leader peptide or the mouse Qdm peptide were used to evaluate this possibility. Tetramer generated with an R5K-substituted HLA-A2 leader peptide was used as a negative control. HLA-E tetramers containing either the HLA-A2 leader peptide or

**FIGURE 3.** P5 and P8 serve as contact residues for HLA-E binding to CD94/NKG2. A. Fresh PBMC from two healthy human donors were stained with HLA-E tetramers folded with the A2 leader sequence peptide VMAPRTLVL (A23–11 V8K) or lysine-substituted peptides: KMAPRTLVL (A23–11 V1K), VMAKRTLVL (A23–11 P4K), VMAPKTLVL (A23–11 R5K), and VMAPRTLKL (A23–11 V8K). Histograms are gated on CD56+ , CD3− lymphocytes. B. Fresh PBMC were stained with HLA-E tetramers folded with multiple HLA leader peptides that varied at the P8 contact residue: VMAPRTLFL (LFL), VMAPRTLIL (LIL), VMAPRTLVL (LVL), and VMAPRTVLL (VLL). Histograms are gated on CD56+ , CD3− lymphocytes.
particularly stringent, with almost exclusive preference of Leu at this
mary anchor positions. The requirement at P9 appears to be par-
dues at positions 2 and 9, consistent with their assignment as pri-
side chains.

Qdm bound equivalently to human and nonhuman primate NK
cells, but they did not stain mouse NK cells (Fig. 4A). Conversely,
Qa-1/Qdm tetramers bound to a subset of mouse NK cells, but not
human or rhesus macaque NK cells (Fig. 4B), confirming and ex-
tending a previous report showing that soluble HLA-E tetramers
bearing the HLA-G-derived leader peptide do not bind mouse NK
cells and Qa-1/Qdm tetramers do not bind human NK cells (39).
Thus, the CD94/NKG2 receptor contact surface is clearly different
on HLA-E and Qa-1. The contact surface on CD94/NKG2 is con-
served within primates, but divergent in mice. It is interesting that
while primate CD94/NKG2 receptors recognize HLA-E bearing
the HLA-A2 peptide (VMAPRTLVL) and Qdm (AMAPRTLLL)
equally, mouse CD94/NKG2 selectively recognizes Qa-1/Qdm
complexes and not Qa-1 bearing the human leader peptide (Fig.
4B). These two peptides differ at P1 and P8. In contrast to humans,
mouse MHC class Ia leader sequences are invariant at the P8 po-
tion. Thus, the mouse CD94/NKG2 may be highly selective for
recognition of Leu at this position, as noted previously (39),
whereas primate CD94/NKG2 must cross-react with leader se-
quence-derived peptides that are polymorphic at this position (Fig.
5) (6, 7, 10, 17–19).

Discussion
In the present study, the peptide-binding specificity of HLA-E was
investigated using an approach based on in vitro folding assays
measuring the incorporation of selected peptides from limited pep-
tide libraries randomized at individual anchor positions. We de-
veloped an immunoblot assay to measure the binding of peptides to
purified HLA-E. Lysine-substituted peptides were used to identify
anchor positions required for binding of the nonamer peptide
Qdm to HLA-E. Lys substitutions at positions 2, 6, 7, and 9 sub-
stantially reduced binding affinity for HLA-E. By contrast, position
1, 3, 4, 5, and 8 Lys substitutions only slightly reduced peptide-
binding affinities. These results are consistent with the reported
crystal structure of HLA-E complexed with a human class I leader
sequence-derived peptides that bind HLA-E (top box).

Two distinct conformations were observed for the peptide po-
tion 5 Arg side chain in the two copies of the HLA-E complex
present in the crystal structure by O’Callaghan et al. (33). In one
molecule, the side chain was fully exposed to solvent, extending
directly out of the center of the peptide-binding groove. In the
other molecule, this side chain was held down through a salt bridge
to negatively charged Glu152 on the top surface of the HLA-E α2
α helix. Our results demonstrate that there is a strong preference
for positively charged residues at P5, with Arg favored over Lys.
Thus, electrostatic interaction with Glu152 appears to promote the
initial assembly of HLA-E-peptide complexes. The possibility that
there may be significant preference for selected amino acids at
other nonanchor positions remains to be fully explored. Stevens et
al. (40) observed preference for hydrophobic amino acids at non-
anchor positions 4 and 9 on analysis of peptides eluted from
HLA-E folded in the presence of a fully random nonamer library.
In the latter study, a preference for Leu at the major P9 anchor
position was observed, but neither Met nor Leu was enriched at the
P2 anchor position, and, in general, the degree of enrichment ob-
served in Edman analysis of the pooled peptide eluate was rather
subtle (40). Our own attempts to fold HLA-E with a fully random-
ized nonamer peptide library were unsuccessful, under the same
conditions that were successful for the mouse class Ib molecule,
Qa-1 (26). The efficiency of in vitro folding was probably ham-
pered by a low abundance of peptides with sequences compatible
with optimal binding to HLA-E.

A striking feature of the HLA-E peptide-binding motif analysis
presented in this work is the observed preference for amino acids
found in corresponding positions in class Ia leader peptides. The
greatest selectivity is observed at the dominant anchor positions,
Met at P2 and Leu at P9. These positions are invariant in HLA-
E-binding class Ia leader peptides (5–7, 10, 17). Invariant residues
Ala at P3, Arg at P5, and Thr at P6 are strongly selected in in vitro
folding experiments. Leu and Val are present at the P7 position in
class Ia leader sequences, and these amino acids were preferen-
tially selected in our folding experiments. Thus, it appears that
HLA-E has a highly selective peptide-binding motif that is opti-
mized for binding class Ia leader peptides. Nevertheless, it is ev-
ident from our results that there is considerable promiscuity at the
P3 position and that selected substitutions at other anchor positions
are tolerated. The peptide-binding motif reported in this work may
be useful in identifying candidate epitopes for HLA-E-restricted T
cells. Such epitopes are of interest because they might represent
universal T cell epitopes, given the low extent of polymorphism in
HLA-E in the human population (2). A number of studies have
reported TCR recognition of HLA-E, although peptide specificity
has not been defined in most situations (27–30, 32). Li et al. (31)
reported the establishment of HLA-E-restricted CD8+ T cells gen-
erated by in vitro priming with peptides derived from TCR Vβ
sequences containing Leu at major anchor positions P2 and P9. In

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** HLA-E peptide-binding motif. Numbers depict the amino
acid positions of HLA-E-binding peptides. Upward and downward point-
ing arrows show the position of contact and anchor residues, respectively.
Major and minor positions are reflected by the length of the arrow. Experimentally
determined amino acids that are preferred at each anchor residue (top box) are shown in comparison with residues in endogenous HLA class
Ia leader sequence-derived peptides that are polymorphic at this position (bottom box).
addition, a peptide from human heat shock protein 60 was demonstrated to bind HLA-E (14). These peptides did not contain the preferred residues identified in the present study at the minor anchor positions. Two viral peptides that have sequences quite divergent from class Ia leader peptides have been identified that stabilize HLA-E (19, 34). The EBV-derived peptide BZLF-1 (SQAPLPCVL) contains Gln at the P2 major anchor position. Our results demonstrated preference for Gln, secondary only to Met and Leu, at the P2 position. Cys may be a preferred amino acid at the P7 position; Cys was not included in our peptide libraries to prevent oxidative cross-linking. Therefore, the BZLF-1 sequence diverges from motif identified in the present study only at the nonanchor P5 position and P6, because Pro was not selected at this position in our in vitro folding experiments. The influenza-derived peptide M59–67K62 (ILGKVFTLT) diverges considerably from the motif identified in this study. In particular, Thr at P9 seems to violate a requirement for Leu at this position. It is possible that this peptide binds HLA-E with relatively low affinity, because quantitative binding analysis has yet to be performed. One must also consider the possibility that selectivity at a given anchor position may be influenced by the identity of amino acids at other positions, through effects on the conformation of bound peptide or HLA-E. In the present study, selectivity at specific positions was analyzed under conditions in which the sequence framework of the peptide was fixed. The impact of cooperative interactions between anchor residues will require further investigation.

The role of specific amino acid side chains in the HLA-E-bound peptide as contacts for interaction with CD94/NKG2 was also investigated using HLA-E tetramers generated with HLA-A2 leader peptides substituted with Lys at each of the nonanchor positions. Tetramers generated with peptides substituted at P5 or P8 were observed to have substantially reduced binding to human NK cells, whereas little or no effect was observed with substitutions at P1 or P4. Thus, the P5 and P8 side chains appear to be directly involved in the contact interface with CD94/NKG2 receptors. Several previous studies have provided evidence that the peptide bound to HLA-E influences recognition by CD94/NKG2 (10, 14, 17, 18). Llano et al. (17) reported that a leader peptide with Ala instead of the usual Thr at P6 stabilizes HLA-E, but the resulting complexes were poor ligands for the inhibitory receptor. Because the side chain of P6 is accommodated in a shallow pocket, this observation could be explained by direct recognition of Thr at position 6 by CD94/NKG2, or by an indirect effect on the conformation of the HLA-E complex. Borrego et al. (10) reported that the HLA-B27 leader sequence, containing Thr instead of Met at the major P2 anchor position, stabilizes HLA-E without inducing resistance to NK-mediated lysis. However, peptides with Thr at P2 have been reported to have very low affinity for HLA-E (6), and it is evident from the current study that Thr is not a preferred amino acid at this position. Thus, the reduced functional potency of the HLA-B27 leader peptide might be attributable to dissociation of the peptide from HLA-E during the cytotoxicity assay. Vales-Gomez et al. (18) used surface plasmon resonance to measure the binding of recombinant CD94/NKG2A and CD94/NKG2C to HLA-E complexes to various human leader sequence-derived peptides. For both inhibitory and activating isoforms, binding affinity was observed to be dependent on peptide sequence. Affinity was substantially reduced by substitution at the P3 (Glu) or P6 (Ala) minor anchor positions. In addition, lesser differences in affinity were observed to be dependent on polymorphisms in the peptide C-terminal residues, with the hierarchy LFL > LIL > VLL (18). In the present study, differences in staining of human NK cells with tetramerized HLA-E complexes were observed to consistently follow the hierarchy LFL > LIL > LVL > VLL.

A striking result was the observation that a conservative replacement of Arg with Lys at P5 in the leader peptide completely abrogated binding of tetramerized HLA-E complexes to human NK cells. This substitution had little effect on HLA-E-binding affinity; indeed, Lys was demonstrated to be a preferred amino acid at P5 for in vitro assembly of HLA-E complexes. It was recently demonstrated that a Val substitution at P5 abrogates CD94/NKG2-dependent inhibition of NK-killing assays (14). Thus, P5 Arg side chain appears to act as a dominant contact for interaction with CD94/NKG2 receptors. It remains to be determined whether CD94/NKG2 specificity recognized the conformer in which the P5 Arg side chain lies flat across the top surface of HLA-E, interacting with an acidic residue in the α2 helix, as opposed to the alternative conformation in which the side chain fully extends out of the HLA-E-binding groove (33). An appealing possibility is that electrostatic interactions involving P5 Arg and the α2 helix promote initial assembly of the HLA-E-peptide complex, and that subsequent CD94/NKG2 receptor binding is accompanied by a rearrangement in the conformation of the Arg side chain involving transfer of electrostatic interaction from HLA-E to CD94/NKG2.

The HLA-E peptide-binding motif determined in this study is remarkably similar to that previously obtained using a very similar experimental approach with the murine class Iib molecule Qa-1 (see Table I) (26). Qa-1 is the major, and possibly the exclusive, ligand for mouse CD94/NKG2 receptors (22), and thus it is the functional counterpart to HLA-E. The observation that humans and mice share CD94/NKG2 receptors and not other NK cell MHC receptors suggests that this NK recognition system arose before the divergence of primate and mouse ancestors (22, 23). However, Qa-1 and HLA-E are not recognizable orthologs at the level of overall amino acid sequence identity. There is greater sequence identity between HLA-E and other human class Ia molecules (∼76%) than between HLA-E and Qa-1 (65%), and vice versa. However, in comparing the α1 and α2 domains, which form the peptide-binding groove, this distinction becomes less clear. The residues that form the F pocket, which accommodates the dominant anchor Leu residue at P9 (33), are nearly identical in HLA-E and Qa-1. However, there are substantial differences in the amino acids that form the other pockets in the peptide binding sites of these two class Iib molecules. Given our results showing substantial overlap in the specificity of HLA-E and Qa-1 at each anchor position, it appears that the general structure and chemical character of each pocket are conserved despite amino acid differences.

Given the high degree of conservation in the peptide-binding specificity of HLA-E and Qa-1, we were interested in determining whether there was similar conservation in the surface that interacts with CD94/NKG2 receptors. Using MHC tetramer staining as a measure of receptor binding, no cross-recognition was observed between human and mouse CD94/NKG2 receptors and species-mismatched MHC class Iib ligands. By contrast, cross-species compatibility was observed between rhesus macaque and humans, such that human HLA-E tetramers efficiently stained rhesus NK cells. Khakoo et al. (41–43) previously demonstrated that chimpanzee CD94/NKG2 interacts with human HLA-E, noting the high degree of structural conservation in CD94, NKG2, and MHC-E among primates. In contrast to CD94/NKG2 and MHC-E, killer cell Ig-like receptors and their MHC class Ia ligands have diverged considerably among primates (41, 44). It has been proposed that sequence similarity among HLA-E and E-like sequences, including Qa-1, has arisen through convergent evolution driven by strong positive selection (45). It is likely that CD94 and NKG2 family members derive from common ancestral genes that predate the divergence of mouse and human ancestors (23). The genes encoding these proteins are syntenic, clustered within the human NK...
complex on chromosome 12 and the mouse NK complex on chromosome 6, and the overall genomic organization is very similar (23, 46). Strong selective pressures appear to have been in play to maintain the peptide-binding specificity of HLA-E and E-like molecules to favor selective assembly with MHC class Ia leader sequence-derived peptides. Conversely, despite the rapid evolution and diversification of MHC class Ia sequences, the leader sequence is highly conserved in a subset of class Ia molecules, at least in mammals. The contact surfaces of CD94/NKG2 and E-like proteins have diversified to a greater extent, while maintaining complementarity within a given species. Thus, the maintenance of this ancient MHC recognition system must have involved coevolutionary forces acting to influence selected structural features of all three components, HLA-E and its functional counterparts, MHC class Ia signal sequences, and the CD94/NKG2 receptors.

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References