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HLA-F Is a Predominantly Empty, Intracellular, TAP-Associated MHC Class Ib Protein with a Restricted Expression Pattern1

Shane D. Wainwright,* P. Andrew Biro,† and Christopher H. Holmes2*  

HLA-F is currently the most enigmatic of the human MHC-encoded class Ib genes. We have investigated the expression of HLA-F using a specific Ab raised against a synthetic peptide corresponding to amino acids 61–84 in the α1 domain of the predicted HLA-F protein. HLA-F is expressed as a β2-microglobulin-associated, 42-kDa protein that shows a restricted tissue distribution. To date, we have detected this product only in peripheral blood B cells, B cell lines, and tissues containing B cells, in particular adult tonsil and fetal liver, a major site of B cell development. Thermostability assays suggest that HLA-F is expressed as an empty heterodimer devoid of peptide. Consistent with this, studies using endoglycosidase-H and cell surface immunoprecipitations also indicate that the overwhelming majority of HLA-F contains an immature oligosaccharide component and is expressed inside the cell. We have found that IFN-γ treatment induces expression of HLA-F mRNA and HLA-F protein, but that this does not result in concomitant cell surface expression. HLA-F associates with at least two components of the conventional class I assembly pathway, calreticulin and TAP. The unusual characteristics of the predicted peptide-binding groove together with the predominantly intracellular localization raise the possibility that HLA-F may be capable of binding only a restricted set of peptides. The Journal of Immunology, 2000, 164: 319–328.

1 Department of Clinical Medicine, Division of Obstetrics and Gynaecology, University of Bristol, St. Michael’s Hospital, Bristol, United Kingdom; and 2 Department of Immunology, St. Bartholomew’s and Royal London School of Medicine and Dentistry, Queen Mary Westfield College, London, United Kingdom

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† Address correspondence and reprint requests to Dr. C. H. Holmes, University of Bristol, Division of Obstetrics and Gynaecology, St. Michael’s Hospital, Southwell Street, Bristol BS2 8EG, U.K. E-mail address: chris.holmes@bristol.ac.uk

‡ Abbreviations used in this paper: H chain, heavy chain; IEF, isoelectric focusing; 1D-IEF, one-dimensional IEF; B-LCL, EBV-transformed B-lymphoblastoid cell line; β2m, β2-microglobulin; ER, endoplasmic reticulum; Endo-H, endoglycosidase-H; MAP, multiantigenic peptide; RAHC, rabbit antiserum against monomorphic determinants on denatured HLA class I heavy chains.

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were subjected to three rounds of cloning by limiting dilution. The isotype Tween-20 in PBS), the wells were incubated with culture supernatant for
After a blocking step (10% (w/v) bovine skimmed milk powder, 0.2% (v/v) HLA-F associates with TAP.
In this study, we describe the use of one such reagent to examine
Peptides were synthesized on a multiantigenic peptide core (MAP) using
Ab production
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MATERIALS AND METHODS
Ab production
Peptides were synthesized on a multiantigenic peptide core (MAP) using
Tissues, cell lines, and Ab
All tissues and cells were obtained following informed consent and with
Northern analysis of IFN-γ-treated cells
The cell lines .221, HOM-2, and JEG-3 were cultured in the presence or
cells, were solubilized at 1 x 10^7 cells/ml in Mg^{2+}-free and Ca^{2+}-free PBS containing 1% (v/v) Triton X-100, 20 mM PMSF, 50 μg/ml leupeptin, and 50 μg/ml antipain, and incubated on ice for 30 min. Insoluble material was removed by centrifugation at 64,000 g for 10 min at 4°C. The lysate was incubated at 4°C under rotation for 30 min with mouse IgG agarose (Sigma), followed by protein G-Sepharose (GammaBind G; Pharmacia, Uppsala, Sweden). The precleared lysate was incubated for 1 h at 4°C with the relevant Ab. Protein G-Sepharose was added and the incubation continued for an additional 1 h, after which the pellet was washed five times with 0.1% (v/v) Triton X-100 in Mg^{2+}-free and Ca^{2+}-free PBS. Immunoprecipitates to be digested with Endo-H were resuspended in 20 μl of 0.2% (w/v) SDS, 2 mM PMSF, and 100 mM sodium citrate phosphate, pH 5.5, and boiled for 1 min. The tubes were cooled on ice for 5 min and then digested overnight at 37°C with 8 μl of Endo-H (Oxford GlycoSciences, Abingdon, U.K.). All immunoprecipitates were solubilized by boiling in Laemmli (40) sample buffer containing 5% (v/v) 2-ME, and separated by SDS-PAGE. Immune complexes were recovered with protein G-Sepharose, as described above.

Cell lysates were subjected to immunoprecipitation with mAb W6/32. The immunoprecipitates were digested with sialidase (Oxford GlycoSciences) following the manufacturer’s method. IEF was then performed using a Bio-Rad Protein II system run overnight at 800 V. 10 mA, according to the method of Neefjes et al. (41). Immunoblotting was conducted according to the method of Kao and Riley (42). Briefly, the gel was washed with agitation four times for 15 min each in 1% (w/v) SDS, 0.2% (v/v) methanol, and 5 mM Tris-HCl, pH 8. A final 15-min wash was conducted in electrophoretic transfer buffer before the gel was immunoblotted with mAb Fpep1.1, as described above. Membranes were stripped of Ab complexes in 100 mM 2-ME, 2% (w/v) SDS, and 62.5 mM Tris-HCl, pH 6.7, for 30 min at 50°C. The stripped membranes were then incubated successively with washing buffer and blocking buffer as above, and reprobed with RAHC. For both Fpep1.1 and RAHC, binding was detected using biotin-conjugated protein G, followed by StreptABCComplex/HRP (Dako, Carpenteria, CA). Binding was visualized using the ECL Western blotting system (Amersham).

**Results**

### Identification of HLA-F protein

A peptide corresponding to amino acids 61–83 of the α1 domain in the deduced protein sequence of HLA-G has been successfully used to raise mAb against HLA-G (28, 29). The peptide sequence (27) corresponds to amino acids 61–83 of the deduced protein sequence of the HLA-F gene product was used to search the SwissProt protein sequence database held at SEQNET (Daresbury, U.K.). No homologies greater than five linear amino acids were found with any other human protein, including class I. Peptides corresponding to amino acids 61–83 of the predicted sequences of HLA-F, HLA-E, and HLA-G were synthesized on a MAP core and used to raise rabbit antisera designated αF, αE, and αG, respectively. In addition, the HLA-F peptide was used to raise mAb: one IgG2b reagent was selected for its specific binding to the immunizing peptide by ELISA and designated Fpep1.1. The reactivity of all these Abs on dot blots of the immunogens is shown in Fig. 1A. Antiserum αF and mAb Fpep1.1 reacted with the HLA-F peptide, but not with HLA-E or HLA-G peptides. The αE and αG antisera also reacted specifically with their respective immunogens and are awaiting further characterization. The reactivity of these reagents only with their respective immunogens also shows that they do not bind to the MAP core.

The mutant cell line .221 does not express HLA-A,-B,-C, or -G (26, 44), but does express HLA-E (17). It has been suggested that a βm-associated HLA-F-H chain may be expressed at a very low level in .221 (26), and therefore, this cell line was used as a potential source of HLA-F protein. Antiserum αF and mAb Fpep1.1 both detected a 42-kDa component, the expected m.w. of HLA-F, in immunoblots of SDS-PAGE-separated .221 cell membranes (Fig. 1B, results for Fpep1.1 only are shown). To confirm that the 42-kDa product was indeed a class I protein, Fpep1.1 and αF were used to immunoblot the βm-associated class I proteins precipitated from .221 using the monomorphic mAb W6/32 and BB7.7. Both reagents precipitated a 42-kDa product reactive with αF and Fpep1.1 (Fig. 1C, left panel, results for Fpep1.1 only are shown).

Thus, Fpep1.1 and αF detect βm-associated class I proteins in .221. To examine the class I proteins expressed by .221, the W6/32 precipitates were subjected to immunoblotting with RAHC, a rabbit antiserum recognizing a monomorphic determinant on all mature class I H chains (36). RAHC identified three components of ~46, 44, and 42 kDa in .221 (Fig. 1C, right panel): only the lower, 42-kDa component comigrated with the 42-kDa class I product detected by Fpep1.1. Thus, .221 appears to express three βm-associated class I products, and only one of these is detected by Fpep1.1.

The W6/32-immunoprecipitated class I proteins identified by Fpep1.1 and RAHC in .221 were examined further by one-dimen-

sional IEF (1D-IEF). By immunoblotting, Fpep1.1 identified two acidic bands in .221 (Fig. 2, .221 panel). When this blot was stripped and reprobed with RAHC, however, three bands were detected (Fig. 2, .221 panel). In addition to the two acidic components detected by Fpep1.1, RAHC also identified a further, more alkaline band (Fig. 2). The profile of RAHC-reactive bands observed in Fig. 2 appears to resemble closely the 1D-IEF profile described for W6/32 immunoprecipitates obtained from metabolically labeled .221 cells in a previous report (17). In this case, the single alkaline band was identified as HLA-E. On this basis, the RAHC-positive/Fpep1.1-negative alkaline band in Fig. 2 appears to represent HLA-E. We confirmed that this was indeed HLA-E by
repeating the above experiments using mAb DT9 reported to detect HLA-E in .221 (27); only the upper RAHC-reactive component was precipitated from .221 by DT9 (data not shown). Taken together, these results show that .221 expresses HLA-F protein in addition to HLA-E protein, and that HLA-F is common with some other class I products migrates as a doublet in 1D-IEF.

HLA-F mRNA has previously been detected in B cell lines, but not in T cell lines (22, 23). The above experiment was repeated using the B-LCL SF-LCL and the T cell line Jurkat. Fpep1.1 detected the characteristic HLA-F doublet in SF-LCL, but did not react with the abundant HLA-A, -B, -C proteins precipitated from these cells by W6/32 (Fig. 2, SF-LCL panel, compare Fpep1.1 and RAHC tracks, respectively). As expected for a T cell line, Jurkat expressed considerably less class Ia protein than B-LCL, and RAHC detected only one major class Ia product in this cell line (Fig. 2, Jurkat panel, RAHC track). Fpep1.1 showed no reactivity with Jurkat (Fig. 2, Jurkat panel, Fpep1.1 track). Thus, Fpep1.1 specifically detects HLA-F in the B cell lines .221 and SF-LCL.

Expression of HLA-F in different cell lines and tissues
To date, we have been unable to detect the HLA-F protein efficiently by immunoprecipitation or immunohistochemistry using the Fpep1.1 or Ab, most likely because these reagents can detect their target protein only under reducing conditions. The expression of HLA-F in different cells and tissues was therefore examined by immunoblotting SDS-PAGE-separated cell membranes with Fpep1.1. The 42-kDa HLA-F product was detected by Fpep1.1 in all 12 B-LCL tested (the different B-LCL, which encompass a variety of MHC specificities, are listed in Materials and Methods). Two of these, SF-LCL and DW-LCL, are shown in Fig. 3A. For these two B-LCL, fibroblast cell lines derived from the same individuals and designated SF-FIB and DW-FIB, respectively, were also available. These showed no reactivity with Fpep1.1 (Fig. 3A). In addition, Fpep1.1 did not react with the T cell lines MOLT-4, Jurkat, and HUT-78, or with the erythroleukemic cell line K-562 (Fig. 3B). Similarly, the epithelial cell lines HT-29 (colonic adenocarcinoma), 293 (embryonal kidney), G-401 (Wilm’s tumor), and JEG-3 (choriocarcinoma) were unreactive (Fig. 3B); the choriocarcinoma cell lines BeWo and Jar were also negative (data not shown). In these experiments, it is possible that HLA-F may be expressed below the level of detection in non-B-LCL. To examine this, class I products were purified by immunoprecipitation using W6/32 and the anti-β2m mouse Ab and then subjected to immunoblotting with Fpep1.1. Although the mAb...
Reactivity of mAb Fpep1.1 with different cell lines. Fpep1.1 was used to probe immunoblots of membranes prepared from the cell lines indicated at the top. In each case, 50 μg protein/track was separated on an 8% polyacrylamide gel. Binding was detected using HRP-conjugated goat anti-mouse IgG and developed using the ECL system. The position of the 45-kDa marker protein is indicated at the left. A, Immunoblot of cell membranes prepared from the EBV-transformed B cell lines SF-LCL and DW-LCL, and the fibroblast cell lines SF-FIB and DW-FIB derived from the two individuals SF and DW, respectively. B, Immunoblots of cell membranes prepared from the T cell lines MOLT-4, Jurkat, and HUT-78; the erythroleukemic cell line K-562; and the epithelial cell lines HT-29 (colonic adenocarcinoma), 293 (embryonal kidney), G401 (Wilm’s tumor), and JEG-3 (choriocarcinoma).

HC10 readily detected HLA-B, -C H chains in these immunoprecipitates, there was no reactivity with Fpep1.1: results for Jurkat only are shown in the lowest panel of Fig. 4B.

Freshly isolated PBL were prepared and fractionated into B cell, T cell, and B/T cell-depleted populations using magnetic beads coated with mAb to CD19 (B cells) and CD3 (T cells). The 42-kDa HLA-F product was readily detected by immunoblotting in B cell membranes (Fig. 4A). However, Fpep1.1 did not react with an equivalent amount of membrane material prepared either from T cells or from the B/T cell-depleted populations (Fig. 4A).

We previously detected HLA-F mRNA in human fetal liver tissue (24). To examine the expression of HLA-F protein in this tissue, Fpep1.1 was used to immunoblot the class I products immunoprecipitated by W6/32 and BBM1 from detergent lysates of first and second trimester fetal liver. Fpep1.1 detected the 42-kDa HLA-F product in BBM1 immunoprecipitates from both first and second trimester fetal liver (Fig. 4B, left panels). However, no reactivity was observed with the class I products precipitated by W6/32, even though HC10 readily detected HLA-B, -C proteins in these precipitates (Fig. 4B, compare W6/32 tracks in left [Fpep1.1] and right [HC10] panels, respectively). This result was unexpected because W6/32 immunoprecipitated HLA-F from B-LCL (see Fig. 2). It is possible that HLA-F is expressed with different characteristics in cultured compared with freshly derived cells, or in fetal compared with adult tissues. To explore this further, we examined cells prepared from fresh adult tonsil, a rich source of B cells. The 42-kDa HLA-F protein was detected by immunoblotting in both W6/32 and BBM1 immunoprecipitates made from adult tonsil cells (Fig. 4B, left panel). These data show that W6/32 can readily detect its target epitope on HLA-F expressed in B cell lines and tonsil cells, but that this epitope is not available on HLA-F expressed in fetal liver.

Previous studies have shown that HLA-F mRNA is expressed at much lower levels than mRNA for HLA-A, -B, -C (23). Our data also suggest that the HLA-F protein is expressed at much lower levels than classical class I. This is evident for SF-LCL in Fig. 2, in which the HLA-F doublet identified by Fpep1.1 in 1D-IEF is below the level of detection by RAHC. However, RAHC readily identified the abundant HLA-A, -B, -C in these cells, as well as identifying HLA-F in .221. Data presented in Fig. 4B also suggest that the level of HLA-F protein detected by Fpep1.1 in fetal liver and tonsil is low compared with the expression of HLA, -B, -C detected by HC10. (In Fig. 4B, the HC10 immunoblot for tonsil was underexposed to allow visualization of discrete 45-kDa products.)

Cellular localization of HLA-F

MHC-encoded class I molecules bind peptides and carry them to the cell surface for interaction with effector T cells. However, previous studies have failed to detect HLA-F at the cell surface of HLA-F transfectants (22, 23, 26). We investigated the cellular localization of HLA-F in .221, B-LCL, and tonsil cells. In the first instance, we set out to determine whether HLA-F can be detected among the class I proteins immunoprecipitated from intact cells. For this, W6/32 was used to immunoprecipitate class I proteins...
from an equivalent number of intact and detergent-solubilized cells, and the precipitates immunoblotted using Fpep1.1. Experiments were conducted at 4°C to inhibit intracellular transport. To control for membrane integrity, parallel precipitations were conducted at 4°C to inhibit intracellular transport. To further examine the cellular localization of HLA-F, class I proteins precipitated from intact cells were low in these experiments, we could not exclude the possibility that HLA-F precipitating from intact cells could actually originate from dead or dying cells.

Maturing class I proteins acquire complex N-linked oligosaccharides only when they pass through the medial Golgi, at which point they become resistant to digestion with Endo-H. Class I proteins within the ER are therefore immature and Endo-H sensitive. To further examine the cellular localization of HLA-F, class I proteins were immunoprecipitated from detergent lysates of .221, SF-LCL, and tonsil cells, subjected to Endo-H digestion, and immunoblotted with Fpep1.1. Endo-H digestion reduced the m.w. of HLA-F from ~42 to 40 kDa in equivalent numbers of .221, SF-LCL, and tonsil cells: no Endo-H-resistant HLA-F was detected (Fig. 6A, left panel). The amount of HLA-F precipitated from SF-LCL was low when compared with the amounts precipitated from .221 and tonsil cells (Fig. 6A). To increase the amount of HLA-F precipitating from SF-LCL, the input cell number was raised 10-fold, and the conditions for W6/32 precipitation were adjusted accordingly. Endo-H-resistant HLA-F was not detected in three separate experiments conducted under these conditions, although, in a further experiment, an apparently Endo-H-resistant HLA-F protein was detected at very low level in SF-LCL (Fig. 6A, right panel).

Our inability to consistently detect Endo-H-resistant HLA-F prompted us to examine the Endo-H sensitivity of the HLA-F proteins apparently precipitated from intact cells (see Fig. 5A). Class I proteins precipitated from intact .221 and SF-LCL by W6/32 were subjected to Endo-H digestion and then immunoblotted with Fpep1.1. In both cell lines, the HLA-F proteins apparently immunoprecipitated from the cell surface were found to be Endo-H sensitive: no Endo-H-resistant HLA-F was detected in immunoprecipitates from either intact .221 or SF-LCL (Fig. 6B). These results suggest that the HLA-F detected in cell surface immunoprecipitations does not represent mature cell surface HLA-F. When taken together, therefore, our data show that HLA-F is a predominantly intracellular protein.

Previous investigators have reported that W6/32 does not bind to the cell surface in .221 (26, 44). More recently, however, a low level of W6/32 binding has been reported at the surface of these
immunoprecipitated from IFN-γ.221 and the B-LCL HOM-2 (Fig. 7A, right panels and HC10 (mJEG-3, .221, and SF-LCL cells and probed with mAb Fpep1.1 (left panel). We set out to examine whether IFN-γ protein G and StreptABComplex/HRP and developed using the ECL system. Immunoprecipitates were separated on an 8% polyacrylamide gel and immunoblotting with either Fpep1.1 (left panels) or RAHC (right panels). Binding was detected using biotin-conjugated protein G and StreptABComplex/HRP and developed using the ECL system. Arrows indicate the position of the 45-kDa marker protein. Note that the SF-LCL and tonsil cell precipitates immobilized with RAHC (right-hand panels) were under- exposed to allow visualization of discrete 45-kDa class I proteins. Under these conditions, HLA-F is below the level of detection for RAHC.

FIGURE 7. Effect of IFN-γ on HLA-F expression. Cells, as indicated, were grown for 72 h in the presence (+) or absence (−) of 1000 U/ml of IFN-γ. A, Northern analysis was conducted on RNA prepared from .221, JEG-3, and HOM-2 cells using an HLA-F-specific oligonucleotide probe (HLA-F, left panel) and a pan class I oligonucleotide probe (Pan class I, right panel). B, Immunoblot of membranes prepared from IFN-γ-treated JEG-3, .221, and SF-LCL cells and probed with mAb Fpep1.1 (left panel) and HC10 (right panels). A total of 50 μg protein/tracks was separated on an 8% polyacrylamide gel before immunoblotting. Binding was detected using HRP-conjugated goat anti-mouse IgG and developed using the ECL system. C, Endo-H sensitivity of IFN-γ-induced HLA-F. Class I proteins immunoprecipitated from IFN-γ-treated and untreated .221 and SF-LCL cells were incubated in the presence (+) or absence (−) of Endo-H. Immunoprecipitates were separated on an 8% polyacrylamide gel and immobilized with Fpep1.1. Binding was detected using biotin-conjugated protein G and StreptABComplex/HRP and developed using the ECL system. Arrows in B and C indicate the position of the 45-kDa marker protein.

cells, and this reactivity was suggested to represent a sialated form of HLA-F (27). In contrast, our data consistently show that HLA-F expressed in .221 is Endo-H sensitive and intracellular: we have been unable to detect mature, Endo-H-resistant HLA-F characteristic of a cell surface-expressed product in this cell line. To investigate this further, RAHC was used to detect both HLA-E and HLA-F in Endo-H-digested class I proteins purified from .221 by W6/32. Of the three components identified by RAHC, only the 46-kDa protein was Endo-H resistant: the 44- and 42-kDa products were both Endo-H sensitive (Fig. 6C). Because the 42-kDa product is HLA-F, the 46- and 44-kDa products most likely represent HLA-E proteins having both mature and immature oligosaccharides, respectively. These data suggest that the W6/32 reactivity previously reported at the surface of .221 is not HLA-F, but rather that it represents a mature 46-kDa class I protein, most likely HLA-E.

Effect of IFN-γ on HLA-F expression

We set out to examine whether IFN-γ stimulation induces HLA-F and could lead to cell surface expression of this protein. By Northern analysis, IFN-γ was found to increase HLA-F mRNA in both .221 and the B-LCL HOM-2 (Fig. 7A, left panel). Exposure to IFN-γ also resulted in an increase in HLA-F protein in membranes prepared from .221 and SF-LCL, as assessed by immunoblotting using Fpep1.1 (Fig. 7B, left panel). This increase was in line with the IFN-γ-stimulated increase in HLA-B, -C in SF-LCL, but was modest when compared with the increase in HLA-C observed in IFN-γ-treated JEG-3 choriocarcinoma cells (Fig. 7B, right panels). We next used Endo-H digestion to determine whether IFN-γ-induced HLA-F could reach the cell surface. IFN-γ increased the amount of W6/32-precipitable HLA-F heterodimers in both .221 and SF-LCL (Fig. 7C, compare IFN-γ (+)/Endo-H (−) tracks with IFN-γ (−)/Endo-H (−) tracks, respectively). However, Endo-H-resistant HLA-F heterodimers were not detected in IFN-γ-treated cells (Fig. 7C, see IFN-γ (+)/Endo-H (−) tracks). Thus, the increase in HLA-F induced by IFN-γ does not result in cell surface expression of the HLA-F protein.

Peptide loading of HLA-F

Under normal conditions, class I proteins can reach the cell surface only if the peptide-binding groove is occupied. HLA-F may be predominantly intracellular because it fails to acquire peptide or because, having acquired peptide, it is unable to exit the ER. Peptide loading of class I molecules results in a change in their thermostability: at 37°C, loaded class I molecules are stable, while empty class I molecules are unstable (43, 46). To examine the thermostability of HLA-F, detergent lysates of .221, SF-LCL, and tonsil cells were incubated at either 4°C or 37°C before immunoprecipitation with mAb W6/32. Immunoprecipitated class I proteins were separated on an 8% polyacrylamide gel and subjected to immunoblotting with either Fpep1.1 (left panels) or RAHC (right panels). Binding was detected using biotin-conjugated protein G and StreptABComplex/HRP and developed using the ECL system. Arrows indicate the position of the 45-kDa marker protein. Note that the SF-LCL and tonsil cell precipitates immobilized with RAHC (right-hand panels) were under-exposed to allow visualization of discrete 45-kDa class I proteins. Under these conditions, HLA-F is below the level of detection for RAHC.

FIGURE 8. Thermostability of HLA-F. Detergent lysates prepared from .221, SF-LCL, and tonsil cells were incubated at either 4°C or 37°C before immunoprecipitation with mAb W6/32. Immunoprecipitated class I proteins were separated on an 8% polyacrylamide gel and subjected to immunoblotting with either Fpep1.1 (left panels) or RAHC (right panels). Binding was detected using biotin-conjugated protein G and StreptABComplex/HRP and developed using the ECL system. Arrows indicate the position of the 45-kDa marker protein. Note that the SF-LCL and tonsil cell precipitates immobilized with RAHC (right-hand panels) were under-exposed to allow visualization of discrete 45-kDa class I proteins. Under these conditions, HLA-F is below the level of detection for RAHC.

Thermostable class I molecules were detected in all these cell populations when precipitates were immunoblotted with RAHC (Fig. 8, right panels). However, the 42-kDa HLA-F protein evident in .221, SF-LCL, and tonsil cells at 4°C was not detected at 37°C (Fig. 8, left panels). These results suggest that the peptide-binding groove of HLA-F is empty in .221, SF-LCL, and tonsil cells.

Interestingly and consistent with our previous Endo-H results (see Fig. 6), the mature 46-kDa HLA-E product identified by RAHC in .221 was detected at 37°C, while the immature 44-kDa HLA-E product was not detected at 37°C (Fig. 8, top right-hand
Peptide loading generally occurs in a multimeric complex containing class I H chain, β2m, TAP1, TAP2, tapasin, and calreticulin. HLA-F may be devoid of peptide because it does not participate in this complex. To determine whether HLA-F associates with TAP, .221 and SF-LCL were solubilized in digitonin to maintain class I/TAP interactions, immunoprecipitated with antisera to TAP1 and TAP2, and immunoblotted using Fpep1.1. To confirm the preservation of TAP associations, we first showed that anti-TAP Ab were capable of coprecipitating calreticulin from these lysates (Fig. 9A, top panels). The 42-kDa HLA-F protein coprecipitated with both TAP1 and TAP2 from .221 (Fig. 9A, middle panel at left). Similar results were obtained in SF-LCL, although the amount of coprecipitating HLA-F was relatively low (Fig. 9A, middle panel at right). This may be due to competition for TAP between HLA-F and class Ia molecules, the latter identified by HC10 in these precipitates (Fig. 9A, bottom right panel). Immunoprecipitations were also conducted on Triton X-100 lysates of .221 and SF-LCL by an antisera to calreticulin (αCRT) and by normal rabbit serum (NRS). Coprecipitated proteins were immunoblotted with Fpep1.1 and HC10. Precipitating and immunoblotting Ab are indicated at the top and left-hand side, respectively. The positions of the 66-kDa (αCRT panel) and 45-kDa (Fpep1.1 and HC10 panels) marker proteins, respectively, are indicated at the right. B, Immunoblot of proteins coprecipitated from Triton X-100 lysates of .221 and SF-LCL by an antisera to calreticulin (αCRT) and by normal rabbit serum (NRS). Coprecipitated proteins were immunoblotted with Fpep1.1. The position of the 45-kDa marker protein is indicated by the arrow. In both A and B, coprecipitated proteins were separated on an 8% polyacrylamide gel. Anti-CRT binding was detected using biotin-conjugated protein G and StreptABComplex/HRP, and Fpep1.1 and HC10 binding was detected using HRP-conjugated rabbit anti-mouse IgG. Immunoblots were developed using the ECL system.

**FIGURE 9.** Association of HLA-F with TAP and calreticulin. A, Immunoblot of proteins coprecipitated from digitonin lysates of .221 and SF-LCL cells by rabbit antisera against TAP1 (αTAP1) and TAP2 (αTAP2), and by normal rabbit serum (NRS). Coprecipitated proteins were immunoblotted with an antisera to calreticulin (αCRT) and with mAb Fpep1.1 and HC10. Precipitating and immunoblotting Ab are indicated at the top and left-hand side, respectively. The positions of the 66-kDa (αCRT panel) and 45-kDa (Fpep1.1 and HC10 panels) marker proteins, respectively, are indicated at the right. B, Immunoblot of proteins coprecipitated from Triton X-100 lysates of .221 and SF-LCL by an antisera to calreticulin (αCRT) and by normal rabbit serum (NRS). Coprecipitated proteins were immunoblotted with Fpep1.1. The position of the 45-kDa marker protein is indicated by the arrow. In both A and B, coprecipitated proteins were separated on an 8% polyacrylamide gel. Anti-CRT binding was detected using biotin-conjugated protein G and StreptABComplex/HRP, and Fpep1.1 and HC10 binding was detected using HRP-conjugated rabbit anti-mouse IgG. Immunoblots were developed using the ECL system.

**panel.** This suggests that .221 contains immature and empty HLA-E together with a mature HLA-E product having an occupied peptide-binding groove.

Peptide loading generally occurs in a multimeric complex containing class I H chain, β2m, TAP1, TAP2, tapasin, and calreticulin. HLA-F may be devoid of peptide because it does not participate in this complex. To determine whether HLA-F associates with TAP, .221 and SF-LCL were solubilized in digitonin to maintain class I/TAP interactions, immunoprecipitated with antisera to TAP1 and TAP2, and immunoblotted using Fpep1.1. To confirm the preservation of TAP associations, we first showed that anti-TAP Ab were capable of coprecipitating calreticulin from these lysates (Fig. 9A, top panels). The 42-kDa HLA-F protein coprecipitated with both TAP1 and TAP2 from .221 (Fig. 9A, middle panel at left). Similar results were obtained in SF-LCL, although the amount of coprecipitating HLA-F was relatively low (Fig. 9A, middle panel at right). This may be due to competition for TAP between HLA-F and class Ia molecules, the latter identified by HC10 in these precipitates (Fig. 9A, bottom right panel). Immunoprecipitations were also conducted on Triton X-100 lysates prepared from .221 and SF-LCL using an antisera to calreticulin. The 42-kDa HLA-F protein coprecipitated with calreticulin in both cell lines, as assessed by immunoblotting with Fpep1.1 (Fig. 9B). These results suggest that HLA-F can associate with the multimeric complex involved in peptide loading.

**Discussion**

We have shown that the HLA-F protein can be detected in B cells, B cell lines, and B cell-containing tissues, in which it occurs in a predominantly intracellular, unstable, and immature form. Our studies have also shown that HLA-F can associate with TAP, but that the protein does not appear to bind peptide and is expressed in an empty configuration.

Previous investigators have drawn attention to unusual features in the predicted peptide-binding groove of HLA-F (22, 23). Despite their variability, human class Ia molecules possess 10 highly conserved amino acid residues that point into the Ag recognition site. The class Ib proteins HLA-E and HLA-G retain 8 and 9 of these residues, respectively. However, only 5 of the 10 residues are conserved in HLA-F. On this basis, it has been suggested that HLA-F may have a different biological function from that of other class I proteins. Some non-MHC-encoded class Ib products have modified peptide-binding grooves that reflect their specific biological functions. The peptide-binding groove of the IgG transporter FeRn, for example, is closed, while that of CD1b is specialized to accommodate nonpeptide ligands (48, 49). However, a recent structural analysis concluded that the residues lining the putative binding groove of HLA-F are consistent with peptide binding (6). Our own preliminary modeling analysis (unpublished observations) also supports this view. We therefore believe that the HLA-F peptide-binding groove is likely to be a peptide receptor.

MHC-encoded class I proteins are expressed at the cell surface in a mature, Endo-H-resistant form only after they have acquired peptide in the ER lumen. In previous studies, HLA-F was not detected at the cell surface in HLA-F transfectants (22, 23, 26). To date, however, there has been no information on the cellular localization of HLA-F in normal cells. This requires reagents that can specifically identify HLA-F among the abundant classical class I proteins normally expressed in somatic cells. The anti-HLA-F reagent Fpep1.1 has allowed us to examine the cellular localization of HLA-F both in cell lines and in freshly isolated human cells. Comparison of the class I proteins immunoprecipitated from intact cells and cell lysates indicated that the overwhelming majority of HLA-F is expressed inside .221, SF-LCL, and tonsil cells. Nevertheless, the detection of limited amounts of HLA-F among the class I proteins immunoprecipitating from intact cells raised the possibility that some HLA-F may reach the cell surface. Surprisingly, however, we were consistently unable to detect Endo-H-resistant HLA-F, characteristic of cell surface class I expression, in whole lysates of .221 and tonsil cells. Similar results were obtained in SF-LCL, although, on one occasion only, we did identify at low level an apparently Endo-H-resistant HLA-F product in these cells. The significance of this latter observation is currently unclear, especially since all the apparent cell surface HLA-F observed immunoprecipitating from intact cells is expressed in the form of empty immature heterodimers, but this seems inherently unlikely. Also, further work is required to determine whether HLA-F can, under some circumstances, acquire mature oligosaccharides and reach the cell surface. Nevertheless, when taken together, our data clearly indicate...
that HLA-F is an empty and intracellular class I protein in normal cells.

Our results on the cellular localization of HLA-F in .221 are in contrast with a recent report by Braud et al. (27), who suggested that HLA-F is expressed at the surface of these cells. Unlike previous investigators (18, 26), these authors reported a low level of W6/32 reactivity at the surface of .221 by flow cytometry. This was proposed to be cell surface HLA-F because it was stated that a sialated, mature HLA-F protein was detectable in these cells using pulse-chase and IEF analysis, although the criteria used to identify HLA-F in these studies were not given. By contrast, the present study used an HLA-F-specific reagent to demonstrate that .221 expresses only immature and empty HLA-F, characteristic of an intracellular class I protein. Our study further reveals that sialidase-treated (immature) HLA-F migrates as a doublet in IEF gels. It is possible that in pulse-chase experiments analyzed by IEF, in which sialidase is not used, these products may appear to represent immature and sialated forms of HLA-F.

Although these data show that HLA-F is not expressed at the cell surface in .221, our studies reveal that this cell line does express low levels of an Endo-H-resistant, thermostable 46-kDa class I protein. This product shows no reactivity with the mAb Fpep1.1, and therefore does not represent HLA-F. Because the 46-kDa product has the properties of a mature cell surface class I protein, it may be responsible for the low level of W6/32 reactivity observed by Braud et al. on the cell surface of .221 (27). Moreover, the low level expression of this product could explain why other groups have reported that .221 is W6/32 negative by flow cytometry (18, 26). On the other hand, we have not used flow cytometry and cannot rule out the possibility that this apparently loaded class I product fails to reach the cell surface in .221.

We have not identified the 46-kDa class I protein unequivocally, although, because .221 expresses only HLA-E and HLA-F, it seems reasonable to propose that it represents cell surface HLA-E. This observation is surprising given that two recent studies have reported that HLA-E does not reach the cell surface in .221 (18, 27). In both studies, different mAb detecting HLA-E failed to bind to .221 in flow cytometry. In addition, in one report, no Endo-H-resistant class I proteins were detected in pulse-chase analysis of W6/32 precipitates from these cells (18). In the second report, sialated and hence mature HLA-E was not detected in .221 by pulse-chase and IEF analysis (27). The reasons for the discrepancy between our results and those of others are not clear. However, the 46-kDa product may be at the limit of detection in flow cytometry not only for W6/32, but also for anti-HLA-E reagents, especially DT9, which was raised against cotton top tamarin MHC class I molecules (27) and, in our hands, cross-reacts only weakly with HLA-E. The 46-kDa protein may also fall below the level of detection in pulse-chase analysis: our approach accesses the entire population of W6/32-precipitable class I molecules, while pulse-chase experiments identify only the proportion of class I proteins synthesized during isotopic labeling.

Our detection of an apparently mature HLA-E protein in .221 is intriguing given that HLA-E is incapable of binding peptides derived from the signal sequences of either HLA-E itself or of HLA-F (17). On this basis, HLA-E would not be expected to reach the cell surface in .221. However, the detection of apparently loaded and mature HLA-E in .221 suggests that this molecule can bind peptides other than those derived from MHC class I signal sequences, as already suggested by others (19).

HLA-F may be empty because it is unable to interact with or acquire peptides from TAP. Although we have not yet conducted pulse-chase experiments to determine the steps involved in its assembly, our studies nevertheless show that HLA-F associates with calreticulin and TAP. It therefore seems likely that HLA-F participates in the multimeric complex involved in class I peptide loading. This raises the possibility that HLA-F is not being loaded, and hence released, from TAP. Interestingly, it has recently been found that some alleles of HLA-C exhibit a stable interaction with TAP (50). This appears to occur because HLA-C molecules are more selective than HLA-A, -B in the range of peptides they bind. Because of this restricted peptide binding, most HLA-C molecules are retained in the ER and not transported to the cell surface. It has been reported that the amounts of intracellular HLA-C are similar to those of HLA-A, -B (50). In contrast, previous studies have shown that the level of HLA-F mRNA is low (23), and our own work now shows that HLA-F protein expression is also low. A combination of low level expression and restricted peptide binding could therefore account for the predominantly intracellular, empty HLA-F observed in the present study.

HLA-F shows a restricted tissue distribution. To date we have detected HLA-F protein only in B cells, B cell lines, and tissues containing B cells, in particular adult tonsil and fetal liver, a major site of B cell development. Our results are broadly in line with two previous studies in which the distribution of HLA-F mRNA was examined using RNase protection assays (22, 23). In each case, HLA-F transcripts were detected in B cell lines, but not T cell lines. These studies also raised the possibility that HLA-F mRNA was expressed in peripheral blood T cells. In contrast, we did not detect HLA-F protein in T cells. In one study, the HLA-F mRNA detected in PBMC was found to be reduced following PHA activation, and this was attributed either to a drop in the proportion of B cells or to the expression of HLA-F mRNA by resting, but not activated, T cells (23). In another report, HLA-F mRNA was detected in T cells enriched from PBMC by nylon wool fractionation (22). It is possible that T cells may express untranslated HLA-F mRNA or, alternatively, the previous detection of HLA-F transcripts in T cell preparations may reflect low level B cell contamination. Taken together, however, our results show that the HLA-F protein has a restricted tissue distribution that, by analogy with some other class Ib proteins, may indicate a specialized function for this molecule.

Our studies have revealed an apparent difference in the characteristics of HLA-F expression between fetal and adult life. We found that W6/32 immunoprecipitated HLA-F from B cell lines and tonsil cells, but not from fetal liver. By contrast, W6/32 readily immunoprecipitated other MHC class I proteins from this tissue, and mAb to β_2m also precipitated HLA-F from fetal liver. This suggests that the W6/32-defined epitope on HLA-F is unavailable in fetal liver. Our own previous work together with that of others has shown that W6/32 epitopes are not available in class I/calreticulin complexes (45, 47). One possibility therefore is that, by contrast with HLA-F in adult cells, HLA-F in fetal liver is associated with calreticulin, or with an as yet unidentified protein that masks the W6/32 epitope. Alternatively, a conformational change in the HLA-F proteins expressed in fetal liver may abrogate W6/32 binding. It remains to be determined whether these differences are reflected in the function of HLA-F during fetal and adult life.

The possibility cannot be excluded that HLA-F has an intracellular function. For example, it might be speculated that, like HLA-DM, which has a role in peptide loading of MHC class II, HLA-F could be involved in peptide loading of other HLA molecules in the TAP complex of B cells. It is also possible that, instead of entering the class I secretory pathway, HLA-F may carry ligands to another cellular compartment, although our inability to detect loaded HLA-F makes this unlikely. Our data show that HLA-F associates with TAP, and it is therefore possible that this class I
protein is awaiting appropriate ligands to allow cell surface expression in B cells. Such putative ligands may not normally be present in B cells, but may become available following infection of B cells, or during normal B cell differentiation or activation.

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References