Impaired Intracellular Transport and Cell Surface Expression of Nonpolymorphic HLA-E: Evidence for Inefficient Peptide Binding

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Summary

The assembly of the classical, polymorphic major histocompatibility complex class I molecules in the endoplasmic reticulum requires the presence of peptide ligands and β2-microglobulin (β2m). Formation of this trimolecular complex is a prerequisite for efficient transport to the cell surface, where presented peptides are scanned by T lymphocytes. The function of the other class I molecules is in dispute. The human, nonclassical class I gene, HLA-E, was found to be ubiquitously transcribed, whereas cell surface expression was difficult to detect upon transfection. Pulse chase experiments revealed that the HLA-E heavy chain in transfectants, obtained with the murine myeloma cell line P3X63-Ag8.653 (X63), displays a significant reduction in oligosaccharide maturation and intracellular transport compared with HLA-B27 in corresponding transfectants. The accordingly low HLA-E cell surface expression could be significantly enhanced by either reducing the culture temperature or by supplementing the medium with human β2m, suggesting inefficient binding of endogenous peptides to HLA-E. To analyze whether HLA-E binds peptides and to identify the corresponding ligands, fractions of acid-extracted material from HLA-E/X63 transfectants were separated by reverse phase HPLC and were tested for their ability to enhance HLA-E cell surface expression. Two fractions specifically increased the HLA class I expression on the HLA-E transfectant clone.

A variety of the molecules involved in vertebrate immune recognition are encoded by the MHC. In humans these include three highly polymorphic class I transplantation antigens (HLA-A, -B, -C). These so-called classical class I MHC molecules are composed of a MHC-encoded transmembrane heavy chain (Mr ~44,000; 44K) noncovalently associated with β2-microglobulin (β2m).1 They present antigens to CTL, due to their capacity to bind peptides from intracellularly degraded proteins, and expose them on the cell surface (1, 2). Moreover, intracellular transport and cell surface expression of classical class I molecules are controlled by the efficient assembly of this trimolecular complex and depend on the availability of high affinity peptides within the endoplasmic reticulum (3–9). Whether or not the less polymorphic nonclassical class I loci (HLA-E [10, 11], -F [12], and -G [13, 14]) also function in antigen presentation in humans is unknown.

Like the classical HLA class I genes, HLA-E, localized between the HLA-C and the HLA-A loci, is highly transcribed in most tissues (10, 11, 15; M. Ulbrecht, manuscript submitted for publication). Conflicting results have been obtained concerning the cell surface expression of the HLA-E-encoded class I heavy chain. Upon transfection of a HLA-E 6.2-kb HindIII subclone of cosmid B1.1 (E*0102) into an HLA class I-negative mutant B-LCL, only an intracellular 41 K heavy chain could be coprecipitated with human β2m (huβ2m) (10, 16). In contrast, the transfer of a 16-kb EcoRI subclone derived from cosmid RSS was reported to result in a detectable cell surface expression when transfected into mouse L cells, which also express huβ2m (17). We recently found low cell surface expression of HLA-E antigen in the mouse myeloma cell line P3X63-Ag8.653 (X63) transfected with cosmid cd3.14 (E*0105) and huβ2m. The discrepancy between the size of the metabolically labeled HLA-E heavy chain (Mr, 41 K) and the size of the iodinated surface HLA-E chain (Mr, 42 K) precipitated from these cells suggests a defect in N-linked glycan maturation. Here we present evidence that the lack of productive cell surface expression of HLA-E is the result of a low stability of the HLA-E molecule during transport and on the cell surface, which may be due to its defective binding of available endogenous peptides.

1 Abbreviations used in this paper: BFA, brefeldin A; β2m, β2-microglobulin; huβ2m, human β2-microglobulin; endo H, endoglycosidase H.
Materials and Methods

Cell Culture. P3X63-Ag8.653 (X63) transfected with either human β2m, human β2m and HLA-E, or human β2m and HLA-B27, as described (M. Ulbrecht, manuscript submitted for publication), was maintained in RPMI 1640 (10% FCS, 1% penicillin/streptomycin; all from Gibco Laboratories, Grand Island, NY) at 37°C in 6% CO2.

10⁶ cells per microtiter plate well were kept for 12 h in 200 μl of RPMI 1640 at either 37, 26, or 37°C in the presence of 12 μg/ml purified human β2m (Sigma Chemical Co., St. Louis, MO).

2 × 10⁶ cells of each fraction were preincubated for 30 min at 37°C in 200 μl of RPMI 1640 containing 10 μg/ml brefeldin A (Sigma Chemical Co.). Cells were then kept for 5 h either at 37°C with or without the addition of 12 μg/ml of purified human β2m, or at 26°C.

Fractions of reverse phase HPLC-separated acid-extracted material were dissolved in RPMI 1640. One-half of each fraction was added to 1.5 × 10⁶ cells of either X63/HLA-E, huβ2m, or X63/HLA-B27, huβ2m transfecants. Incubation was performed for 7 h at 37°C in microtiter plate wells in a total volume of 200 μl of RPMI 1640.

Antibodies. Supernatants of hybridomas producing mAbs specific for human class I, B9.12.1 (IgG2a [18]); human β2m, BBM.1 (IgG2b; American Type Culture Collection, Rockville, MD); purified mAbs specific for human CD4, M-T151 (IgG2a), and M-T242 (IgG2b); and human class I heavy chain, A1.4 (IgG2b [19]; United Biomedical Inc., Hauppauge, NY) were used for immunofluorescence and immunoprecipitation.

Immunofluorescence Staining and FACS Analysis. 1–2 × 10⁶ cells per microtiter plate well were washed twice with ice-cold PBS, then incubated with 40 μl of BBM.1 or B9.12.1 hybridoma supernatant or 40 μl of A1.4 at a concentration of 20 μg/ml for 30 min on ice. Cells were washed thrice with PBS and then incubated with 40 μl dichlorotriazinyl aminofluorescein (DTAF)-conjugated goat anti-mouse Ig-specific antiserum (Dianova) at a concentration of 25 μg/ml for 30 min on ice. After four washes with PBS, samples were fixed with 1% paraformaldehyde in PBS and analyzed on a FACScan flow cytometer (Becton Dickinson & Co., Mountain View, CA). 5,000 viable cells were measured. Mean fluorescence was obtained with logarithmic amplification of fluorescence intensity expressed in channels, where 4 logs span 1,024 channels (256 channels per log). An increase in fluorescence intensity of 160 channels represents a 4.2-fold increase in expression, and 120 channels indicates at 26°C. At the time points indicated aliquots were removed and lysed.

Preparation of Radiolabeled Proteins. For metabolic labeling, cells (1–3 × 10⁶/ml) were preincubated for 1.5 h in methionine-free MEM supplemented with 10% dialyzed FCS (all from Gibco Laboratories) at 37°C. Cells were then pulse labeled with 20 μCi/ml [35S]methionine (>1,000 Ci/mmol; Amersham Corp., Arlington Heights, IL) for 10 min at 37°C and chased in the presence of a 200–400-fold molar excess of cold methionine at 37°C, or where indicated at 26°C. At the time points indicated aliquots were removed and lysed.

Isolation of cell surface molecules was carried out using the lactoperoxidase-glucose oxidase method (20). In brief, 10⁶ cells cultured at either 37 or 26°C for 24 h were washed twice with PBS and labeled in 1 ml of PBS with 5 μl lactoperoxidase (L-0515; Sigma Chemical Co.), 6 μl glucose oxidase type V (Sigma Chemical Co.), and 1 μCi of carrier-free Na125I (17.4 Ci/mg; DuPont Co., Wilmington, DE) in the presence of 0.01 mM KI and 5 mM D-glucose for 20 min at room temperature. Cells were washed five times in PBS/10% FCS and lysed.

Immunoprecipitation and SDS-PAGE. [35S]-Methionine metabolically labeled cells were lysed at 4°C for 30 min in 0.6 ml lysis buffer (1% [wt/vol] NP-40, 2 mM benzamidine HCl, 2 mM PMSF, 2% [vol/vol] aprotinin in PBS). After sedimentation of nuclei and debris at 15,000 g for 15 min at 4°C, lysates were supplemented with 50 μl of FCS and preclared at 4°C one to three times for 12 h with 100 μl horse serum-saturated Sepharose 6B (Pharmacia Fine Chemicals, Piscataway, NJ), one to two times for 2 h with 50 μl of protein A-Sepharose CL-4B (Sigma Chemical Co.), and once for 1 h with 20 μl of isotype control mAb (M-T151 or M-T242). After a last preclaring step with 20 μl of protein A-Sepharose for 1 h, lysates were incubated for 1 h at 4°C with 20 μl of either A1.4- or B9.12.1-protein A-Sepharose. Immunoadsorbents were washed six times in PBS containing 0.2% (wt/vol) NP-40, resuspended in 50 μl of SDS sample buffer (4.2% SDS, 0.1% 2-ME, 18% glycerol), boiled for 3 min, and analysed by SDS-PAGE on 9, 10, or 12% gels. Gels were treated with Amplify (Amersham Corp.), dried, and fluorographed at ~85°C.

For 125I-labeled proteins a modification of this protocol was adopted. After preclaring at 4°C for the first 15 h with 100 μl of horse serum-saturated Sepharose and then for 2 h with 25 μl of protein A-Sepharose, lysates were sequentially immunoprecipitated (1 h at 4°C) using the following reagents: 20 μl isotype control M-T151–protein A-Sepharose, 20 μl of B9.12.1-protein A-Sepharose, 20 μl of isotype control M-T242–protein A-Sepharose, 30 μl of A1.4–protein A-Sepharose, and finally 20 μl of BBM.1–protein A-Sepharose. Between each round lysates were celled 20 μl of protein A-Sepharose for 2 h at 4°C. Further treatment of immunoadsorbents was as described. For analysis, 10 or 12% SDS-PAGE was used.

Endo H Treatment of Radiolabeled Proteins. After washing with PBS containing 0.2% (wt/vol) NP-40, immunoadsorbents were split into two equal aliquots, resuspended in 26 μl of sodium phosphate buffer (50 mM, pH 5.5) supplemented with 0.1 M 2-ME and 0.02% SDS, boiled for 1 min, and then cooled on ice. 1 μl of 200 μM PMSF and 8 μl of endo H (1 mU/μl; Boehringer Mannheim Biochemicals) were added (control samples receiving 8 μl PBS), and digestion was performed at 37°C for 15 h under gentle agitation. Samples were analyzed as described by 10 or 12% SDS-PAGE.

Acid Extraction of Peptides and HPLC Separation. 3 × 10⁶ human β2m/HLA-E–transfected X63 cells kept at 37°C and washed twice with PBS were resuspended in 0.1% (vol/vol) TFA. Extraction was performed as described (21). Lyophilized material was resuspended in 0.1% TFA and subjected to a Sephadex G-25 gel filtration column (Pharmacia Fine Chemicals). Material of M<10 K was collected and dried by vacuum centrifugation. The extract was dissolved in 0.1% TFA and separated by HPLC (LKB Instruments) on a reverse phase column, Hibar LiChrospher (Merck), using (a) 0.1% TFA/H2O, and (b) 0.1% TFA/acetonitrile as a solvent system. A linear gradient from 0 to 70% (vol/vol) of b in a was applied over a period of 60 min at room temperature. The flow rate was adjusted to 1 ml/min. The effluent was monitored at 206 nm. Fractions of 1 min were collected and dried by vacuum centrifugation.

Results

Impaired Intracellular Transport of HLA-E Molecules. Post-translational modifications and intracellular transport of HLA-E and HLA-B27 heavy chains were investigated by pulse chase experiments (Fig. 1). Comparing X63 transfecants expressing only huβ2m, HLA-E and huβ2m, or HLA-B27...
and huβ2m, revealed that the HLA-B27 molecules showed extensive glycan modification during the chase period of 90 min (Fig. 1 a) and expressed predominantly mature (endo H-resistant) glycans at 120 min (Fig. 1 b). In contrast, HLA-E molecules did not increase in size (Fig. 1 a) and remained endo H sensitive (Fig. 1 b). In biosynthetically labeled cells, both HLA-E and HLA-B27 heavy chains were precipitated at least partially complexed with β2m (see Fig. 5), irrespective of the mAb used (B9.12.1 [18] or A1.4, which also recognizes denatured class I heavy chains [19]).

Enhancement of HLA-E Surface Expression at Low Temperature. A defect in carbohydrate side chain processing and intracellular trafficking of classical MHC class I molecules, which has been observed in the absence of peptide ligands, could be compensated for by a reduction in temperature (4, 22). Culturing the X63 transfectants at 26°C resulted in a three- to eightfold increase of human class I-specific cell surface immunofluorescence of the HLA-E transfectant, whereas no effect was observed on the HLA-B27 expression (Fig. 2). Maximum expression was reached after a 4-h culture period and persisted up to 48 h. Labeling of cell surface class I molecules and immunoprecipitation confirmed the increase of HLA-E molecules on the cell surface (Fig. 3). Whereas in metabolically labeled cells a M, 41 K HLA-E heavy chain remained endo H sensitive, HLA-E antigen expressed on the cell surface at either 37 or 26°C was endo H resistant. A polypeptide of M, 37 K coprecipitated with cell surface HLA-E polypeptides and with HLA-B27 heavy chains not complexed with β2m. The origin of this protein is not clear. It might represent a proteolytically shortened class I heavy chain or another, possibly irrelevant molecule coprecipitated with human class I polypeptides.

Enhancement of HLA-E Surface Expression by Exogenous huβ2m. Sequential immunoprecipitation using mAb B9.12.1 followed by A1.4 indicates that the majority of HLA-E and a substantial fraction of HLA-B27 are present on the cell surface of the X63 transfectants as free class I heavy chains, not complexed with β2m (Fig. 3 b). Some HLA-E molecules are complexed with β2m on the cell surface when immunoprecipitated with anti-β2m (BBM.1; Fig. 3 b). Since immunoprecipitation of metabolically and cell surface-labeled cells was performed under the same conditions with concentrated cell lysates, the great reduction of β2m-associated HLA-E polypeptide in cell surface-labeled lysates should not be due to a subsequent enhanced dissociation of the iodinated HLA-E molecule (23).
Exogenous β2m has been shown to stabilize free heavy chains on the cell surface (24). Incubation of the X63 transfectants for 12 h at 37°C in the presence of exogenous huβ2m led to the same increase in HLA-E cell surface expression as observed after 12 h at 26°C (Fig. 2). No increase was detected after a 1-h incubation with huβ2m. An increased level of huβ2m at the cell surface of the HLA-E transfectant was only observed when huβ2m was in the medium. Again, neither treatment affected HLA-B27 cell surface expression. The association of exogenous huβ2m with mouse cells has been described to be rapid (24), reaching saturation after 15 min and reflecting the presence of a large pool of free class I heavy chains on the mouse cells tested. The prolonged time required for enhanced HLA-E expression suggests that free HLA-E is not present on the cell surface in a conformation undetectable by the reagents used, but that HLA-E polypeptides reaching the cell surface are continuously stabilized. This is confirmed by the lack of any increase of HLA-E cell surface expression in the presence of brefeldin A (BFA), which inhibits further intracellular transport (Fig. 4) (4, 25). In contrast, purified mouse β2m did not enhance HLA-E cell surface expression (data not shown).

Low Temperature Stabilizes Mature HLA-E Molecules. To correlate the enhancement of HLA-E surface expression at low temperature with the underlying processes of intracellular transport, transfectants were pulse labeled at 37°C and equal aliquots chased at either 37 or 26°C. Low temperature reduces the turnover both of immature M, 41 K HLA-E heavy chains and HLA-E molecules of M, 42 K with mature glycans, though the effect on the processed form predominates (Fig. 5). The mature M, 42 K form of HLA-E is abundantly detectable when chased at 26°C, whereas only trace amounts precipitate after a 4-h chase at 37°C. This is not a result of an increase in processing rate, since equal amounts of immature HLA-E α chains are precipitated after a 4-h chase at both temperatures. In contrast, reduction of the chase temperature had little influence on the turnover and the oligosaccharide maturation of the HLA-B27 heavy chain. That the residual immature form can be detected after 4 h at 37°C, whereas only a mature product is seen in cells chased at 26°C, might indicate that HLA-B27-linked glycan modification was accelerated at 26°C. At either chase temperature the HLA-E molecules failed to show complete oligosaccharide maturation even after 8 h, implying a reduced rate of intracellular transport. The experiments suggest that after reaching the cell surface at 37°C, the mature form may dissociate from β2m and be rapidly degraded. Since the mAb A1.4 also recognizes denatured HLA class I heavy chains, the turnover of the HLA-E polypeptide at 37°C should not be due to irreversible denaturation. Incubation at 26°C or the presence of exogenous huβ2m stabilizes the conformation of HLA-E and thus inhibits its degradation.

Enhancement of HLA-E Cell Surface Expression by Eluted Peptide Material. The observation that cell surface expression of the HLA-E antigen was only detected upon transfection into X63 cells might indicate that these cells contain a low
Figure 4. BFA inhibits enhancement of cell surface expression of the HLA-E class I molecules at low temperature or by excess huβ2m in the medium. Aliquots of huβ2m+, huβ2m/HLA-E+, huβ2m/HLA-B27+ X63 transfectant clones were preincubated for 30 min at 37°C in the presence of BFA. Cells were then kept for 5 h either at 37°C, with or without addition of 12 μg/ml of purified huβ2m, or at 26°C. Cells were then stained with mAb AL4 by indirect immunofluorescence. Fluorescence intensities are indicated as mean channels.

amount of self-peptides capable of binding to this class I molecule, or that they are permissive to the cell surface transport of empty, otherwise retained, class I molecules. To analyze whether HLA-E has the ability to bind peptides and to identify the corresponding ligands, acid extracts of the X63/huβ2m, HLA-E transfectant were prepared as described, and peptides of M, < 10 K were further fractionated by reverse phase HPLC (21, 26). We reasoned that the small amount

Figure 3. Increase of mature, endo H-resistant surface HLA-E molecules at low temperature. X63 cells expressing huβ2m (a, lanes 5 and 6; b, lanes 2 and 7), HLA-B27/huβ2m (a, lanes 7 and 8; b, lanes 1, 5, 6, and 12), or HLA-E/huβ2m (a, lanes 1–4; b, lanes 3, 4, 8–11, and 13) were kept for 24 h at 26 or 37°C, as indicated, before surface-labeling by iodination and lysis. Lysates were sequentially immunoprecipitated with mAbs B9.12.1 (a, lanes 1–8; b, lanes 12 and 13), A1.4 (a, lanes 5–11), and BBM.1 (b, lanes 1–4). Equal aliquots of B9.12.1 (a) and A1.4 immune complexes (b) were either endo H (+) or mock (−) digested. Analysis was performed by SDS-PAGE on 10% (a) and 12% (b) gels.

Figure 5. Posttranslational modifications of HLA-E α chains induced at low temperature. HLA-B27/huβ2m* (lanes 1–6) or HLA-E/huβ2m* (lanes 7–12) X63 transfectants were cultured and metabolically labeled for 10 min at 37°C. Equal aliquots were chased for the times indicated at either 37 or 26°C, and lysed. HLA class I molecules were immunoprecipitated with mAb B9.12.1 (lanes 2–6 and 8–12) and analyzed by 9% SDS-PAGE. Representative unspecific immunoprecipitates obtained with isotype control M-T151 during preclearing are shown for the 0-min time points (lanes 1 and 7). The upper bands in the HLA class I immunoprecipitates were endo H resistant (data not shown) and represent the mature form of the class I heavy chains. Trace amounts of mature HLA-E molecules after a 240-min chase at 37°C are indicated ( ). A short exposure visualizes oligosaccharide maturation of the HLA-B27 molecules. ( ) Small amounts of immature HLA-B27 molecules present after a 240-min chase at 37°C. To demonstrate co-precipitating β2m, remaining immunoprecipitates were analyzed by 12% SDS-PAGE (bottom). Due to the pipetting scheme, the 0-min time points contained fewer cells.
of β2m-associated mature HLA-E antigen on the cell surface might be peptide ligand associated. Individual peptide fractions were tested for the enhancement of HLA-E or HLA-B27 cell surface expression (Fig. 6). Two fractions (23 and 24) significantly enhanced the HLA class I expression on the HLA-E transfectant clone. None of the fractions had an effect on the HLA-B27 transfectant (data not shown). Adopting the same elution protocol, purified huβ2m eluted from the reverse phase HPLC column in fraction 38.

Discussion

These findings suggest that the impaired expression of the HLA-E antigen may be the result of insufficient binding of peptide ligands (3, 7) or of complexing longer peptides, giving rise to unstable molecules (27). Further experiments are needed to determine whether the extracted ligands were originally peptide ligand associated. Individual peptide fractions were tested for the enhancement of HLA-E or HLA-B27 cell surface expression (Fig. 6). Two fractions (23 and 24) significantly enhanced the HLA class I expression on the HLA-E transfectant clone. None of the fractions had an effect on the HLA-B27 transfectant (data not shown). Adopting the same elution protocol, purified huβ2m eluted from the reverse phase HPLC column in fraction 38.

A specific physiological role for nonclassical MHC class I molecules is only emerging now. In the mouse, such molecules can present synthetic peptides to T cells, and it has been postulated that they display a greater specialization of peptide binding than the classical MHC molecules (37–40). Several reports have suggested that TL region class I gene products might be involved in antigen presentation to γ/δ receptor-bearing T cells (38, 41). The influence of heat-shock proteins on Qa-1 cell surface expression (42), and the finding that several γ/δ T cell lines recognize heat-shock proteins (43), have led to the hypothesis that the TL molecules and other nonclassical MHC class I molecules might, as a primary line of defense, present peptides derived from an endogenous stress-response protein to γ/δ T cells (42). In addition, since they are not saturated with endogenous peptides, they might preferentially bind environmental antigens that can be recognized by the immune system. The observed stabilization of HLA-E class I antigens at reduced temperature might be of physiological significance in the skin, where the temperature is lower than elsewhere in the body.

It is also conceivable that HLA-E serves a more "ancient" function intracellularly as a "modulator" of endoplasmic peptide loading. This may be related to the function of heat-shock proteins (hsp70), in which the hypothetical structure of the peptide binding domain is similar to the MHC an-

Figure 6. Enhancement of HLA-E cell surface expression by eluted peptide material. X63 transfectants expressing either huβ2m and HLA-E, or huβ2m and HLA-B27, were incubated for 7 h at 37°C with 50% of the reverse phase HPLC-separated fractions of acid extracts from X63/HLA-E, huβ2m transfectants, and tested for the cell surface expression of HLA class I heavy chains by flow cytometry using mAb A1.4. (∆ mean channels) Specific fluorescence obtained by subtracting background staining (no first mAb) from A1.4 staining. The number of the HPLC fractions is indicated at the bottom. The two bars at the right show A1.4 binding to cells incubated with medium alone for 7 h at either 37 or 26°C.
tigen binding site (44, 45). Since HLA-E is retained and accumulates in the class I “peptide-loading” compartment, the endoplasmic reticulum, it might influence the spectrum of peptides presented by classical class I molecules through low affinity binding and easy release of peptides, thus modulating the concentration of free peptides available. Finally, HLA-E might be involved in the postulated process of trimming preprocessed peptides to the biologically active length of eight to nine amino acids.

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