HLA-F Complex without Peptide Binds to MHC Class I Protein in the Open Conformer Form

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HLA-F has low levels of polymorphism in humans and is highly conserved among primates, suggesting a conserved function in the immune response. In this study, we probed the structure of HLA-F on the surface of B lymphoblastoid cell lines and activated lymphocytes by direct measurement of peptide binding to native HLA-F. Our findings suggested that HLA-F is expressed independently of bound peptide, at least in regard to peptide complexity profiles similar to those of either HLA-E or classical MHC class I (MHC-I). As a further probe of native HLA-F structure, we used a number of complementary approaches to explore the interactions of HLA-F with other molecules, at the cell surface, intracellularly, and in direct physical biochemical measurements. This analysis demonstrated that HLA-F surface expression was coincident with MHC-I H chain (HC) expression and was downregulated upon perturbation of MHC-I HC structure. It was further possible to directly demonstrate that MHC-I would interact with HLA-F only when in the form of an open conformer free of peptide and not as a trimeric complex. This interaction was directly observed by coimmunoprecipitation and by surface plasmon resonance and indirectly on the surface of cells through coincident tetramer and MHC-I HC colocalization. These data suggest that HLA-F is expressed independently of peptide and that a physical interaction specific to MHC-I HC plays a role in the function of MHC-I HC expression in activated lymphocytes. The Journal of Immunology, 2010, 184: 6199–6208.

In the human system, MHC class I (MHC-I) proteins comprise a group of molecules that perform a variety of functions, extending from the classical Ag-presenting molecules HLA-A, -B, and -C to distantly related proteins including MHC-I–related chain A that resides in the MHC and interacts with the activating immune receptor NKG2D (1–4). Classical MHC-I molecules are expressed as trimeric complexes that consist of H chain (HC), β2-microglobulin (β2m) and peptide, usually 8–10 aas in length, embedded in the polymorphic binding groove of the HC. Bound peptides are derived from intracellular proteins that have been cleaved by the proteosome and other cytoplasmic peptidases and transported into the endoplasmic reticulum (ER), usually by the transporters associated with Ag processing (TAPs). In the ER, peptides are loaded onto MHC-I via an assembly complex, and the trimeric complex is subsequently expressed on the cell surface (5, 6).

Whereas a large majority of studies have focused on MHC-I complexes as the structural component, MHC-I molecules are also expressed on proliferating lymphoid cells as a stable pool of MHC-I HC (7). These HCs are expressed devoid of peptide and/or β2m and appear to be derived from fully mature MHC-I molecules rather than arising from protein misfolding (8, 9). They were originally detected using mAbs that do not react with MHC-I complex, but do react with denatured MHC-I. The reactivities of most of these Abs have been mapped to epitopes located between residues 55 and 87 in the α1 domain, covering sequences that directly interact with peptide in the complex structure and thus not available as Ab epitopes when peptide is bound. Whereas functional studies of MHC-I molecules have understandably focused on MHC-I complex structures for several decades, recent work has begun to examine potential alternative functions for MHC-I HC. These so-called open conformers have been implicated in a number of interactions with other receptors both in trans and in cis, including the formation of homodimers, on the cell surface (10).

Also resident in the MHC, and more recently diverged from the classical MHC-I than is MHC-I–related chain A, are the nonclassical MHC-I molecules HLA-E, HLA-F, and HLA-G. HLA-G has been studied extensively after expression was identified exclusively in the placental trophoblast in alternative protein forms, each form derived from alternative splicing of the primary mRNA (11–13). A function for HLA-G as an inhibitor of NK activity through interaction with ILT2 or ILT4 NK receptors has been suggested (14, 15). HLA-E function has been elucidated through its interaction with CD94/NKG2 receptors (16). The interaction with such CD94/NKG2 heterodimers can augment, inhibit, or have no effect on NK cell-mediated cytotoxicity and cytokine production (17). Surface expression of HLA-E requires nonamer peptides—including those derivative from the signal sequences of other HLA class I molecules including HLA-A, -B, -C, and -G, but not HLA-F (18)—and also forms stable complexes with limited numbers of structurally unrelated peptides (19). Similar to MHC-I, both HLA-G and HLA-E are expressed in complex forms with bound peptide, but are also expressed as HC under certain circumstances. Indeed, HLA-G free HC is expressed on the surface of trophoblasts and may modulate the efficiency of the CD85J/LIR-1 and HLA-G interaction (20). An Ab specific for HLA-E free HC has been described, and expression has been observed on activated lymphocytes and B lymphoblastoid cell lines (B-LCLs) although no function has yet been suggested (18, 21).

The third nonclassical molecule HLA-F has been less studied, and neither its native structure nor function is known. Evidence of a physical association of HLA-F and TAP was reported (22), but
surface expression was not reduced in TAP-negative mutant lines (23). Unlike classical MHC-I, the HLA-F cytoplasmic tail can be required for export from the endoplasmic reticulum, implicating a function for HLA-F independent of peptide-loading in the ER (24). Our work using new mAbs reactive with HLA-F showed that, although HLA-F was not surface expressed on most cell lines that contained intracellular protein, HLA-F was expressed on the surface of B and some monocyte cell lines and in vivo on extravesicular throphoblasts that had invaded the maternal decidua (12, 23). Further examination of PBLs demonstrated intracellular HLA-F protein expression in all resting lymphocyte subsets, including B, T, NK, and monocyte cells, and HLA-F surface expression was upregulated upon activation for all cell types (25).

HLA-F has low levels of polymorphism in humans and is highly conserved among primates (26, 27); combined with the structural similarities of HLA-F and other HLA class I, this suggests a conserved function in the immune response possibly along broadly similar lines to those of HLA-E or -G. In this study, we explored the structure of HLA-F on the surface of B-LCLs and activated lymphocytes by testing for similarities in peptide binding between native HLA-F and classical class I and its closer cousins HLA-E and HLA-G, which bind relatively restricted sets of peptides (18, 28). As a further probe of native HLA-F structure, we used a number of complementary approaches to explore the interactions of HLA-F with other molecules, at the cell surface, intracellularly, and in direct physical biochemical interactions.

Materials and Methods

Cells and cell culture

Cell lines NKL, OSP2, Daudi, MT2, Jurkat, Hut78, THP-1, Sup1, H9, Mol3, and U937 were all obtained from the American Type Culture Collection (Manassas, VA) and cultured according to the product information sheet provided. B-LCLs (AMAI, BM9, BM15, Bolet, BSM, JY, Steinlin) were previously collected and studied by the International Histocompatibility Workshops and Conference and obtained directly from the International Histocompatibility Working Group in Seattle (29). B-LCL 721.221 was obtained from the American Type Culture Collection and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and 1 mM sodium pyruvate. Other B-LCLs were grown in a culture medium of RPMI 1640 supplemented with 10% FBS, 100 mM penicillin, and 100 mM streptomycin.

Abs

MHC-I mAbs used in this study included: W6/32, specific for pan HLA class I MHC complex; HCA2, specific for a subset of allelic HLA class I HC s (30); LA45, specific for a different subset of HLA class I HCs (31); 3D12, specific for HLA-E complex; 7G3, specific for HLA-E HC without HCs (32); LA45, specific for a different subset of HLA class I HCs (31); 4B4, specific for intracellular HLA-F as described in this study; and 16G1, reactive with soluble HLA-G COOH terminus (33) and used as a control Ab for IgG1 isotype mAbs.

Immunofluorescence staining and FACS analysis

Cell surface expression of proteins was measured by indirect immunofluorescence staining as previously described (18). Cells were preincubated with saturating concentration of mAb followed by washing and labeling with FITC-conjugated goat F(ab)2 anti-mouse Ig (BioSource, Camarillo, CA). Samples were analyzed on a FACS cytometer (Becton Dickinson, Mountain View, CA) and analysis was performed using FlowJo (Tree Star, Ashland, OR). For tetramer staining, 1 μg refolded H2-Dβ (control) or HLA-F monomer was incubated with 1.5 μg R-P-E conjugated streptavidin (SAPE; Invitrogen, Carlsbad, CA) per reaction at room temperature for 30 min. H2-Dβ and HLA-F tetramers were synthesized in vitro using previously described protocols and constructs (23, 33, 34). Recombinant MHC-IHC and beta2-microglobulin were expressed in Escherichia coli and purified from the inclusion body. The peptide/ MHC complex was refolded by dilution of the proteins, and subsequently purified by gel filtration. The complex was biotinylated using BirA enzyme (Affinity, Denver, CO) and the biotinylated complex was repurified by gel filtration. Tetramer was formed by mixing biotinylated peptide/MHC complex with PE or Alexa Fluor 647-conjugated Streptavidin (Invitrogen) at a 4:1 ratio.

Acid treatment of cells was performed by resuspension of ~2.0 × 10^6 cells in 1 ml RPMI 1640 plus 10% FBS (pH 2.2) for 90 s, followed by the addition of 13 ml RPMI 1640 plus 10% FBS (pH 7.4). The cells were spun down, washed two times, and resuspended in cold FACS buffer (PBS plus 2% BSA, 0.1% NaN₃) for staining. CD3 stimulation was performed using solid phase bound anti-CD3 mAb UCHT1 (Biolegend, San Diego, CA); 1.0 × 10^6 cells were incubated for 20 h in separate wells of a 96-flat well plate coated with anti-CD3 or isotype control Ab. After 20 h, the cells were washed twice in cold FACS buffer and stained with the appropriate reagents. N-ethyl maleimide (NEM) treatment was performed by culture of ~1.0 × 10^5 cells in culture medium plus 100 μM NEM for 20 min (35), with or without the presence of mAb HCA2 or isotype control. The cells were spun down, washed an additional two times, and resuspended in FACS buffer for staining.

Measurement of peptides bound to HLA-F

HLA-F was isolated from the detergent lysate of PLH cells as described previously (18), using three different HLA-F specific immunofluorescence columns constructed with each of 3D11, 4A11, and 4B4, an HLA-E specific 3D12 column, and a W6/32 column. A total of three separate isolations were performed using different affinity column combinations connected in series: 1) 4A11, 4B4, and 3D12; 2) 3D12, 4D11, and 4B4; and 3) 4A11, 4B4, 3D12, and W632. MHC complexes were eluted with 2N acetic acid, and peptides were recovered in 2.5 N acetic acid by ultrafiltration using a Microcon filter with a 10,000 M.W. cutoff (Millipore, Billerica, MA) and concentrated by vacuum centrifugation. Eluted peptides were subjected to tandem mass spectrometry (MS/MS) performed by the Fred Hutchinson Cancer Research Center Proteomics Resource.

Mass spectrometry

The liquid chromatography/mass spectrometry setup consisted of a trap column (100 μm × 2 cm) made from an IntegraFrit (New Objective, Woburn, MA) packed with Magic C18AQ resin (5 μm, 200A particles; Michrom Bioreources, Auburn, CA), followed by an analytical column (75 μm × 25 cm) made from a PicoFrit (New Objective) packed with Magic C18AQ resin (5-μm, 100A resin; Michrom Bioreources). The columns were connected in-line to an Eksigent 1D+nano-HPLC (Eksigent Technologies, Dublin, CA) in a vented column configuration to allow fast sample loading (36). The HPLC column setup was coupled to an LTQ-Orbitrap (Thermo Fisher Scientific, Waltham, MA) hybrid mass spectrometer using the nano-electrospray source. The MHC class peptide samples were analyzed by the liquid chromatography-quadrupole mass spectrometer using a 60-min gradient from 2 to 35% acetonitrile with 0.1% formic acid (against water with 0.1% formic acid) at a flow rate of 300 nL/min. A spray voltage of 2.25 kV was applied to the nanospray tip. The mass spectrometer experiment consisted of a full MS scan in the Orbitrap (AGC target value 1e6, resolution 60k, microscan, and injection time 500 ms) followed by up to 5 MS/MS spectra acquisitions in the linear ion trap. The five most intense ions from the Fourier-transform full MS scan were selected for fragmentation in the linear ion trap by collision-induced dissociation with a normalized collision energy of 35% (isolation width of 2 m/z; target value of 1e4, and injection time of 100 ms). Selected ions were dynamically excluded for 15 s with a list size of 100, a repeat count of 1, and a repeat duration of 1.5 s. The dynamic exclusion mass width around each precursor ion was ~0.55 m/z to +1.55 m/z. Charge-state screening was used, allowing any ions with an identified charge state (+1 or higher) to be selected for MS/MS.

Database identification

The liquid chromatography-quadrupole mass spectrometer data files were converted to the mzXML format and subsequently searched using the database search program X!Tandem (www.thegpm.org) (37) contained in the CPAS analysis system (38). The default scoring algorithm in X!Tandem was replaced with the k-score scoring algorithm contained in CPAS (39). The data were searched against the IPI human database (v. 3.59; European Bioinformatics Institute, Hinxton, England, U.K.), in which three trypsin (porcine and bovine) sequences have been added (80,131 total sequences). The database search program X!Tandem expectation value X!Tandem protein scoring algorithm contained in the CPAS analysis system (38). The default scoring algorithm in X!Tandem was replaced with the k-score scoring algorithm contained in CPAS (39). The data were searched against the IPI human database (v. 3.59; European Bioinformatics Institute, Hinxton, England, U.K.), in which three trypsin (porcine and bovine) sequences have been added (80,131 total sequences). The database search was performed with no enzyme specificity and a maximum peptide length of 50 aas. The database search results were analyzed using PeptideProphet (Institute for Systems Biology, Seattle, WA) (40) and also manually filtered for likely MHC class peptides using the following criteria: mass accuracy <10 ppm; peptide length ≥13 aas; X!Tandem expectation value <2.0. The purpose of the filtering criteria was to identify candidate peptide MS/MS scans that were then manually inspected to determine whether the MS/MS fragment ions matched the identified peptide sequence.
Immunoprecipitation and Western blotting

Immunoprecipitation was performed as described (18, 23) with modifications. Briefly, $1 \times 10^7$ PLH cells were lysed in 1% NP-40, 140 mM NaCl, 200 mM PMSF, 10 mg/ml peptatin, 14 mg/ml aprotinin in 10 mM Tris pH 7.8, precleared by control Ab, equally divided into three parts. Each part was subjected to a sequential immunoprecipitation by HLA-F specific Ab coupled sephasore 4B in three distinct sequences: 1) 4B1, 3D11 then 4A11; 2) 4A11, 3D11 then 4B4; 3) 3D11, 4A11 then 4B4. After extensive washing with lysis buffer, Ag was eluted by 0.05 M diethylamine gel pH 11.2, neutralized by 1M Tris pH 6.8, separated on a 10% Tris-Glycine gel (Novex, San Diego, CA), and electroblotted as described (18). HLA protein was detected by mAb followed by HRP-labeled goat antihuman IgG (BioSource) at 1:5000 dilution and finally with an ECL system (Amersham Biosciences, Arlington Heights, IL). Densitometry was performed by scanning the radiograph film with a Sharp JX-320 scanner (Sharp Electronics, Mahwah, NJ) and quantified with Image Quant 5.0 software (University of Virginia ITC–Academic Computing Health Sciences).

Expression and purification of recombinant HLA proteins

Construction of plasmid, protein expression, and refolding was described previously for both HLA-E and HLA-F (21, 23). The DNA sequences used for the glycine-serine linker and a BirA substrate peptide have been described (41). $\beta_2$m in pHN1+ was provided by D.C. Wiley (Harvard University, Cambridge, MA) and expressed in E. coli strain XA90.

Both HC and L chain $\beta_2$m inclusion bodies were isolated from cell pellets, washed repeatedly in detergent, and solubilized in 8 M urea, 25 mM MES (pH 6.0), 10 mM EDTA, and 0.1 mM DTT (solubilization buffer) as described (42). Refolding was accomplished using a variation of the method by O’Callaghan et al. (43), by dilution of 12 mg/ml proteins (in 30 ml solubilization buffer) into 500 ml of 0.01 M Tris-Cl, 150 mM NaCl, 1mM EDTA, and 0.02% NaN3 on a Superdex 200 SEC column (Amersham Biosciences) in HBS-EP buffer (10 mM sodium acetate (pH 4.5) and 10 mg/ml $\beta_2$m in 10 mM sodium acetate (pH 5.0) were used for subsequent analyses. Injections of 85 ml containing various concentrations of HC and control solutions were performed at 20 ml/min, followed by a dissociation phase in buffer. Raw sensograms were corrected by subtracting the reference flow cell response.

Results

Peptide elution suggests that native HLA-F is expressed as an empty MHC-I

Previous molecular modeling of HLA-F suggested a structure similar to other MHC-I complexes with possibly a partially open-ended peptide binding groove (46). However, an examination of the sequence of HLA-F suggested fundamental structural differences from other MHC-I, because 5 of 10 residues conserved in both human and mouse MHC-I and pointing into the Ag-recognition site were substantially altered in HLA-F (47). In addition, the altered cytoplasmic domain of HLA-F can direct surface expression through pathways independent of loading with peptides in the ER (24). In these respects, HLA-F is unique among MHC-I HCs, indicating that HLA-F might also be unique in regard to peptide binding characteristics.

To examine HLA-F for peptide binding, we used B-LCLs because they express HLA-F on the cell surface (23). LCL PHH1 was chosen because we had measured surface levels of HLA-F that were highest among several LCLs examined. This cells also express HLA-E and HLA-A, B, and C class I abundantly, providing convenient and important controls for the experimental variables. HLA-E and MHC-I also provided examples of two distinct ranges of peptide binding of MHC-I, where HLA-E had been shown to bind small numbers of highly specific peptides that are derived from the available HLA class I expressed by the LCL being examined (18). MHC-I as assessed by pan–MHC-I Ab W6/32 will contain hundreds to thousands of distinct peptides with peptide motifs predicted from those HLA class I expressed in the LCL (48).

In the first experiment, lysate from $2 \times 10^7$ cells was passed through columns constructed with Abs 4A11 (anti–HLA-F) and 4B4 (anti–HLA-F intracellular form) arranged in series. After acid treatment and elution, samples were analyzed by mass spectrometry to characterize eluted peptides. To confirm these results, we repeated the experiments two additional times using different sequential Ab columns and with the addition of the anti–HLA-F reagent 3D11 (anti–HLA-F all forms), which was not tested in the first experiment. Positive controls were provided by the addition of W6/32 at the end of the third series, and 3D12 (anti–HLA-E) in the second and third series. Sequential column arrangements and the number of specific peptides identified in each experiment are reported in Table I.

After filtering nonspecific results from complex mixtures of peptides, 789 putative peptides eluted from the W6/32 column were

<table>
<thead>
<tr>
<th>Table I. Peptide elution summary</th>
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<tbody>
<tr>
<td><strong>Serial MHC-I Isolation</strong></td>
</tr>
<tr>
<td>4A11</td>
</tr>
<tr>
<td>4A11 → 4B4</td>
</tr>
<tr>
<td>3D12 → 3D11 → 4B4</td>
</tr>
<tr>
<td>4A11 → 4B4 → 3D12 → W632</td>
</tr>
<tr>
<td>Peptide intersection</td>
</tr>
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</table>

*Selection criteria were dMass < 0.001; scan no. ≤12,000; peptides with length ≤13.*
identified (Table I). Given the HLA type of PLH as A*0301, B*4701, Cw*0602, these peptides were analyzed using the HLA Peptide Binding Predictions Web site (http://www-bimas.cit.nih.gov/molbio/hla_bind/), which provides access to software that ranks potential 8-mer, 9-mer, or 10-mer peptides based on a predicted half-time of dissociation to HLA class I molecules (49). Of the 789 putative peptides eluted from the W6/32 column, 265 had the motif for HLA-A*0301 and 98 showed the HLA-Cw*0602 motif. Several of these peptides were confirmed by MS/MS analysis. The motif for B*4701 was not available for comparison. In two experiments, we were able to unambiguously identify the expected peptides bound to HLA-E, including nonamers derived from HLA-A*0301 and B*4701 both expressed on the target LCL (Table II). In addition, six peptides not previously reported as binding to HLA-E, some of which have motifs substantially distinct from the class I derived nonamers, were identified independently from both separate anti–HLA-E columns. Six of these peptides were confirmed as binding to HLA-E through in vitro refolding and three of these through subsequent peptide elution analysis (data not shown).

Using the same filtering criteria, from 3 to 53 peptides were identified from the repeat runs of the total of six anti-F columns, on the order of the numbers identified from the HLA-E columns. However, no peptides with a molar abundance relative to HC within 20-fold of those obtained for HLA-E were found from any of the anti–HLA-F columns (Fig. 1). In addition, none of the peptides identified in anti–HLA-F Ab columns were identical between different runs, neither between Abs in the same serial isolation nor between the same Ab from different isolations (Table I). The results for HLA-E and pan MHC indicate a sound technical approach, supporting these negative results from the anti–HLA-F columns analyzed within the same experiments.

**MHC-I HC modulates HLA-F surface expression**

Because it was possible to refold HLA-F with β2m we initially attempted to identify a receptor for HLA-F by analyzing differential patterns of HLA-F tetramer binding to a diverse panel of cell lines, followed by attempts to generate Abs that would replicate these patterns and block tetramer binding. Despite repeated attempts, we were unable to obtain any Abs producing the expected binding patterns. At the same time, we were investigating blocking of tetramer using existing Abs, and we observed a potential interaction between MHC-I HC Abs and HLA-F. Reactivities of two of these Abs, HCA2 and LA45, have been well characterized with HCA2 reacting preferentially with a subset of HLA-A locus HC (30), where the LA45 epitope includes approximately half the alleles of both HLA-A and -B loci HCs (31). Our initial experiments indicated that HCA2 or LA45 Ab binding interfered with HLA-F Ab binding, because prior addition of either Ab resulted in reduced binding of the anti–HLA-F reagents (Fig. 2A). However, neither HC Ab blocked the levels of F tetramer binding, and none of the anti–HLA-F reagents interfered with HCA2 or LA45 binding (Fig. 2A). The effect of HC Abs was specific, because LCLs that expressed different combinations of HCA2 and LA45 epitopes affected HLA-F detection on these cells in accord with the presence or absence of those epitopes (Fig. 2B). The observation that 3D11 did not block HCA2 binding and that HCA2 did not interfere with F-tetramer binding suggested the possibility that HCA2 and LA45 were instead downmodulating HLA-F or changing surface levels through other means, such as HLA-F proteolytic release.

To test for proteolytic release of HLA-F, we examined supernatants for HLA-F protein after treatment with HCA2 incubation. No HLA-F protein was detected in supernatants of cells treated with HCA2, as assessed with an HLA-F-specific ELISA, suggesting the alternative possibility that HLA-F is internalized in response to HC Ab. Accordingly, we repeated these experiments in the presence of 100 mM NEM, which inhibits transcytosis and endocytosis and prevents the uptake of surface proteins (50). Whereas HCA2 substantially downmodulated HLA-F expression on B-LCL AMAI, the same experimental conditions resulted in no change of HLA-F levels when NEM was present at appropriate concentrations (Fig. 2C). These results further suggested that HLA-F is downmodulated upon the addition of HCA2 rather than HCA2 blocking 3D11 binding.

**Increased HCA2 binding on cells correlates with increased HLA-F tetramer binding**

Mild acid treatment of resting cells is known to destabilize class I MHC complexes on the cell surface, causing peptide and β2m to dissociate generating MHC-I HC (51). We thus reasoned that if HLA-F is interacting with MHC-I HC, specific binding of the F tetramer should increase with increased levels of MHC-I HC. We first examined cells that are negative for HLA-F expression and for MHC-I HC, including the T cell lymphoma cell lines Jurkat, Molt-3, HUT-78 and H9, and mononuclear cell line U937, as well as class I bare, β2m-deficient Burkitt’s lymphoma cell line Daudi. All these cells were analyzed for HCA2 binding and HLA-F tetramer binding before and after mild acid treatment (Fig. 3A, 3B). On untreated cells, no HLA-F tetramer binding was observed, whereas after mild acid treatment all cell lines except class I bare cell line Daudi became both HCA2 reactive and bound HLA-F tetramer in a manner closely associated with the intensity of HCA2 staining (Fig. 3B). The control HLA-A*0201 tetramer did not bind to either untreated or acid treated cells (not shown).

A second measure of coincidence between HCA2 expression and HLA-F tetramer binding was taken by activation of cell line OSP2, which can be activated upon treatment with anti-CD3 Ab. Consistent with the results obtained after acid treatment, upregulation of HCA2 was observed and was found coincident with increased F tetramer binding (Fig. 3C). Essentially similar results were obtained with a T cell clone after activation and expansion, and activated T cell clone 1C7-7 stained with both HCA2 and HLA-F tetramer showed coincident expression of the two signals, as did H9 cells after acid treatment (Fig. 3D). These latter experiments showed that although HCA2 is not always coincident with HLA-F Abs or with F tetramer, the latter are always found overlapping with HC.

**Commmunoprecipitation of HLA-F with MHC-I HC including HLA-E**

If MHC-I HC does bind directly to HLA-F it might be possible to isolate both proteins as part of a complex, depending on the strength of the interactions. To test this possibility, we performed sequential

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**Table II. Peptides eluted from HLA-E and pan MHC-I**

<table>
<thead>
<tr>
<th>Ab</th>
<th>Peptides</th>
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<tbody>
<tr>
<td>3D12</td>
<td>VMAPRTLLL (HLA-A*0201)</td>
</tr>
<tr>
<td></td>
<td>VMAPRTLIL (HLA-B*0301)</td>
</tr>
<tr>
<td></td>
<td>ILGPPPSFb</td>
</tr>
<tr>
<td></td>
<td>ILKQNKVPILa</td>
</tr>
<tr>
<td></td>
<td>VMISKLASFLa</td>
</tr>
<tr>
<td></td>
<td>RFPPTPPLe</td>
</tr>
<tr>
<td></td>
<td>YYVAGMIlb</td>
</tr>
<tr>
<td></td>
<td>VMSVGFILLb</td>
</tr>
<tr>
<td>W6/32</td>
<td>265 with motif x(MVL)xxxxx(YK) (HLA-A*0201)</td>
</tr>
<tr>
<td></td>
<td>98 with motif xDxxxxxL (HLA-C*0602)</td>
</tr>
</tbody>
</table>

*aConfirmed binding by elution.

*bConfirmed binding by refolding.
immunoprecipitations (IPs) of HLA-F from LCL PLH with three anti-F reagents (Fig. 4). Earlier experiments had not detected any other MHC-I protein coprecipitating with HLA-F, but were limited in their experimentation with different detergents for lysis and had not included all available Abs that were reactive with HLA-F (23). One Ab that we isolated, 4B4, does not react with surface HLA-F on any cell lines tested, but does IP HLA-F protein. In addition, Abs 3D11 and 4A11 also have distinct reactivities at specific times after activation of lymphocytes (25), indicating that each of the three anti–HLA-F Abs react with distinct forms of HLA-F.

These considerations motivated experimentation with a variety of detergents and further testing of available anti-F reagents. In an experiment designed under conditions similar to the peptide isolations described above, we performed sequential IPs with Abs 4B4, 3D11, and 4A11 in each of the three possible sequences followed by Western blot analysis with anti-HC Abs. Representative results of these experiments are presented in Fig. 4. Three features of these results are noteworthy. First, within each of the HLA-F Ab IP series, it was possible to coprecipitate HCA2-reactive proteins with Ab 4B4 regardless of the order in IP sequence. This IP product included HLA-F (Fig. 4, left) and three bands reactive with HCA2. Control experiments showed that HLA-F is not reactive with HCA2 under the conditions used for Western blot analysis (data not shown). Of the three HCA2 reactive bands observed, the lower HCA2 reactive band corresponds in size to soluble HLA class I and can be produced in cells as a result of proteolytic cleavage as described (52). Second, blotting of an identical IP with anti–HLA-E–HC Ab 7G3 showed that the middle, fainter band identified by HCA2 was in fact HLA-E (Fig. 4, right). A third feature is the higher m.w. band that is reactive with 3D11, found only in the 3D11 IP, and is present despite running samples under reducing conditions.

Direct measurement of HLA-F and MHC-I HC interactions

As an additional and more direct method for measuring HLA-F and MHC-I HC interactions, we used label-free SPR-based technology for studying these biomolecular interactions in real time. Our hypothesis to this point was that HLA-F physically interacts with MHC-I HC but not with the complex form. This was based on the coincidence of HLA-F and MHC-I HC in cell binding assays and modulation of HLA-F surface levels as described above. However, a direct measurement required producing the HC open conformer presumably without peptide in a homogeneous and reproducible manner. Because it was not possible to refold sufficient quantities of HLA-A2 or -A3 without peptide, we took advantage of the method of conditional MHC ligands to produce the appropriate empty class I molecules (44). This method was developed to allow for refolding of the stable complex, which can then be treated with UV to produce empty or at least peptide-receptive MHC molecules that can then be charged with a peptide of choice (45). After refolding HLA-A2 and -A3 with peptides that can form corresponding complexes but that can be cleaved upon ultraviolet irradiation, we were able to produce homogeneous preparations of putative empty MHC-I molecules after purification by SEC. It is possible that a small fragment remains, depending on its affinity, after UV treatment. However, considerable data on MHC-I peptide binding indicates that no peptides of 5 aas have been demonstrated to stabilize classical MHC-I, supporting the likelihood that our UV-treated complexes are indeed empty. But even if complexes do have some portion of peptide bound, when contrasted with peptide-bound complexes where there is little or no binding, the affinity for HLA-F is clearly and markedly changed (Fig. 5). Furthermore, these results are consistent with the HLA-E binding in which we have direct evidence of empty HLA-E.
To examine direct binding, we tested a surface coupled with HLA-F refolded with b2m. Because we were interested in distinguishing between MHC-I in complex with peptide versus empty MHC-I binding, we passed HLA-A2 refolded with conditional ligand before and after UV treatment over the HLA-F/b2m surface. HLA-A2 UV-treated protein showed binding to the HLA-F/b2m surface and increasing amounts (2.5 and 7.5 μM) of the HLA-A2 UV-treated protein resulted in proportional increases in SPR signals (Fig. 5A, left). Furthermore, no binding was observed when HLA-A2 was used before the UV treatment or when the HLA-A2 was refolded with a conventional ligand. To demonstrate the specificity of the binding to HLA-F/b2m, a second surface coupled with HLA-E G/b2m was used, and 2.5 μM HLA-A2 was injected after UV treatment and over both surfaces, resulting in significantly stronger binding to the HLA-F/b2m surface (Fig. 5A, right). The HLA-E[G]/b2m surface signal accounts for nonspecific binding of HLA-A2 to the surface. Essentially similar results were obtained with HLA-A3, in which the signal from the UV-treated complex was measured with increasing protein amounts, and no background binding to HLA-F was observed without UV treatment or when conventional peptide was used for refolding. Again, the binding was specific for HLA-F as evidenced by background levels of HLA-A3 binding to the empty HLA-E coated surface (Fig. 5B).

Motivated by the identification of HLA-E in the coimmunoprecipitations described above, we extended these results to include a comparative binding of HLA-E refolded with and without peptide. Our prior work had shown that it was possible to refold sufficient quantities of HLA-E without peptide, obviating the need for a similar conditional ligand strategy (21). Refolded forms of both allelic variants were available for analysis. When either allele was refolded with conventional nonamer peptides derived from either HLA-A2 or HLA-G signal sequences (18), binding was detected at background levels on either HLA-F or control HLA-E surfaces, whereas strong binding was evident when empty versions of either HLA-E allele were exposed to the HLA-F surface (Fig. 5C, 5D). The binding patterns of the HLA-E[G] and E[R] alleles were essentially similar at the level of analysis performed. Although binding of HLA-E[R] appears quantitatively stronger in the results shown, variation in protein preparation and purification prevent a quantitative comparison between experiments.

Discussion

Our findings combined with previous data demonstrate that HLA-F is expressed independent of bound peptide, at least in regard to peptide complexity profiles similar to those of either HLA-E or classical MHC-I. In addition, we were able to demonstrate that not only was HLA-F surface expression coincident with MHC-I HC expression, but that HLA-F surface expression was downregulated upon perturbation of the MHC-I HC structure. It was further possible to directly demonstrate that MHC-I would only interact with HLA-F in the form of an open conformer, probably free of...
peptide and not as peptide bound complex. This interaction was directly observed through coimmunoprecipitation and SPR and indirectly on the surface of cells through coincident tetramer and MHC-I HC colocalization. These data suggest that HLA-F is expressed independently of peptide and that a physical interaction specific to MHC-I HC plays a role in the functional consequence of MHC-I HC expression in activated lymphocytes.

Although our results suggest that HLA-F is expressed as HC without peptide, IPs using our panel of HLA-F specific Abs coprecipitate β₂m in subequimolar amounts (23, 25). Based on these data and the differential coimmunoprecipitation experiments reported in this study (Fig. 4), it appears likely that more than one complex form of HLA-F is expressed, with a portion complexed with β₂m, and some or all complexed with MHC-I HC. In total there is evidence of at least three different forms of HLA-F based on differential staining of surface HLA-F using Abs 4A11 and 3D11 over the course of lymphocyte activation and the unique 4B4 binding pattern. In light of this, it is also possible that HLA-F...
can bind larger peptides, beyond the limits of our analysis of 13mers, or it can be stabilized by interactions with proteins other than MHC-I or other biomolecules such as phospholipids similar to CD1d (53). Interestingly, the HLA-F protein is entirely dependent on its cytoplasmic tail for export from the ER, and the HLA-F cytoplasmic sequence contains motifs that are overlapping with both CD1d (24, 54).

It is possible to refold HLA-F with β2m without peptide which forms a stable structure that can be used in tetramer binding (55) and Fig. 3). Open conformer MHC-I are relatively unstable in comparison but are upregulated by cold treatment, whereas HLA-F is not, suggesting that there is likely a threshold of stability that MHC-I HC must reach to traffic to and remain on the cell surface. The possibility that HLA-F is required for the formation of the open conformer is raised considering their coincident surface expression and physical interaction. Because HLA-F is otherwise expressed intracellularly in resting lymphocytes, it is also possible that the formation of MHC-I HC upon activation induces complex formation between HLA-F and MHC-I HC, which then signals transport to the surface.

When free HC Abs bind to MHC-I HC on the cell surface, the conformation of the HC may be changed to more closely resemble MHC complex with peptide. Whether HLA-F recycles with MHC-I HC, as suggested by internalization upon addition of HC Abs, would clearly add an important dimension to studies of the trafficking of MHC-I HC. As an interesting supplement, HLA-F does have a tyrosine-based internalization motif (TSQA) similar to that proposed to regulate MHC-I endocytosis and intracellular trafficking, whereas HLA-C and HLA-G do not (56). This might be relevant to HLA-C and HLA-G cycling in trophoblasts cells in light of the coincident expression of these molecules in conjunction with HLA-F in the placental environment (12). It is not known whether HLA-C is expressed as a free HC in trophoblasts, but evidently HLA-G is (15, 20) in trophoblasts that have invaded the maternal decidua, HLA-F and HLA-G are coexpressed on the surface. It would be interesting to explore the idea that the lack of the internalization motif on HLA-C and HLA-G was directly related to their expression in the placental environment.

Our data do not address whether HLA-F interacts with all MHC-I HC, because we were able to analyze only those MHC-I for which a conditional ligand was available (HLA-A2 and -A3) or that we were able to refold without peptide (HLA-E). Considering the extensive polymorphism of MHC-I, it is possible that HLA-F interacts with only a subset of MHC-I alleles or, more generally stated, that the affinity between HLA-F and different MHC-I alleles will differ substantially. Our analysis of the three MHC-I HC was not performed under conditions that allowed for precise quantitative measurement; however, differences could be detected between HLA-A2 or HLA-A3 and HLA-E. We do not have detail on the interaction points between HLA-F and MHC-I HC, but a logical presumption from this work would be that they are restricted to regions exposed upon removal of peptide.

The reactivities of MHC-I HC-specific mAbs have been mapped to epitopes formed by residues within positions 57–84 in the α1 domain that are specific to the open conformer (10). These residues are in contact with peptide or otherwise hidden in the peptide-bound molecule, but become exposed in the MHC-I HC conformer. HCA2 has been mapped to residues 77–84, probably ruling this segment out because HLA-F and HCA2 do not compete for binding to MHC-I, but a similar comparative analysis with other MHC-I HC specific Abs could be useful in mapping the HLA-F contact residues. Further insight might come from comparative sequence analysis with HLA-F homologs expressed in other primates. For example, HLA-F homologs are found in macaques with as few as 10 aa differences in the α1 to α3 domains (26).

These findings leave open the possibility that HLA-F binds to a specific receptor or that HLA-F and MHC-I HC interactions can occur in trans between cells. Addressing the former possibility, whereas our immunoprecipitations with 3D11 and 4A11 did not co precipitate equimolar amounts of MHC-I as did 4B4, it is unlikely that this evidence argues against an interaction of HLA-F and MHC-I HC on the surface. Subequimolar amounts were detected with both Abs, and it is not clear that this was due to experimental conditions (e.g., the detergent used) and possibly compounded by Ab specificities. In fact, there is a strong correlation with binding of HLA-F tetramer and the presence of surface MHC-I HC (Fig. 3). In
addition, surface HLA-F is internalized upon addition of free HC Abs, which could implicate an interaction between the two molecules prior to the addition of HC Ab. If heterodimers of MHC-I HC and HLA-F or similar complexes of homodimers of each species exist on the surface, it would suggest the possibility for interaction with a unique receptor in trans, perhaps another polymorphic locus or group of genes.

MHC-I interactions with other proteins have been demonstrated in cis, including insulin and epidermal growth factor receptors, and evidence points to that interaction occurring exclusively with MHC-I HC (57–59). Indeed a relatively long list of reported cis-associations between MHC-I and other surface receptors and proteins can be compiled from the literature (10). Whether all of these associations are formed between the MHC-I open conformer remains to be determined, but the discovery reported in this study might place a new light on these interactions and might refocus some emphasis on this aspect of MHC-I function.

Additional functional data will clarify the role that interaction between HLA-F and MHC-I plays in class I presentation by activated cells. However, the high conservation of HLA-F and the binding to MHC-I HC together suggest a role as chaperone to stabilize MHC-I HC expression in the absence of peptide. This escort function could be operating in both directions, bringing MHC-I HC to the surface and internalizing MHC-I HC after appropriate signals have been delivered, although we have no direct evidence in support of the latter possibility. The coincident internalization of HLA-F may therefore suggest an involvement in internalization of MHC-I upon an encounter with the formation of an alternative stable complex.

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Disclosures
The authors have no financial conflicts of interest.

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