

Soluble Nonclassical HLA Generated by the Metalloproteinase Pathway

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ABSTRACT: Soluble human leukocyte antigens (HLA-A, -B, and -C) proteins can be generated by a membrane-bound metalloproteinase (MPase). The MPase-mediated pathway produces soluble nonconformed HLA proteins susceptible to further degradation, and also HLA proteins with high affinity peptides stable at physiologic temperatures. Accessibility of classical HLA to the MPase cleavage inversely correlates with stability of heavy chain (HC) interactions with β_2 -microglobulin (β_2 m). Whether a MPase is involved in release of soluble nonclassical HLA or CD1 proteins is unknown. We have investigated this question with transfectants expressing full-length HLA proteins. Native surface HLA-E and -G complexes, similar to HLA-A2, were unstable at low pH and dissociated giving rise to β_2 m-free HC. Furthermore, HLA-E and -G proteins, similar to HLA-A2, were readily

released from cell surface into supernatants as soluble 37-kilodalton β_2 m-free HC. However, the stability of surface CD1d complexes was not affected by pH changes and no soluble CD1d was detected. Because β_2 m-free CD1d HC were expressed on cells, the lack of cleaved soluble products cannot be explained by high stability of native complexes. Instead, absence of a CD1d-specific MPase in these cells or its impaired interactions with substrate HC may be responsible. *Human Immunology* 64, 802–810 (2003). © American Society for Histocompatibility and Immunogenetics, 2003. Published by Elsevier Inc.

KEYWORDS: soluble nonclassical HLA; CD1d; β_2 -microglobulin; metalloproteinase

ABBREVIATIONS

APC antigen-presenting cell
 β_2 m β_2 -microglobulin
HC heavy chain
HLA human leukocyte antigens
kD kilodalton
mAb monoclonal antibody
MFI mean fluorescence intensities

MHC major histocompatibility complex
MIIC MHC class II compartment
MPase metalloproteinase
PMA phorbol 12-myristate 13-acetate
SDS-PAGE sodium dodecyl sulfate polyacrylamide
 gel electrophoresis

INTRODUCTION

The classical major histocompatibility complex (MHC) class I molecules, or human leukocyte antigens (HLA), are assembled as trimolecular complexes comprised of a 43-kilodalton (kD) highly polymorphic heavy chain (HC) noncovalently associated with antigenic peptide

and a 12-kD β_2 -microglobulin (β_2 m) in the endoplasmic reticulum [1]. The HLA-A, -B, and -C complexes are then transported to the cell surface in widespread tissues and function in presentation of antigens to the CD8⁺ T cells. The best studied proteins in the family of nonclassical HLA are HLA-E and -G molecules, displaying similar assembly and high homology to classical HLA class I [2]. A structurally distinct group of non-MHC encoded CD1 proteins is also assembled in heterodimeric HC/ β_2 m complexes [3].

Tissue expression of these nonclassical HLA class I and CD1 proteins is more restricted in comparison to ubiquitously expressed HLA-A, -B, and -C. HLA-E is expressed in many fetal and adult tissues whereas HLA-G, although confined mainly to fetal placental tissues, is also expressed on thymic epithelial cells and activated

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antigen-presenting cells (APC) during viral infections [4–6]. CD1a-c proteins are found on cortical thymocytes, skin Langerhans cells, dendritic cells, and on a subset of B cells, whereas CD1d proteins are expressed mainly on gastrointestinal epithelium and also on bone marrow derived cells [7, 8]. Certain structural features shared by these proteins, for example, antigen-binding grooves formed by N-terminus domains, broadly determine their functions as antigen-presenting proteins albeit with certain distinctions in comparison to classical HLA class I proteins. Thus, HLA-E complexes can present self and viral peptides and are capable of interactions with T-cell receptors leading to stimulation of specific effector T cells [9–11]. However, HLA-E is a ligand for CD94/NKG2 receptors mediating cytolytic activities of natural killer (NK) cells [12]. Similarly, HLA-G complexes contain bound peptides and can be recognized by specific T cells in animal models [13–15]. HLA-G molecules have also been found to interact with multiple NK cell receptors and their main functions appear to be induction of immune tolerance, which are different from classical antigen-presenting functions of HLA-A, -B, and -C [4, 16, 17]. Finally, mature CD1 proteins can survey various endosomal compartments for lipid and glycolipid bacterial antigens for presentation to specific T-cell subsets (reviewed in [3]).

It was discovered three decades ago that classical HLA class I molecules can be expressed as soluble proteins [18, 19]. Truly soluble HLA proteins are generated by two major mechanisms: alternative splicing of primary mRNA transcripts producing HC which lack transmembrane regions, and proteolytic release of surface HC containing only extracellular domains [20, 21]. The proteolytic processing is mediated by a membrane-bound metalloproteinase (MPase). We have reported previously that native HLA complexes expressed on activated, but not resting, cells spontaneously dissociate and give rise to β_2m -free HC [22]. This process of generation of surface β_2m -free HC remains poorly defined, but it may involve internalization of HLA complexes via clathrin-coated pits followed by their dissociation in endosomal compartments and recycling of β_2m -free HC to the cell surface [22–25]. Surface β_2m -free HC are then cleaved by a MPase, which releases soluble non-conformed HC, accounting for the majority of soluble HLA proteins generated by the MPase pathway. The MPase also releases peptide-conformed HC that can re-associate with β_2m in solution, thus producing soluble HLA complexes stable at physiologic temperatures because they contain high affinity peptides [21, 26, 27]. Alternative splicing of mRNA transcripts generating soluble forms of HLA-G and potentially soluble variants of CD1a, c, and d proteins has also been described [28–31]. However, whether soluble nonclassical HLA class I and CD1 pro-

teins can be generated by the MPase pathway is unknown.

In this article we report that native HLA-E and -G complexes expressed on transfected cells are unstable at low pH and readily dissociate, giving rise to β_2m -free HC. Expression of β_2m -free HLA-E and -G HC on these cells, similar to HLA-A2, is regulated by a membrane-bound MPase that releases soluble HC containing extracellular domains. In contrast, the stability of CD1d complexes is not affected by pH, and no soluble CD1d proteins are released. Nevertheless, β_2m -free CD1d HC are expressed on transfected cells. Lack of their proteolytic processing can reflect absence of a CD1d-specific MPase in these cells or may result from impaired interactions of MPase with its substrate.

MATERIALS AND METHODS

Antibodies and Reagents

The monoclonal antibody (mAb) HCA2 (IgG1) is specific for β_2m -free HLA class I HC [32]; the epitope spanning amino acids 77–84 and recognized by this mAb is also present on HLA-G HC [33]. The mAb W6/32 (IgG2a) recognizes HLA-A, -B, -C, and -G complexes, and mAb BBM.1 (IgG2b) reacts with human β_2m . The mAb DT9 (IgG2b) reacts with HLA-E HC irrespective of their association with β_2m [9], and was kindly provided by Dr. V. Braud (John Radcliffe Hospital, Oxford, United Kingdom). The mAb A1.4 (IgG1) recognizes an ubiquitously expressed nonpolymorphic epitope in the $\alpha 3$ domain of HLA-A, -B, -C HC irrespective of their association with β_2m [34] and data not shown; this mAb also reacts with HLA-E HC [35]. The mAb 75.10.1 (IgG2a) and 51.1.3 (IgG2b) recognize β_2m -free CD1d HC and CD1d complexes, respectively [36]. All mAb were used as purified proteins at 10 $\mu\text{g/ml}$, except for the mAb 75.10.1, which was used at 25 to 50 $\mu\text{g/ml}$.

Phorbol 12-myristate 13-acetate (PMA) was from Sigma Chemical Co. (St. Louis, MO, USA). The hydroxamate derivative and inhibitor of MPases, BB-2116, was a gift from British Biotech Pharmaceuticals Ltd. (Oxford, United Kingdom).

Transfected Cell Lines

A panel of transfected cell lines was originated by introducing MHC class I genes into a parental mutagenized HLA-A, -B, -C-negative 721.221 (.221) lymphoblastoid cell line [37]. Stable HLA-A2 transfectants were produced by electroporation of .221 cells with 25 μg of HLA-A0201 subcloned into pCDNA3 plasmid and selection of positively stained variants with mAb W6/32 in the presence of antibiotic G418 (1.0 mg/ml; Sigma Chemical Co.). Similarly produced HLA-G transfectants

were described elsewhere [16]. The expression of HLA-E was driven by transfection of .221 cells with the HLA-A2 leader/HLA-E mature HC construct [38]. CD1d-containing plasmid (kindly provided by R. Blumberg, Harvard Medical School, Boston, MA, USA) was introduced into .221 cells by electroporation, and positively stained with mAb 51.1.3 transfectants were selected in the presence of antibiotic G418. Parental .221 cell line and subcloned transfectants were maintained in RPMI 1640 medium supplemented with 10% FBS (HyClone Laboratories, Inc., Logan, UT, USA), glutamine and antibiotics.

Immunofluorescence Staining Analysis

For flow cytometric analysis, cells (2×10^5) were washed and incubated for 30 minutes on ice in 100 μ l PBS/1% FBS/10 mM sodium azide (hereafter, washing buffer) plus appropriately diluted mAb. After two washes with cold washing buffer, affinity-purified FITC-conjugated goat antibody to mouse IgG (Zymed Laboratories, Inc., San Francisco, CA, USA) was added for an additional 30 minutes on ice. Cells were then washed, fixed in 2% formaldehyde, and analyzed by flow cytometry with a FACScaliber (BD Biosciences, San Jose, CA, USA). Negative controls included cells incubated with an isotypic control (IgG2a irrelevant mAb) and FITC-conjugated goat antimouse IgG.

pH Treatment of Cells

Native HLA-A, -B, -C complexes on the surface of cells treated with low pH can dissociate [39]. This property of HLA proteins was utilized for probing the stability of HC interactions with β_2m . Stripping of class I HC from bound peptides and β_2m was carried out by resuspending the pellet of washed cells in the pH 3.0 citrate-phosphate buffer (131 mM citric acid/66 mM Na_2HPO_4 /1% [w/v] bovine serum albumin) for 3 minutes on ice followed by washes in RPMI 1640/20 mM HEPES, pH 7.4. Viability of cells was consistently >95%. Treated cells were stained with mAb and prepared for flow cytometric analysis as above. In some experiments, cells were similarly treated with high pH buffer (200 mM Na_2CO_3 /1% [w/v] bovine serum albumin, pH 12.0) followed by washes in RPMI 1640 as above.

Biochemical Analysis

Release of HC from the surface of transfected cells into supernatants was examined with radioiodinated cells. Cells were labeled with 1 mCi of Na^{125}I (ICN Biomedicals, Inc., Irvine, CA, USA) by the lactoperoxidase method [22]. After three washes with cold PBS (pH 7.4), labeled pelleted cells (5×10^5 per sample) were either detergent-solubilized on ice with lysis buffer (0.5%

Nonidet P-40/10 mM iodoacetamide/5 mM EDTA/1 mM phenylmethylsulfonyl fluoride/10 μ M leupeptin and pepstatin A in PBS, pH 8.3) or resuspended in RPMI 1640/1% FBS/20-mM HEPES, pH 7.4 (hereafter, assay medium) for further incubations as described below. For evaluation of cell surface expression, aliquots of cell lysates were precleared on ice with sepharose-protein A beads (Zymed Laboratories, Inc.) precoated with mAb OKT4 (anti-CD4) and rabbit antimouse IgG, and then immunoprecipitated at 4° C with sepharose-protein A beads precoated with anti-MHC class I mAb. In some experiments, pelleted labeled cells were treated at low pH on ice as described above prior to solubilization with detergent and immunoprecipitation with mAb. In experiments evaluating the release of soluble HC by the MPase, labeled cells were incubated for 1 hour at 37° C in assay medium alone, and in the presence of PMA (80 nM) or mixture of PMA and BB-2116 (5 μ M). Supernatants were harvested and precleared on ice with irrelevant mAb and rabbit antimouse IgG as above. Aliquots of precleared supernatants were immunoprecipitated at 4° C with sepharose-protein A beads precoated with anti-MHC class I mAb. Immunoprecipitates were washed with cold lysis buffer, reduced with 2% (vol/vol) 2-mercaptoethanol, and subjected to SDS-PAGE on 14% gels followed by autoradiography.

RESULTS

Native HLA-A2, -E, and -G Complexes Are Unstable at Low pH and Readily Dissociate Although Stability of CD1d Molecules Is Not Affected

Native classical HLA heterodimers are unstable under low pH conditions and give rise to β_2m -free HC, which become susceptible to the MPase-mediated proteolytic cleavage. We have demonstrated previously, for a mutant HLA-A2 allele, that the association of surface HC with β_2m protects them from proteolysis as effectively as the MPase inhibitor BB-2116 [27]. Low pH conditions that alter the stability of classical HLA molecular complexes and result in their reversible dissociation are relevant in some cellular compartments, such as acidic endosomes. Acidic endosomes have been found to contain recycling surface HLA complexes [23, 40]. Internalization and recycling of HLA-G and CD1d complexes have also been reported [41, 42]. We investigated here the stability of native HLA-E, -G and CD1d complexes expressed on the cell surface at physiologic and low pH.

A panel of MHC class I negative 721.221 cells transfected with cDNA encoding for full-length HLA proteins was utilized. Transfectants were evaluated for the expression of surface proteins by flow cytometric analysis after staining with specific mAb recognizing either β_2m -

free or β_2m -complexed HC. As seen in Figure 1, low pH treatment of transfected cells resulted in almost complete dissociation of native HLA-A2 and -G complexes recognized by mAb W6/32 (Figure 1, panels a and c), and also native HLA-E complexes reacting with mAb BBM.1 (Figure 1, panel e). The mAb BBM.1 indeed reacts predominantly, albeit not specifically, with HLA-E on these transfectants as demonstrated by comparison with stained parental nontransfected cells (Figure 1, panel f). In addition, mAb BBM.1 reacted with the 42/12-kD heterodimer in the lysates of radioiodinated HLA-E transfectants but failed to immunoprecipitate this heterodimer from the lysates of CD1d-transfectants (Figures 2B and 2C). Following dissociation of native complexes on the cell surface at low pH, a dramatic increase in the expression levels of β_2m -free HC for both classical HLA-A2 and nonclassical HLA-G was observed, as illustrated by staining with mAb HCA2 (Figure 1, panels b and d). These data were confirmed by biochemical analysis. As characterized for HLA-A2 and HLA-G molecules immunoprecipitated from radioiodinated cell lysates and analyzed by SDS-PAGE, most of native complexes expressed on the cell surface at pH 7.4 and recognized by mAb W6/32 could dissociate at pH 3.0 and generated an abundance of β_2m -free HC reacting with mAb HCA2 (Figures 2A and 2B). Similarly, because the majority of native HLA-E complexes on cells dissociated following the pH shift from physiologic to acidic, less HLA-E molecules were immunoprecipitated by mAb BBM.1. In this case, the pH shift appeared to reduce the overall levels of HLA-E HC immunoprecipitated by both mAb BBM.1 and A1.4. However, the dissociation of HLA-E complexes at low pH was clearly evident because the proportion of the mAb A1.4-reactive HLA-E HC in cell lysates increased significantly over that immunoprecipitated by mAb BBM.1 (Figure 2B).

In contrast, native complexes of another distinct non-MHC encoded protein, CD1d, appeared to be stable at low pH. Thus, the expression levels of CD1d complexes recognized by mAb 51.1.3 were unaltered at pH 7.4 and 3.0 (Figure 1, panel g), and the staining of cells with mAb BBM.1 was only marginally diminished (Figure 1, panel i). This result was corroborated by a marginal increase in staining with mAb 75.10.1, which recognizes β_2m -free CD1d HC (Figure 1, panel h). Relatively high stability of other CD1 family members, namely CD1b and CD1c, demonstrated by lack of dissociation of these complexes in solution at low pH or under high ionic strength conditions has been reported [43, 44]. We have also tested the stability of CD1d complexes expressed on cell surface at high pH. Whereas exposure of cells to pH 12.0 reproducibly increased the expression of β_2m -free HLA-A2 HC by 60% with no significant effect on cell viability, no increase in expression of β_2m -free CD1d

HC was detected (data not shown). Thus, native CD1d complexes expressed in these cells remain stable at both low and high pH.

β_2m -Free HLA-E, -G, and CD1d HC Are Constitutively Expressed on the Cell Surface

β_2m -free HC on the surface of activated cells expressing classical HLA alleles originate from native HLA molecules by yet poorly defined mechanism [22, 25]. Flow cytometric analysis clearly indicated that nonclassical β_2m -free HLA-G and CD1d HC are expressed on the cell surface under physiologic conditions (Figure 1, panels d and h). Biochemical analysis confirmed this conclusion concerning β_2m -free HLA-G and CD1d HC, and also revealed the presence of β_2m -free HLA-E HC recognized by mAb A1.4 on appropriate transfectants (Figures 2B and 2C). Anti-HLA-E mAb DT9 immunoprecipitated a similar radioiodinated 42-kD protein, albeit it reacted weakly with this antigen as compared to mAb A1.4 (data not shown).

In complete agreement with flow cytometric data, low pH treatment did not alter the levels of surface β_2m -free and β_2m -complexed CD1d HC expressed on transfectants and immunoprecipitated from cell lysates with mAb 75.10.1 and 51.1.3, respectively. In addition, similar levels of 48/12-kD heterodimers were recognized by mAb BBM.1 in lysates derived separately from cells treated at physiologic or low pH (Figure 2C). As illustrated here, mAb BBM.1 immunoprecipitates contained considerably less 48-kD HC than mAb 53.1.3 immunoprecipitates. This result may reflect differences in interactions of β_2m with classical HLA class I and CD1 HC [45].

Soluble β_2m -Free HLA-E and -G HC, But Not CD1d, Are Released From Cell Surface by a MPase

Instability of native classical HLA complexes at low pH conditions leads to dissociation of β_2m and appearance of β_2m -free HC on the surface of activated cells. We have reported previously, for cells with endogenous and transfected classical HLA alleles, that expression of β_2m -free HC invariably results in their cleavage mediated by a membrane-bound MPase and subsequent release of soluble HC [21, 22, 26, 27]. Nonclassical HLA-E and -G as well as classical HLA-A2 molecules appeared to have similar patterns of β_2m -free HC expression and stability of native complexes (Figures 1 and 2). However, the CD1d molecules displayed a different pattern. Thus, native CD1d complexes remained stable at low pH, but surprisingly, β_2m -free CD1d HC were clearly expressed on transfectants (Figure 1 [panels g–i] and Figure 2).

We next investigated whether β_2m -free HLA-E, -G, and CD1d HC can be processed by a MPase and released as soluble proteins into cell supernatants. Supernatants

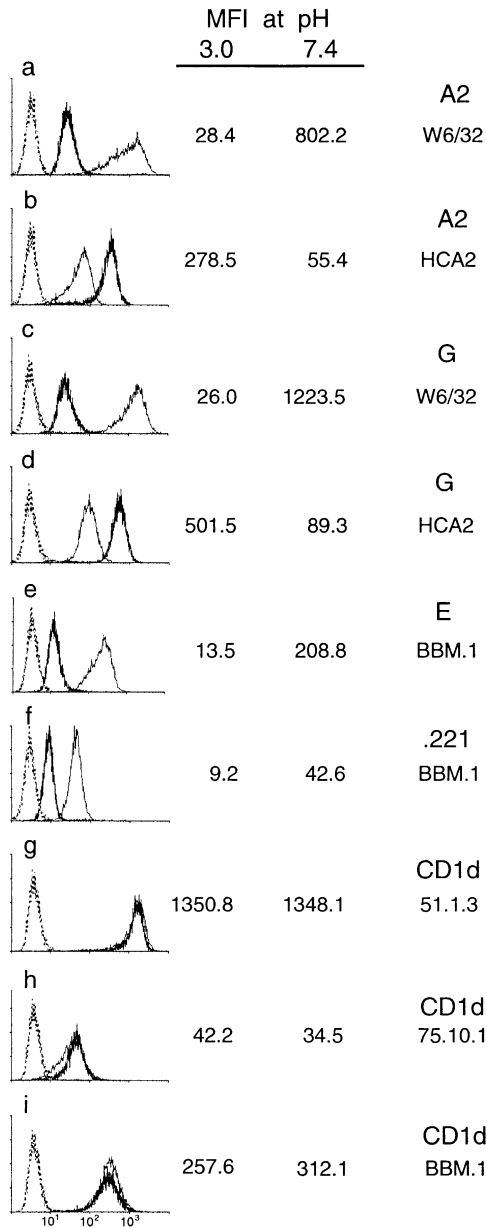


FIGURE 1 Instability of human leukocyte antigen (HLA) class I native complexes at low pH upregulates expression of surface β_2 microglobulin (β_2 m) free heavy chain (HC). Parental (.221) and transfected with HLA class I and CD1d gene constructs cells were either treated at low pH as described in Materials and Methods section or stained directly on ice with anti-MHC class I mAb specific for β_2 m-free or β_2 m-complexed HC and FITC-conjugated goat antimouse IgG followed by flow cytometric analysis. Mean fluorescence intensity (MFI) values for cells stained with specific monoclonal antibodies (mAb) at physiologic pH (light line) and after low pH treatment (bold line) are illustrated. Mean fluorescence intensity (MFI) values for cells expressing HLA-A2, -G, -E, and CD1d proteins, and for parental cells stained with irrelevant mAb at physiologic pH (dotted line) were 3.1, 3.3, 3.4, 4.1, and 3.0, respectively. MFI values for parental cells stained with mAb W6/32, HCA2, 51.1.3, and 75.10.1 were 12.2, 5.3, 3.8, and 4.4, respectively. Data are representative of three experiments.

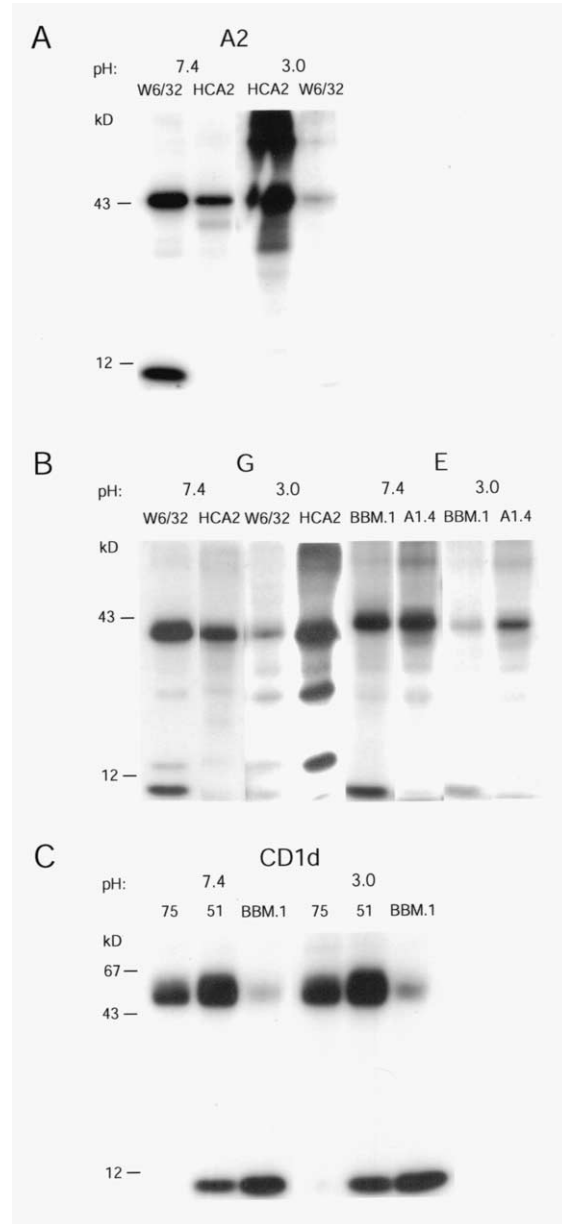
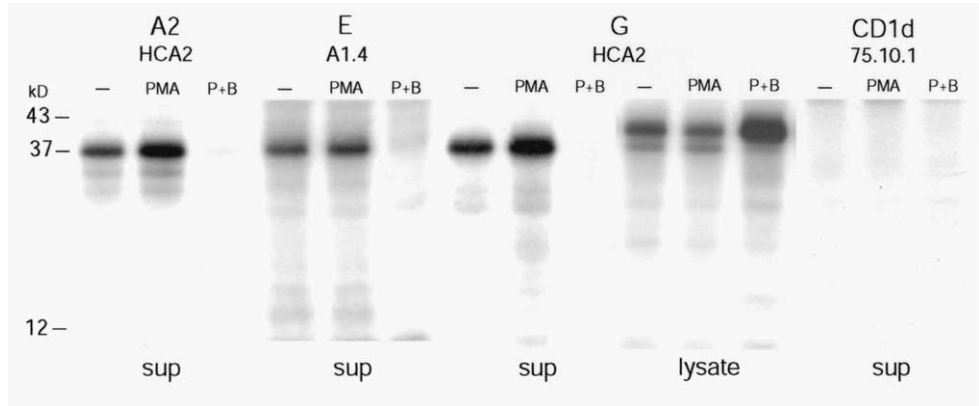


FIGURE 2 Expression of β_2 microglobulin (β_2 m) free and β_2 m-complexed heavy chain (HC) on the surface of transfectants at physiologic and low pH. Lysates derived from radioiodinated human leukocyte antigen (HLA)-A2, -G, -E, and CD1d expressing cells, either untreated (7.4) or treated at low (3.0) pH, were immunoprecipitated with monoclonal antibodies (mAbs) recognizing β_2 m-free or β_2 m-complexed HC followed by SDS-PAGE of immunoprecipitates and autoradiography. The 40-kD β_2 m-free HLA-G HC are susceptible to partial proteolysis at acidic pH as illustrated by the appearance of lower molecular weight bands coimmunoprecipitated with mAb HCA2. The pH 3.0 CD1d-precipitates were obtained from 8×10^5 cells per lane, whereas 5×10^5 cells per lane were used for other precipitates. Positions of molecular weight (kD) markers are indicated.

FIGURE 3 Release of soluble β_2 microglobulin (β_2 m) free heavy chain (HC) by the metalloproteinase (MPase). Radioiodinated human leukocyte antigens (HLA)-A2, -E, -G and CD1d expressing cells were incubated in assay medium alone (–), and in the presence of phorbol 12-myristate 13-acetate (PMA) or a mixture of PMA and BB-2116 (P+B) for 1 hour at 37° C. Supernatants (sup) or cell lysates were then immunoprecipitated with monoclonal antibodies recognizing β_2 m-free HC, and immunoprecipitates analyzed by SDS-PAGE and autoradiography. Positions of molecular weight markers and soluble 37-kD HC are indicated.



were collected from radioiodinated cells and immunoprecipitated with specific mAb. SDS-PAGE analysis of immunoprecipitates revealed the presence of soluble 37-kD β_2 m-free HLA-E and -G HC in supernatants (Figure 3). Incubations of cells with the nonspecific MPase activator PMA increased the release of soluble HC, whereas the MPase inhibitor BB-2116 completely, and invariably, abrogated their release. The efficiency of the MPase-mediated release of soluble 37-kD HC was similar for HLA-A2 and -G transfectants expressing similar levels of surface β_2 m-free HC (Figure 1, panels a and c). As exemplified with HLA-G transfectants, the levels of released soluble 37-kD HC inversely correlated with the levels of 40-kD HC remaining on the surface of cells treated with PMA or a mixture of PMA and BB-2116 (Figure 3). No soluble CD1d proteins recognized by mAb 75.10.1 and 51.1.3 could be detected in supernatants derived from CD1d transfectants (Figure 3, and data not shown).

DISCUSSION

These results clearly demonstrated that soluble nonclassical HLA-E and -G proteins can be released from the surface of transfected cells by the MPase-mediated pathway. Surface HLA-E and -G proteins on these cells displayed a pattern of expression similar to that of classical HLA-A, -B, and -C alleles including instability at low pH resulting in the dissociation of native complexes. Moreover, β_2 m-free HLA-E and -G HC constitutively expressed on the cell surface and not protected by β_2 m from the MPase cleavage were released as soluble 37-kD HC into supernatants. The MPase-mediated release was observed with cells at physiologic and low pH (Figure 3 and data not shown). The question of whether the instability of classical and nonclassical HLA native complexes

at low pH has functional consequences and may be important for their pathways of antigen presentation remains unanswered.

It is intriguing that CD1d complexes displayed high stability at both low and high pH. Surprisingly, 48-kD β_2 m-free CD1d HC were clearly present on the surface of cells. The mechanisms governing their expression and that of classical and nonclassical β_2 m-free HC are likely to be quite different. As yet another distinction from HLA class I proteins, no soluble CD1d proteins, either β_2 m-free or β_2 m-complexed HC, were released from cell surface into supernatants. The most plausible explanations for this finding are the absence of a CD1d-specific MPase or, alternatively, impaired interactions between MPase and substrate HC that may exist in transfected .221 cells. CD1d proteins are involved in unique pathways of antigen presentation, and it is possible that interactions of CD1d HC with this MPase are also different from that found for classical HLA class I.

We have demonstrated previously for HLA-A2 and -B7 alleles that soluble HLA proteins released by the MPase can contain high affinity peptides [27]. These soluble HLA proteins, similar to β_2 m-free HC, originate from native complexes expressed on the cell surface. According to the proposed mechanism, native HLA complexes can dissociate in endosomal compartments with acidic environment giving rise to HC with bound high affinity peptides, which are recycled to the cell surface. However, bound peptides alone cannot protect HC not associated with β_2 m from the cleavage. These HC are released by the MPase, but then re-associate with β_2 m in solution producing soluble HLA proteins containing peptides. The release of soluble HLA proteins is enhanced in the presence of protein kinase C activators including PMA [21, 26, 27]. In a slightly modified scenario, internalized HLA class I molecules, similar to

class II molecules, can exchange and bind peptides in intracellular acidic compartments (*e.g.*, MIIC) prior to recycling with acquired peptides to the surface [46]. If this peptide-loading pathway is involved in release of soluble HLA proteins, it could suggest that the cleavage of HC with bound peptides occurs in MIICs before re-association with β_2m . In addition, this would require the presence of a class I processing MPase in these MIICs, or in a close proximity. Interestingly, a membrane MPase capable of processing several unrelated surface receptors, ADAM17, has been reported to undergo internalization and endocytosis in response to activation by PMA [47].

Soluble HLA proteins with bound peptides released by this MPase-mediated pathway may function in presentation of high affinity peptides. A requirement for high affinity peptides is necessary for HC to retain their conformations following β_2m dissociation and allow for cleavage in the MPase pathway [26]. Generation of soluble HLA proteins by alternative splicing is unlikely to have such requirement regarding the affinity of peptides complexed with HC and β_2m . The latter conclusion is suggested by data obtained with cells expressing soluble HLA-A2- and -B7 encoding constructs; endogenous peptides recovered from soluble HLA complexes were similar to the peptides that bind surface class I molecules [48]. It remains to be established whether soluble HLA proteins generated by two distinct pathways, *i.e.*, MPase-mediated and alternative splicing, can present distinct repertoires of antigenic peptides.

The MPase-mediated release of soluble classical HLA-A, -B, -C, and nonclassical HLA-E and -G proteins is consistent with their roles as mediators of immunoregulation in transplantation. As proposed originally for classical HLA class I, soluble β_2m -free and β_2m -complexed HC may have different functions because they are likely to interact with different sets of surface receptors on antigen-presenting and effector cells [26, 27]. Soluble nonclassical HLA-E and -G proteins, similar to their membrane-bound counterparts, may target yet another set of receptors expressed on immune cells.

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