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HLA-F and MHC-I Open Conformers Cooperate in a MHC-I Antigen Cross-Presentation Pathway


Peptides that are presented by MHC class I (MHC-I) are processed from two potential sources, as follows: newly synthesized endogenous proteins for direct presentation on the surface of most nucleated cells and exogenous proteins for cross-presentation typically by professional APCs. In this study, we present data that implicate the nonclassical HLA-F and open conformers of MHC-I expressed on activated cells in a pathway for the presentation of exogenous proteins by MHC-I. This pathway is distinguished from the conventional endogenous pathway by its independence from TAP and tapasin and its sensitivity to inhibitors of lysosomal enzymes, and further distinguished by its dependence on MHC-I allotype-specific epitope recognition for Ag uptake. Thus, our data from in vitro experiments collectively support a previously unrecognized model of Ag cross-presentation mediated by HLA-F and MHC-I open conformers on activated lymphocytes and monocytes, which may significantly contribute to the regulation of immune system functions and the immune defense. *The Journal of Immunology, 2013, 191: 000–000.

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MHC-I OC and HLA-F interaction in this pathway was tested through direct binding of Ag and specific interference with their surface expression and transport. Mutant cell lines that lack a functional endogenous pathway and drugs that interfere with intracellular trafficking and protein degradation were used to distinguish the pathway from endogenous MHC-I presentation. These data collectively support a model for a general mode of exogenous MHC-I Ag uptake and presentation by activated lymphocytes and monocytes that differ in significant detail from the presentation of endogenous Ag, suggesting a role for cooperation between HLA-F and MHC-I OC in this pathway.

Materials and Methods

Cell lines and cultures

NKL and KMA were all obtained from American Type Culture Collection (Manassas, VA) and cultured according to the product information sheet provided. B-LCL cell lines were previously collected and analyzed by the International Histocompatibility Workshops and Conference and obtained directly from the International Histocompatibility Working Group in Seattle (20). LCL 721.221 was obtained from the American Type Culture Collection and maintained in RPMI 1640 medium supplemented with 10% v/v FCS, 2 mM L-glutamine, and 1 mM sodium pyruvate. Genotyping for the presence of the HA-1H and R alleles was carried out as described (21). The CTL clones specific for HIV, CMV, and minor H Ags were derived in prior studies (22–26). Typically, 1–2 x 10⁵ freshly thawed CTL clone cells were added to 25 x 10⁶ irradiated BM. The cells were grown in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 U/ml streptomycin. Genotyping for the presence of the HA-1H and R alleles was carried out as described (21). The CTL clones specific for HIV, CMV, and minor H Ags were derived in prior studies (22–26).

B-LCL cell lines were grown in RPMI 1640 medium supplemented with 10% PBS, 100 U/ml penicillin, and 100 U/ml streptomycin. Genotyping for the presence of the HA-1H and R alleles was carried out as described (21). The CTL clones specific for HIV, CMV, and minor H Ags were derived in prior studies (22–26). Typically, 1–2 x 10⁵ freshly thawed CTL clone cells were added to 25 x 10⁶ irradiated BM. The cells were grown in RPMI 1640 medium supplemented with 10% v/v FCS, 2 mM L-glutamine, and 1 mM sodium pyruvate. Genotyping for the presence of the HA-1H and R alleles was carried out as described (21). The CTL clones specific for HIV, CMV, and minor H Ags were derived in prior studies (22–26).

Abs and reagents

mAbs 3D11, 4A11, 4B4, and 6A4 specific for HLA-F were generated in our laboratory, as previously described (17, 17). HCA-2 was a gift from Thomas Spies (Fred Hutchinson Cancer Research Center, Seattle, WA). Other mAbs were purchased from suppliers, including MA2.1 (American Type Culture Collection), Rab 5 (Abcam, Cambridge, MA), and CMV pp65 Tegument protein (UL83) mAb (Fitzgerald Industries International, Acton, MA). Proteins were obtained from the following sources: recombinant HIV-p24 (24 kDa; Prospecis, Ness-Ziona, Israel) and recombinant pp65 protein (65 kDa; Miltenyi Biotec, Auburn, CA). Type Culture Collection), Rab 5 (Abcam, Cambridge, MA), and CMV pp65 Tegument protein (UL83) mAb (Fitzgerald Industries International, Acton, MA). Proteins were obtained from the following sources: recombinant HIV-p24 (24 kDa; Prospecis, Ness-Ziona, Israel) and recombinant pp65 protein (65 kDa; Miltenyi Biotec, Auburn, CA). Other mAb were previously collected and analyzed by the International Histocompatibility Workshops and Conference and obtained directly from the International Histocompatibility Working Group in Seattle (20). LCL 721.221 was obtained from the American Type Culture Collection and maintained in RPMI 1640 medium supplemented with 10% v/v FCS, 2 mM L-glutamine, and 1 mM sodium pyruvate. Genotyping for the presence of the HA-1H and R alleles was carried out as described (21). The CTL clones specific for HIV, CMV, and minor H Ags were derived in prior studies (22–26).

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Cellular assays

B-LCL cell lines were prelabeled with 50 μCi ⁵¹Cr for 1 h at 37°C and washed and incubated in the absence or presence of 10 μg/ml brefeldin A (BFA; BioLegend), 100 μM N-ethylmaleimide (Sigma-Aldrich, St. Louis, MO), 200 μM chloroguanine (Sigma-Aldrich), or 200 μM leupeptin (Sigma-Aldrich) for 1 h prior to addition of Ag. A total of 5 x 10⁵ Cr-labeled B-LCL cells suspended in 500 μl RPMI 1640 plus 2% BSA was incubated with peptide, vaccinia virus pp65, recombinant protein (p24, pp65), or synthetic peptide and derived from the same viral sequences for 4 h at 37°C. These experiments resulted in establishing a protocol for proteins that required denaturing the proteins prior to the sensitization of cells, similar to that previously described (28). Following incubation with Ag, cells were washed three times and resuspended in RPMI 1640 plus 10% PBS and plated out at 5 x 10⁵ cells/ml with effectors at the indicated ratios. At 4 h, 30 μl supernatant was collected and applied to lumaplates, dried, and counted using a TopCount scintillation counter (Perkin Elmer, San Jose, CA).

Temperature shift experiments were performed in an essentially similar protocol with cells incubated at either 4°C or 37°C for 1 h before labeling with control peptide, p24, or p17 at the indicated concentration for an additional 2 h at 4°C or 37°C. For the centrisor experiments, Ags were prepared by adding a 2:1 concentration of Ag to cells at 10³/ml per ml suspended in ADM-V serum-free medium or RPMI 1640 plus 2% FBS (no differences were observed) for 2 h at 37°C. After incubation, cells were spun down at 1300 rpm, and the supernatant was collected and spun through a 3000 m.w. cutoff centrifuge device. The flowthrough was applied at 1:2 starting dilution to the ³⁵Cr-labeled targets, in parallel to ³⁵Cr-labeled targets pulsed with control peptide or protein at the indicated concentration through serial dilutions. After incubation, the cells were treated and analyzed, as above. In peptide competition experiments, B-LCL or NKL cells were prelabeled with ³⁵Cr for 1 h at 37°C prior to pulsing with Ag. After labeling, the cells were incubated for 1 h with 100 μM A*020 peptide (CIR; Anaspec, Fremont, CA) at 37°C before the addition of positive control peptide or recombinant proteins for an additional 4 h.

Intracellular cytokine staining was performed by directly adding recombinant pp65 to CTL clone IC7-31 or the HA-1H 50-aa protein to CTL clone GAS9#, each resuspended in RPMI 1640 plus 10% human AB serum. The cells were incubated for 1 h at 37°C, followed by the addition of 10 μg/ml BFA and further incubation for 4 h at 37°C. After incubation, cells were fixed and stained for intracellular IFN-γ as per instructions (Intraprep; Beckman Coulter).

Short hairpin RNA knockdown constructs

Lentiviral vector (29, 30) was used to construct β-m and HLA-F-specific knockdowns using synthetic oligonucleotides cloned into the XbaI/EcoRV cloning sites. Short hairpin RNA (shRNA) construct targeting β-m had targeting sequence 5'-CAGCAGAAATGGAATGCTA-3' with forward oligonucleotide 5'-CTAGACAGAGAGATGAAATCTCAGTTCA-3' and reverse oligonucleotide 5'-ATCCAGAAGGATGAGAATCTCAGTTCA-3'. Background sequence was generated for a loop, the construct targeting HLA-F had targeting sequence 5'-GGTTGACTTTCCATTCTCTGCTGTTTCTCAGTTCA-3'. Italicized font represents the loop.

Mass spectrometry

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis of peptides and proteins was carried out at the Proteomics Shared Resource at the Fred Hutchinson Cancer Research Center, essentially as described previously (15).

Immunostaining and confocal microscopy

B-LCL cells (BM9) were incubated with 100 μg/ml biotinylated gp100#2 or HA-1H in RPMI 1640 plus 2% BSA for 4 h at 37°C. After incubation, cells were washed and surface stained with panel class I mAb W6/32 labeled with Alexa-488 (BioLegend, San Diego, CA). The cells were incubated for another 60 min with anti–HLA-F mAb 3D11 or MHC-I H chain mAb HCA2. After incubation, cells were washed and surface stained with W6/32 labeled with Alexa-488 (BioLegend). The cells were then washed, and intracellular staining was performed with anti–IgG1-Dylight 649 (Jackson ImmunoResearch Laboratories, West Grove, PA) for detection of anti–HLA-F and streptavidin Alexa-594 (Invitrogen, Eugene, OR) for detection of intracellular biotinylated Ags, as per instructions (Intraprep; Beckman Coulter, Brea, CA). After intracellular staining, the cells were fixed with 1% paraformaldehyde, washed, resuspended in Prolong Gold antifade reagent with DAPI (Invitrogen), and mounted onto optical slides. The cells were imaged using a DeltaVision RT Wide-Field Deconvolution microscope (Applied Precision, Issaquah, WA), and the images were analyzed with ImageJ.

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helper plasmids into 293T cells by calcium phosphate. Viral particles were harvested at 16 and 40 h, filtered by 0.45 μm pore size, and concentrated 100-fold by PEG-it (SBI, Mountain View, CA). All transductions were performed over consecutive 4 d. At day 1, 60 × 10^4 B-LCL cells were seeded in 24-well plates in 1.5 ml RPMI 1640 with 10% PBS; 20 μl 100-fold concentrated virus supernatant was added in the presence of 8 μg/ml protease; and the plate was centrifuged at 2500 rpm at room temperature for 1 h. A total of 1 ml culture medium was replaced with 1 ml fresh medium containing 20 μl concentrated virus and protease and centrifuged once per day for the next 3 d. GFP-positive cells were analyzed and sorted by flow cytometry.

**MHC-I complex formation**

T2 cells were washed twice with RPMI 1640 medium plus 10% BSA and resuspended in RPMI 1640–10% BSA at a concentration of 50 × 10^5 cells/ml. Cells were homogenized with 15 strokes in a Pyrex glass homogenizer, and 1/10 (v/v) of hypotonic buffer containing 10 mM HEPES (pH 7.5), 15 mM KCl, 1.5 mM MgAc, and 1 mM DTT at a concentration of 35 × 10^5 cells/ml. Cells were homogenized with 15 strokes in a Pyrex glass homogenizer, and 1/10 (v/v) of hypotonic buffer containing 10 mM HEPES (pH 7.5), 700 mM KCl, 40 mM KCl, and 1 mM DTT was added to the final homogenate. After centrifugation at 900 × g for 10 min at 4˚C, the nuclear pellet was washed with 10 mM HEPES (pH 7.5), 85 mM KCl, 5.5 mM MgAc, and 1 mM DTT and collected by centrifugation. Combined postnuclear supernatants were centrifuged at 145,000 × g for 25 min, and the resulting crude membrane pellet was resuspended in 1 ml homogenizer buffer containing 10 mM Tris (pH 8.0), 0.25 M sucrose, and 1 mM EDTA.

A 50% (w/v) sucrose stock solution in 10 mM Tris (pH 7.5), 1 mM EDTA was used to prepare a 50–10% sucrose gradient using 11 dilution steps of 1 ml each. The gradient was equilibrated overnight at 4˚C. The membrane suspension prepared above was layered on top of the gradient and centrifuged at 100,000 × g at 4˚C for 25 min, and the resulting crude membrane pellet was resuspended in 1 ml homogenizer buffer containing 10 mM Tris (pH 8.0), 0.25 M sucrose, and 1 mM EDTA.

**Subcellular fractionation**

KOSE cells were suspended in hypo-osmotic buffer containing 10 mM HEPES (pH 7.5), 15 mM KCl, 1.5 mM MgAc, and 1 mM DTT at a concentration of 35 × 10^5 cells/ml. Cells were homogenized with 15 strokes in a Pyrex glass homogenizer, and 1/10 (v/v) of hypotonic buffer containing 10 mM HEPES (pH 7.5), 700 mM KCl, 40 mM KCl, and 1 mM DTT was added to the final homogenate. After centrifugation at 900 × g for 10 min at 4˚C, the nuclear pellet was washed with 10 mM HEPES (pH 7.5), 85 mM KCl, 5.5 mM MgAc, and 1 mM DTT and collected by centrifugation. Combined postnuclear supernatants were centrifuged at 145,000 × g for 25 min, and the resulting crude membrane pellet was resuspended in 1 ml homogenizer buffer containing 10 mM Tris (pH 8.0), 0.25 M sucrose, and 1 mM EDTA.

**Pull-down and Western blotting**

T2- or T2/B35-transfected cells were conditioned in RPMI 1640 supplemented with 2%/5% protease-free BSA for 30 min at 37˚C with 5% CO2. Cells were incubated with 50 μg/ml biotinylated 50-aa proteins for 2 h, washed with ice-cold Dulbecco’s PBS, and lysed in Dulbecco’s PBS containing 1% Triton X-100 and 1% Nonidet P40, as described previously (15). Proteins of interest were pulled down by streptavidin and eluted by 8 M guanidine HCl (pH 1.5). Plasmaprins were prepared on 10% Bis-Tris gels (Invitrogen, Grand Island, NY) and analyzed by Western blot using indicated Abs.

**Results**

**HLA-F is downregulated via internalization in response to exogenous peptide or protein**

Our previous work demonstrated that HLA-F was downmodulated on the surface of B-LCLs upon addition of MHC-I mAb specific for H chain or OC (15). Because H chain–specific mAb bind within the cleft region of MHC-I, we hypothesized that such mAb binding either structurally mimics peptide binding and thus alters the structure of MHC-I H chain to resemble complex resulting in dissociation and downmodulation of HLA-F, or, alternatively, causes cross-linking of HLA-F/MHC-I heterodimers triggering internalization. Because the postulated structure of MHC-I when associated with HLA-F is open and thus peptide receptive, we considered the possibility that long polypeptides (>30 aa) with internal MHC-I epitopes may also bind to open class-I MHC, and that the binding of long polypeptides containing multiple MHC epitopes would produce cross-linking in the same way as mAb and thus downmodulation of HLA-F. To test this hypothesis, HLA-F levels were compared before and after the addition of denatured viral proteins HIV-1 p24 or HCMV pp65. Marked decreases in HLA-F levels were indeed observed in both cases (Fig. 1A). The observed downmodulation suggested that HLA-F was being internalized in response to the addition of exogenous protein potentially interacting with MHC-I, HLA-F, or a related complex or structure on the cell surface. Fluorescence microscopy was then used to visualize the fate of HLA-F, MHC-I, and exogenous protein directly. A synthesized 50-aa polypeptide (biotinylated gp100#2) derived from melanoma Ag gp100 (33) was incubated with target HLA-F–positive B-LCL cell lines and visualized by costaining with MHC-I–specific mAb HC10 and HLA-F–specific mAb 3D11. The biotinylated gp100 protein colocalized with HLA-F or MHC-I both on the cell surface and within the cell, supporting the idea that the molecules are internalized together (Fig. 1B). The overlapping intracellular signals may also indicate that both molecules remain colocalized during the initial stages of processing.

**Exogenous Ag internalizes into early endosomes, and processing is sensitive to inhibitors of lysosomal enzymes**

Given the colocalization of HLA-F, open MHC, and Ag, we next attempted to trace the passage of Ag from the extracellular space, through internalization, and back to the surface as MHC-I peptide complex. First, we examined complex formation on the surface of T2 cells in the presence or absence of added Ag using conformation-specific mAbs mA2.1 (HLA-A*02 specific) and W6/32 (pan MHC complex specific). These experiments were designed to evaluate an increase in MHC complex formation after addition of Ag as a means of gauging cross-presentation of target Ag and are similar in design to a previously described assay that measured relative MHC-I peptide affinities (34). This assay uses the TAP-deficient T2 cell line to maximize the ability to detect the formation of new complex above background when using the conformation-specific mAbs W6/32 and mA2.1. To optimize the concentrations of control nonamer peptide and target Ag to be used before and after drug treatment, titrations of peptide and protein were carried out in the absence of drugs (Supplemental Fig. 1). The midpoint or half-maximal concentration of Ag for both protein and peptide was used in triplicate experiments, and the mean fluorescence index (MFI) of mAb binding before and after addition of peptide and protein was compared in the presence of two lysosomal inhibitors and BFA. Although the change in MFI for control nonamer peptide and target Ag to be used before and after drug treatment, titrations of peptide and protein to be used before and after drug treatment, titrations of peptide and protein were carried out in the absence of drugs. Although the change in MFI for control nonamer peptide and target Ag to be used before and after drug treatment, titrations of peptide and protein were carried out in the absence of drugs. Although the change in MFI for control nonamer peptide and target Ag to be used before and after drug treatment, titrations of peptide and protein were carried out in the absence of drugs.

Next, we observed the pathway and processing of Ag more directly, we fractionated cells after the addition of exogenous pp65 Ag and analyzed the proteins by Western blot with pp65-specific mAb. Enzyme activity for each fraction was measured to identify the subcellular compartments contained within each fraction. The enzyme markers present in fractions 1–4 and 9–10 were consistent with the presence of early endosomes and lysosomes, respectively. These fractions also contained pp65 protein, which migrated at a reduced m.w. in all fractions. The species of pp65 in the gradient fractions containing lysosomes had further reduced m.w. relative to

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FIGURE 1. Cointernalization of exogenous Ag, HLA-F, and MHC-I. (A) HLA-F surface expression is downmodulated by addition of denatured full-length protein. HLA-F surface expression on B, T, or monocyte cell lines was measured before (solid line) or after (dotted line) addition of denatured recombinant HCMV pp65 protein or HIV-1 p24 protein. For all stains, HLA-F was measured with mAb 3D11 with isotype-matched irrelevant Ab (gray). (B) HLA-F and MHC-I colocalize internally with exogenously added protein. B-LCL BM9 cells were stained with biotinylated gp100 50-aa protein labeled with Alexa-594–labeled streptavidin, anti–MHC-I mAb HC10 (IgG2a) labeled with Alexa-488, and anti–HLA-F mAb 3D11 (IgG1) detected with anti-IgG1 Alexa-647. The overlapping image for the three fluoros are merged on the far right as indicated. The boxed section is separated and expanded to the immediate right, and the Pearson coefficients calculated using ImageJ between the stain and the gp100 image above are listed adjacent. Cells were with DAPI, and the surface with Wheat Germ Agglutinin-Pacific Blue. (C) MHC-I complex formation on T2 cells (TAP and tapasin deficient) was used as a measure of exogenous Ag uptake. The increase in surface MHC-I complexed with peptide and β2m was measured using mAbs W6/32 and mA2.1 before and after addition of exogenous pp65 protein and peptide. The change in MFI using the indicated Abs was measured (average of three independent measurements) with and without the addition of inhibitors, as indicated in the legend between the graphs. (D) Partially processed exogenous pp65 Ag colocalized in cellular fractions containing early endosomes, and further processed proteins colocalized with fractions containing lysosomes. Western analysis was performed with mAb indicated to the left of each gel profile with m.w. markers to the right. Gradient fraction number is indicated above each lane, and relative enzyme activity measured within each fraction is indicated beneath the gels. For β-Hex (β-hexaminidase) and 5′-Ntdase (5′nucleotidase), the relative absorbance from three independent experiments is plotted versus the respective fractions with error bars indicating deviation from the mean. For Rab5 and Rab7, the results of Western analysis are reported according to relative intensities of bands from three independent measurements.
the fractions containing early endosomes, suggesting additional processing of protein (Fig. 1D). These data, taken together with the drug sensitivity data, are consistent with Ag entering cells, passing through early endosomes, and proceeding through lysosomes before the derivative peptide is generated. Derivative peptide then presumably combines with MHC-I, and the resultant complex is expressed on the surface (as detected by W6/32 and mA2.1).

Finally, to confirm and clarify the biochemical evidence for localization, we performed additional fluorescence microscopy using containing with endosomal markers (Fig. 2). Four markers were used, as follows: Rab5, a marker for early endosomes regulating the fusion between endocytic vesicles and early endosomes (35); Rab7, a marker found on a major organelle in the endolysosomal pathway (36); LAMP1, the lysosomal-associated membrane protein 1 (37); and EEA1, the early endosome Ag 1 (38). Initial stains of gp100 with EEA1 and LAMP1 showed isolated surface staining for gp100, but after internalization showed largely overlapping staining with both of the endosomal markers. Furthermore, co-stains of Ag gp100 with 3D11 and either Rab5 or Rab7 showed a clear overlap in signals among the sets of trios, indicating that as HLA-F and gp100 were internalized, they transited from the endocytic vesicles to the lysosomal pathway. Similarly, MHC-I and gp100 could be observed to internalize and colocalize with both Rab5 and LAMP1, indicating their coordinate transit from the endosome to the lysosome pathway.

**Binding of surface MHC-I to exogenous protein is dependent on MHC-I–specific epitopes**

Given coincident internalization of exogenous Ag with MHC-I and HLA-F, we examined whether direct physical interaction occurs between exogenous Ag and MHC-I in an epitope-specific manner. We used a previously established experimental system that identified HLA-A*0201–specific high-affinity mutant peptides derived from tumor Ag gp100 (39). We chose three peptides differing from one another by successive single amino acid changes from that study, that bound with low (ELE), medium (the naturally occurring sequence YLE), or high (YLF) affinities to HLA-A*0201. These peptides were used to measure relative increases in MHC-I complex formation using the T2 system described above. The ability of the peptides to increase levels of MHC-I complex was in direct relation to their binding affinity for HLA-A*0201 as was a corresponding reduction in the levels of MHC-I OC (through complex formation) and HLA-F (Fig. 3A). These data suggested that higher affinity peptides spontaneously formed complexes with MHC-I more readily, resulting in increased levels of HLA-F/MHC-I OC dissociation and consequent reduced levels of surface HLA-F.

To test whether specific MHC epitopes contained within an extended polypeptide affected their ability to bind OC of MHC-I, we synthesized three N-terminally biotinylated 50-aa protein fragments of gp100, as follows: one that included the native nonamer sequence and two derivatives that contained the low- and high-affinity mutant sequences. The relative binding of each polypeptide to T2 cells reflected the binding affinity of the epitope sequence contained within it, suggesting a direct interaction of the extended polypeptide with open MHC-I, as seen with the corresponding nonameric peptides. In support of this interpretation, mAbs against MHC-I OC were able to block relative binding of the high-affinity protein to either T2 cells or B-LCL HOM2 (Fig. 3B). To examine the interaction of the peptide sequence with MHC-I and possibly HLA-F, we used the low- and high-affinity polypeptides in comparative precipitation experiments using T2 cells and T2-HLA-B*35 transfectants. Western blot analysis of precipitated material showed that the high-affinity polypeptide consistently bound to quantitatively higher levels of both MHC-I and HLA-F, reflecting the surface-binding abilities and relative affinities of the epitopes contained within the polypeptides (Fig. 3C).

**Exogenous protein sensitizes HLA-F–positive targets to CTL recognition**

The coincident internalization of exogenous Ag with MHC-I and HLA-F combined with epitope specificity of MHC-I binding suggested a pathway for Ag presentation distinct from existing models for cross-presentation by MHC-I (40–43). To explore a role for the interaction of MHC-I OC and HLA-F in Ag uptake for processing and presentation, we used available CTL clones specific for epitopes HIV-gag p17 and CMV pp65 and tested whether B-LCL target cells that express both HLA-F and the MHC-1–restricting allele could be specifically sensitized to lysis by CTL effectors upon the addition of exogenous Ag. For a number of different combinations of target cell, exogenous protein (HIV-gag p17 and CMV pp65), class I HLA-restricting allele (HLA A*0201, A*0301, B*2705), and CTL, we were able to define conditions in which exogenous proteins optimally sensitized B-LCL to lysis by specific CTL. BFA was used to distinguish between two possibilities that could confer sensitivity to CTL lysis: internalization and processing of exogenous protein or spontaneous formation of MHC-I complex by direct addition of peptide (Fig. 4A). BFA inhibits transport of proteins through the Golgi and induces retrograde protein transport from the Golgi to the ER, thus distinguishing presentation of internalized and processed protein from spontaneous complex formation by direct addition of peptide.

Although inhibition of lysis by BFA discriminated between exogenous protein and direct peptide addition, three additional control experiments were performed to exclude the possibilities that the protein preparations were contaminated with degenerate peptides or were degraded to release corresponding nonamer peptides during the course of the experiments. First, we tested for the presence of small amounts of contaminating peptide by subjecting each protein preparation to LC-MS/MS and examining the spectra for the targeted specific peptides and extended peptides containing the specific peptide sequence (for up to 4 aa extending in both directions). No peptides containing the target epitopes were detectable in any of the preparations (Supplemental Fig. 2A, 2B). Next, to examine the possibility that peptides might be spontaneously generated from proteins during incubation with target cells, we performed a mock incubation identical to experimental conditions used for sensitization, followed by fractionation. Peptide titrations before or after centrincon pass-through were overlapping, demonstrating effective recovery of pM concentrations of peptide. In contrast, the pass-through from long polypeptide preparations was ineffective for sensitization to lysis at all concentrations tested, down to the lowest concentrations of protein effective for sensitization (Supplemental Fig. 2C).

The third control experiment was based on our observation that surface binding of biotinylated p17 and p24 Ag to B-LCL was reduced substantially at 4°C versus 37°C for both polypeptides (Supplemental Fig. 3A). Thus, if Ag uptake and processing were required, sensitization to lysis would be impaired at 4°C, but not at 37°C. Conversely, direct addition of peptide should not be impaired at 4°C as peptide uptake is not required for complex formation (28). For both polypeptides, the ability of exogenous Ag to sensitize B-LCL to lysis was impaired at lower temperature, whereas direct addition of peptide was unaffected (Supplemental Fig. 3B). In combination with the blockade of presentation by BFA, these control experiments support the conclusion that specific peptide is neither present in the original protein preparations nor generated external to cells during the course of the experiments prior to exposure of targets to effectors.
The data support a pathway in which exogenous Ags are internalized, processed, and presented by MHC-I by B-LCLs—one potentially involving HLA-F. However, surface expression of HLA-F is upregulated in most lymphocyte subsets upon activation, including activated T cell clones (17), suggesting that this pathway might also function in other cell types that express HLA-F. To test this in T cells, Ag-specific, HLA-F–positive CTL clones were pulsed with Ag directly in the absence of a target cell line and assayed for their ability to act as self-stimulators. The observed increase in IFN-γ expression after exposure to extracellular Ag suggested that T cell clones apparently acquire, process, and present exogenous Ag, suggesting that the effector cell itself can be involved in expansion of a memory response by cross-presenting Ag once stimulated (Fig. 4B). The ability of activated effectors to recruit Ag via this pathway suggests that Ag cross-presentation of fragmented target proteins may occur at the site of inflammation during effector responses.

**HLA-F and MHC-I OC in cross-presentation**

To examine the involvement of HLA-F in the novel pathway directly, we designed shRNA knockdowns targeting HLA-F, using lentivirus constructs to express the shRNA (29). B-LCL KOSE cells expressing HLA-A*0201 were transfected with the construct and with control vector. We screened four distinct sequence constructs for HLA-F and selected one that effected maximal downregulation of HLA-F. Surface levels of HLA-F were markedly decreased using the F4 shRNA construct, which also coincidentally reduced MHC-I H chain expression (Fig. 5A). The downregulation of MHC-I in the F4 transductants may be related to the intracellular interactions previously detected between HLA-F and MHC-I (15). It was not possible in our experience to separate MHC-I OC expression from HLA-F expression; class I–deficient 0.221 cells do not express surface HLA-F, whereas all precursor B-LCL expressing other MHC-I do coexpress HLA-F. Based on this limitation and their observed biophysical interactions, our working hypothesis is that HLA-F and MHC-I OC are expressed codependently.

Binding of biotinylated proteins to the surface of cells transduced with F4 was examined as an indirect measure of the effect that HLA-F and MHC-I H chain levels might have on Ag binding. Both 50-aa polypeptides derived from gp100 and HA-1H showed marked reductions in surface binding on cells treated with HLA-F–specific shRNA (Fig. 5B). Furthermore, when tested for sensitization to lysis by a pp65-specific CTL clone, uptake and processing of exogenous pp65 protein were significantly impaired in F4 transfectants. This was in contrast to the recognition of target cells that occurred with endogenously synthesized pp65 after vaccinia/pp65 infection or those pulsed with specific peptide. In those cases, both knockdowns compared similarly to vector only–transfected control (Fig. 5C). Sensitization assays incorporating mAb HCA2 or 3D11 blocking also demonstrated that specific interference of MHC-I OC or HLA-F prior to addition of exogenous protein affected Ag uptake and subsequent processing for MHC-I presentation (Fig. 5D).
Processing and presentation of exogenous Ag for MHC-I are independent of TAP and tapasin

The endogenous class I Ag presentation pathway and its dependence on TAP and tapasin are well characterized (44, 45). As a logical step toward characterization of exogenous MHC-I presentation, we examined two Ag sources in which we could compare endogenous presentation and exogenous presentation in TAP and tapasin mutant lines. We first established that the HA-1H Ag was TAP dependent when presented through the endogenous pathway. B-LCL 721 was typed for HA-1 alleles using an established protocol (21) and found to be HA-1H homozygous. Dependence on TAP was confirmed when a panel of 721 and derivative.134 (TAP negative), 0.134C2 (TAP restored), and class II deletion mutant 0.174 (TAP and tapasin deficient) were tested with HA-1H–specific CTL. Only 721 and TAP-restored 0.134C2 cells were sensitive to lysis by HA-1H–specific CTL (Fig. 6A). We next tested the same panel for access by exogenous Ag using the HA-1H 50-aa polypeptide. In contrast to endogenous Ag, HA-1H CTL lysed both 0.134 and 0.174 mutant lines pulsed with HA-1H protein, and, consistent with prior experiments, BfA inhibited presentation of exogenous protein, but not peptide (Fig. 6B).

Confirmation of the independence of this pathway from TAP and tapasin was obtained with pp65-specific CTL in which the presentation of endogenous pp65, expressed with a vaccinia virus construct, could be compared directly with exogenous protein using the

FIGURE 3. Epitope-specific binding of 50-aa polypeptides to MHC-I OC. (A) HLA-A*02–binding peptides differentially upregulate MHC-I complex and downregulate HLA-F and MHC-I OC. Normalized MFI is plotted for each of the indicated mAb stainings of T2 with and without the addition of the indicated peptides. (B) Higher affinity of peptide sequences is mirrored in stronger binding of 50-aa polypeptides to T2 cells. Plotted are MFIs of T2 cells stained with 50-aa biotinylated proteins containing the respective low (ELE)-, medium (YLE)-, and high (YLF)-affinity epitope sequences as defined by direct peptide binding. Binding of 50-aa biotinylated proteins is reduced by prior addition of anti–MHC-I OC mAbs HC10 and HCA2 on both T2 cells and HLA-A*02–expressing LCL HOM2, as indicated. (C) MHC-I and HLA-F specifically interact with 50-aa polypeptides, and relative binding reflects the peptide epitope affinity. Quantitative precipitation of MHC-I and HLA-F protein with 50-aa polypeptides containing the low-affinity and high-affinity peptide sequences reflects their surface-binding affinity. T2 and T2/B35 transfectants were incubated with the indicated biotinylated 50-aa polypeptides, and protein was precipitated, as described in Materials and Methods. Western blot analysis of the fractionated precipitates is shown using HCA2 (MHC-I) and 3D11 (HLA-F).
same panel of LCL mutants. CTL specific for a HLA-A*0201–restricted pp65 epitope only recognized the TAP-restored mutant 0.134C2 infected with vaccinia-pp65, consistent with prior studies showing that endogenous pp65 Ag presentation is TAP and tapasin dependent (46). In contrast, exogenous pp65 protein sensitized all targets, including the mutant cell lines. For all targets, sensitization to lysis by protein was inhibited by BFA, whereas peptide sensitization was not altered (Fig. 6C).

Discussion

Our investigations into the biological function of HLA-F support a novel role for the interaction between HLA-F and OC of MHC-I in the uptake of extracellular Ag for cross-presentation. Several lines of evidence suggest an active role for OC of MHC-I in the uptake of Ag as whole or partial protein that contains epitopes specific to receptors MHC-I expressed on target cells. Exogenous Ag was internalized together with MHC-I, and downmodulation of surface MHC-I resulted in reduced binding, uptake, and presentation of Ag. Differential binding of polypeptides containing low- and high-affinity MHC-I–binding epitopes to MHC-I and HLA-F suggested that exogenous Ag binds to the surface of activated cells to a structure that includes MHC-I OC and HLA-F and that is in contact with the MHC-I–specific epitope sequence found within the extended polypeptide. The requirement of this model for exposure of linear epitopes is consistent with the observation that in our experiments protein required denaturation to enter this pathway, as it did in previously described experiments for cross-presentation of BZLF-1 and pp65 (28). We speculate that MHC-I OC, possibly stabilized by denatured recombinant protein. The p17 Ag was delivered as 50-aa protein containing the respective CTL epitopes, and nonamer peptides were used as positive and negative controls as appropriate for each effect. Results are representative of three or more independent experiments performed against multiple A*02-positive targets, each performed in triplicate with error bars indicating SE of each triplicate. Immediately to the right of each profile are bar graphs reporting sensitization of the corresponding targets to CTL lysis in the presence or absence of BFA. Specific lysis at a single E:T ratio was measured without (solid) or with (hatched) prior treatment of targets with BFA for each peptide and the respective proteins, as indicated. Results are representative of two independent experiments, each performed in triplicate with error bars representing SE of each triplicate. (B) Ag presentation from exogenous protein can occur in T cell and monocyte cell lines. Adding denatured proteins exogenously to corresponding CTL clone cultures elicits IFN-γ production. Fluorescence measurements of IFN-γ are plotted versus forward scatter after the addition of either exogenous viral or minor histocompatibility Ag protein.

It is well known, based on many crystal structures and considerable binding and T cell recognition data, that a folded class I MHC molecule binds 8- to 10-mer peptides, depending on the allele and epitope, and that, in contrast to MHC-II, the ends of the pockets are closed (4). There are few reported exceptions to this rule, but, whereas the suggestion that extended polypeptide chains are capable of binding to MHC-I OC may be considered controversial, peptide length specificity of some HLA class I alleles has been shown to be very broad and includes peptides of up to 25 aa in length (47). The structure of the MHC-I OC is unknown and is most certainly distinct from the classical structure consisting of H chain, β2m, and peptide. Solving the MHC-I OC structure could address possibilities for an alternative Ag-binding mechanism related to our proposed model for interaction of the MHC-I–binding cleft with epitopes within extended polypeptides.

In addition to MHC-I OC, several experiments directly implicated HLA-F in this pathway. First, the observed overlapping internalization and localization of Ag and HLA-F were coincident with the same observation with MHC-I OCs. Secondly, HLA-F was coprecipitated with MHC-I by polypeptide containing MHC-I–binding epitopes at relative levels approximately paralleling those of MHC-I. Third, downmodulation of HLA-F resulted in interference with Ag binding, uptake, and processing for presentation, and, although downmodulation was coincident with downmodulation of MHC-I, blocking with HLA-F–specific mAb alone interfered with Ag cross-presentation. Indeed, the fact that HLA-F and MHC-I OC, and not MHC-I complex, physically interact in cells relates the findings of MHC-I OC function directly to HLA-F (15). One possible role for HLA-F could be in the stabilization and transport of MHC-I OC to, on, and from the surface. The physical interaction between HLA-F and classical MHC-I OC, their coin-
The evidence presented in this work suggests that Ag, MHC-I OC, and possibly HLA-F transit from the surface through the endosomal pathway into lysosomes or lysosome-like structures, where protein is degraded to produce target peptide independently of TAP or tapasin. After complex formation, MHC-I containing specific peptide derived from the exogenous Ag source is transported to the surface. This model has evident similarities with the MHC-II Ag-presenting pathway in particular, and there is already good evidence that MHC-I molecules visit phagolysosomal compartments to acquire peptides prior to surface expression (48, 49). MHC-I proteins have been shown to reside in endosomes and lysosomes of dendritic cells, and exchange of MHC-I between the cell membrane and endosomal compartments has been demonstrated in both T cells and macrophages (50–52).

Furthermore, in the mouse, exogenous MHC-I Ag loading has been associated with endosomal and lysosomal trafficking in dendritic cells (53).

Although previous studies have provided good evidence in support of a pathway for class I loading that is shared with class II molecules (49), our work presents at least three major new findings. First, no evidence implicating the participation of MHC-I OC in the transport of Ags has been accumulated. Second, no studies or proposed models have suggested an allelic dependence on MHC-I, although any including HLA-F has been reported. Second, no studies or proposed models have suggested an allelic dependence on MHC-I, although any including HLA-F has been reported. Furthermore, in the mouse, exogenous MHC-I Ag loading has been associated with endosomal and lysosomal trafficking in dendritic cells (53).
T cells), particularly when a target cell is lacking in MHC expression. Very little detail has been reported regarding how memory CD8+ T cells are activated, including their potential activation by amateur APCs (64).

Whereas the details of cross-presentation for MHC-I have not been well elucidated, several lines of evidence suggest this immune pathway could play a fundamental role in protection from pathogens (40, 43, 45). If MHC-I OC and HLA-F function in MHC-I Ag cross-presentation, then uncovering the biochemical rules for Ag uptake, processing, and presentation particular to this pathway could provide new strategies for the design and optimization of novel immunogens.

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Disclosures
The authors have no financial conflicts of interest.

References

FIGURE 6. Ag presentation through the HLA-F–associated pathway is independent of TAP or tapasin. (A) Endogenous HA-1H Ag presentation is TAP dependent. Specific CTL clone response to endogenous Ag was tested upon exposure to B-LCLs 721 and derivative LCLs 0.134 (TAP negative), 0.134C2 (0.134 TAP transfectant restored), and 0.174 (MHC class II region deletion, including TAP and tapasin). (B) Exogenous HA-1H Ag is processed and presented in TAP and tapasin-negative cells. Both 0.134 and T2 (0.174 chr 6) are sensitive to lysis by the HA-1H–specific CTL clone upon addition of a 50-aa protein containing the HA-1H epitope, but not a similar 50-aa protein representing the alternative HA-1R allele. HA-1H nonamer peptide was included as positive control, and parallel analysis without added peptide served as negative control (see legend). CTL assays are representative of two independent experiments, each performed in triplicate, with error bars indicating SE between triplicates. (C) TAP and tapasin-negative cells pulsed with exogenous pp65 protein, but not endogenous pp65 protein, are efficiently lysed by specific CTL clone. HCMV pp65 protein was delivered endogenously via vaccinia construct or exogenously as denatured recombinant protein, with peptide controls, as indicated. For each set of experiments (A–C), targets are indicated above each profile, and CTL clone effectors and MHC-I specificities are indicated at the top of each set of profiles. Legends are provided at the upper right corner of the first of each set of profiles. Bar graphs immediately to the right of each profile report result from a single E:T ratio of the corresponding effector and target for peptide or protein, as indicated beneath the bars without (solid bar) or with (hatched bar) the prior addition of BFA. CTL assays are representative of three independent experiments with two unique clones each performed in triplicate, with error bars indicating SE between triplicates.
Figure S1, related to Fig. 1B. Peptide titration on T2 cells. T2 cells were analyzed by FACS using the indicated mAbs (see legend) before and after incubation with increasing concentrations of pp65 peptide, and the change in mean florescence index (MFI) is plotted versus peptide concentration.
Figure S2 related to Fig. 3. Sensitization to lysis by whole protein is not due to contaminating peptide. (A) Protein preparations are free of peptide at levels above minimal concentrations effective to sensitize targets. Titrations of specific peptide and corresponding proteins were carried out in Cr release assays and the minimal peptide and protein concentrations that elicited 40%-maximal specific lysis are reported in the peptide conc. and protein conc. columns respectively. The ratio of these numbers is listed in the Pep:pro ratio column. Each protein was examined for the presence of peptide by MS/MS and the minimal detectable amount of peptide is reported / the quantity of protein examined in each experiment yields the limits of detection in the respective quantity of protein (as a molar ratio calculating peptides at 1kD and proteins at 5kD excepting pp65 at 50kD). Minimum detection limits were established using direct analysis of each specific peptide, and ranged from 1 pg (p24) to 100 pg (p17) for the five proteins tested and the indicated amounts of protein were analyzed by LC-MS/MS with no detectable peptide. By combining these data it was apparent that preexisting peptide was not detectable in any protein preparations to levels of from ten to two hundred-fold below the minimum required to

<table>
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<th>peptide conc.</th>
<th>protein conc.</th>
<th>Pep:pro ratio</th>
<th>MS/MS detection</th>
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effect target lysis, with the exception of pp65. For pp65 no peptides were detected, but the lower
detection limit was greater than the minimum required for 40% target lysis. (B) A sample spectra
of 10 pg of each peptide is shown, excluding p17 peptide KIRLRPGGKKK which was only
detectable at 100 pg. (C) Prior incubation of proteins with targets does not spontaneously
generate specific peptide released into culture. A single E:T ratio was tested for each clone
titrating peptide and protein concentrations as indicated. Peptide or protein were incubated with
targets as described in Methods for the appropriate times, up to the point of exposing targets to
effectors, supernatants were collected and portions spun through centricon membranes with
cutoff sizes permissible for peptide pass-through. These two preparations, before and after pass-
through, were then used to sensitize targets as before in titrations spanning the effective limits of
sensitization. For protein, but not for peptide, the centricon eliminated sensitization to lysis
consistent with the absence of specific peptide being generated during incubation of proteins
with targets.
Figure S3, related to Fig. 3. Reduced temperature lowers surface binding of exogenous protein coincident with reduced levels of antigen presentation. (A) HLA-F surface expression and surface binding of p17 and p24 50 amino acid (50-aa) proteins were measured at 4°C (dotted lines) and 37°C (solid lines). N-terminal biotinylated p17 and p24 proteins were detected with streptavidin-PE. (B) Specific lysis of targets SAVC and HOM2 sensitized with increasing concentrations of exogenous peptide or protein at 4°C or 37°C by effectors SR01 (p17 RLR) and 7709 (p24 RKW) as indicated in the legend above each graph.