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# The CD85J/Leukocyte Inhibitory Receptor-1 Distinguishes between Conformed and $\beta_2$-Microglobulin-Free HLA-G Molecules[1]

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**For a proper development of the placenta, maternal NK cells should not attack the fetal extravillous cytotrophoblast cells. This inhibition of maternal NK cells is partially mediated via the nonclassical MHC class I molecule HLA-G. Recently, we demonstrated that HLA-G forms disulfide-linked high molecular complexes on the surface of transfected cells. In the present study, we demonstrate that HLA-G must associate with $\beta_2$m for its interaction with CD85J/leukocyte Ig-like receptor-1 (LIR-1). Although HLA-G free H chain complexes are expressed on the surface, they are not recognized and possibly interfere with CD85J/LIR-1 and HLA-G interaction. The formation of these complexes on the cell surface might represent a novel mechanism developed specifically by the HLA-G protein aimed to control the efficiency of the CD85J/LIR-1-mediated inhibition. We also show that endogenous HLA-G complexes are expressed on the cell surface. These findings provide novel insights into the delicate interaction between extravillous cytotrophoblast cells and NK cells in the decidua.** ***The Journal of Immunology,*** **2005, 175: 4866–4874.**

Human pregnancy presents a unique immune challenge; although implantation of the fetal semiallograft in the uterine wall exposes it to the potentially harmful maternal immune system, the fetus succeeds to survive. The mechanisms by which the fetus escapes immune detection remain enigmatic. One of the major characteristics of the human decidua is the abundant recruitment of maternal NK cells at the site of implantation. These specialized NK cells (termed decidual NK cells) are mostly characterized by the $CD56^{bright}$,$CD16^{-}$ phenotype, whereas most (~90%) of NK cells in the PBL are $CD56^{dim}$, $CD16^{+}$ (1). These different phenotypes are largely reflected in the diverse set of proteins that each of these NK subsets express as well as in other features such as their cytotoxic abilities and cytokine responsiveness (2, 3). The reasons why the decidual NK cells are so different from NK found in PBL are unknown. One possible explanation might be the constant interaction with the special tissue they inhabit (4). In this area of potential maternal-fetal immunological interaction, several mechanisms have evolved to protect the thriving fetus from maternal immune attack (4–6). A key role in maternal tolerance of the fetus has been attributed to the differential expression of MHC class I molecules on trophoblast cells. The extravillous cytotrophoblast (EVT)[3] cells, which are present in direct contact with maternal NK cells, express only small amounts of the classical MHC class I molecules (HLA-A and HLA-B) in the first trimester (reviewed in Ref. 4). In this way, the fetus probably evades maternal T cell-mediated allorecognition (7). However, to prevent decidual NK cell attack, trophoblast cells express the nonclassical class I MHC molecules HLA-G (8), HLA-E (9), CD1d (10), and the classical class I MHC molecule HLA-C (11).

The HLA-G molecule is characterized by tissue-restricted distribution, low allelic polymorphism (12, 13), and seven alternative-spliced isoforms (HLA-G 1–7) (14). It exerts its immunotolerant abilities by interacting with three receptors: CD85J/leukocyte Ig-like receptor-1 (LIR-1) (ILT2), LIR-2 (ILT4/CD85d), and KIR2DL4 (CD158d), although data regarding this receptor are controversial (15–18).

The ability of HLA-G to inhibit lysis of trophoblast cells by NK cells in vivo is a subject of debate as trophoblast cells are very resistant to NK cell lysis independently of MHC class I expression (19). However, HLA-G was shown to modulate the immune system in various manners. Indirect evidence suggests that HLA-G is able to inhibit NK cell cytotoxicity, allogeneic proliferation of T cells, Ag-specific T cell cytotoxicity, and macrophages and mononuclear cell activity (20–22). Therefore, it is possible that in vivo HLA-G might have additional effects other than inhibition of cytotoxicity. For example, HLA-G might play a role in modulation of cytokine secretion to control trophoblast invasion by interacting with the inhibitory receptors. Another possible role of HLA-G in trophoblasts may be to provide nonamer peptide to the HLA-E molecules, which is the ligand for the CD94/NKG2A receptor. Such trophoblastic HLA-E was shown to interact with its receptor present on decidual NK cells (4).

The full-length HLA-G1 protein is composed of H chain, associated $\beta_2$-microglobulin ($\beta_2$m) and nonameric peptide similar to the classical MHC class I structure (23). However, there are

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[3] Abbreviations used in this paper: EVT, extravillous cytotrophoblast; $\beta_2$m, $\beta_2$-microglobulin; LIR-1, leukocyte Ig-like receptor-1; FHC, free H chain.



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HLA-G free H chains (FHCs), which are probably formed by dissociation of $\beta_2$m from the conformed HLA-G protein (24, 25). We and others have demonstrated previously that the HLA-G1 protein is expressed on the cell surface in a unique pattern of disulfide-linked oligomers (26–28). This organization probably increases the avidity of the binding between the NK inhibitory receptor CD85J/LIR-1 and the HLA-G protein, thus enables an efficient inhibition of NK cell cytotoxicity

In this study, we demonstrate for the first time that HLA-G FHC complexes are present on the cell surface; however, they are not important for CD85J/LIR-1 recognition. Importantly, we show that the presence of these complexes or other complexes composed of FHC and conformed HLA-G possibly interrupts with the binding of CD85J/LIR-1 to the HLA-G protein. In addition, we show here the existence of endogenous HLA-G complexes on the cell surface. In summary, our findings further emphasize the uniqueness of the HLA-G protein, demonstrate the nature of the HLA-G complexes on the cell surface, and suggest a possible regulatory mechanism by which the HLA-G protein can modulate the inhibitory effect of the CD85J/LIR-1 receptor.

## Materials and Methods

### *Cells, mAbs, and fusion protein*

The cell lines used in this work are the human B lymphoblastoid MHC class I negative cell line 721.221, 721.221 transfectants (described in Ref. 26), and the human choriocarcinoma cell line Jeg3. Primary NK cells were isolated from PBLs using the human NK cell isolation kit and the autoMACS instrument (Miltenyi Biotec). NK cells were kept in culture as described previously (21).

All mAbs used in this work were generated in mice, including W6/32 anti-mAb (IgG2a), directed against class I MHC molecules, HLA-G mAbs, MEM-G/09 and MEM-G/01, and anti-conformed HLA-E mAbs, MEM-E/08 and MEM-E/06 (all of which are IgG1, produced and characterized in the Prague laboratory), mouse monoclonal HLA-G Ab 4H84 (8) was kindly provided by M. McMaster (University of California, San Francisco, CA), and HCA2 mAb (IgG1) directed against HLA-G and -A FHC (29), HC10 (IgG2a) directed against HLA-B and -C FHC (30, 31), and anti-CD85J/LIR-1 mAb- HPF1 (IgG1; described in Refs. 18 and 32). The production of CD85J/LIR-1 Ig fusion protein by COS-7 cells, its purification on protein G column, and the FACS analysis for its expression were performed as described previously (33).

### *Cell treatments*

Acid treatment was performed by resuspending $1 \times 10^6$ cells with 50 $\mu$l of 1% BSA 300 mM glycine-HCl buffer (pH 2.4) for 2 min at room temperature. The suspension was neutralized by adding 25 ml of RPMI 1640 medium. Protease papain treatment was performed by incubating $1 \times 10^6$/2 ml cells with 0.6 mg of papain from *Carica papaya* (Roche) for 1 h at 37°C in RPMI 1640/10% FCS medium. The suspension was neutralized by adding 100 $\mu$l of FCS and then three washes with RPMI 1640/10% FCS were performed to remove the protease.

### *Cytotoxicity assay*

The cytotoxic activity of NK cells against the various target cells was assessed in 5-h $^{35}$S release assays as described previously (21). In experiments in which mAbs were included, the final mAb concentration was 5 $\mu$g/ml. In all of the presented cytotoxic assays, the spontaneous release was <25% of maximal release.

### *Immunoprecipitation, two-dimensional SDS-PAGE, and Western blot analysis*

721.221-transfected cells with or without the treatments described above were washed four times with cold PBS containing 1 mM $MgCl_2$ and 0.1 mM $CaCl_2$ and then biotinylated with EZ-Link Sulfo-NHS-LC-Biotin (Pierce) for 30 min at 4°C. Cells were washed four times to remove unconjugated reagent and detergent-solubilized on ice in lysis buffer (PBS containing 150 mM NaCl, 50 mM Tris (pH 7.6), 0.5% Nonidet P-40, 9 mM iodoacetamide, 5 mM EDTA, 1:100 aprotinin (Sigma-Aldrich), and 1 mM PMSF). Cell lysates were precleared overnight at 4°C with protein A-Sepharose beads (Zymed Laboratories) that were previously precoated with rabbit anti-mouse IgG. Precleared lysates were then immunoprecipitated for 4 h at 4°C with MEM-G/09 or HCA2 mAbs, followed by protein A-Sepharose beads incubation for 4 h at 4°C. The immunoprecipitates were washed with lysis buffer, and biotinylated proteins were eluted in the presence of SDS under nonreducing conditions. The nonreduced samples were subjected to two-dimensional SDS-PAGE with reduced and nonreduced dimensions. The blotted biotinylated proteins were visualized by streptavidin-HRP conjugate (Amersham Biosciences), and HLA-G proteins were specifically detected by MEM-G/01 mAb, followed by goat anti-mouse Ig HRP (Sigma-Aldrich) using ECL detection.

Jeg3 cells were biotinylated as described above. Cells were washed four times to remove unconjugated reagent and then incubated with MEM-G/09 mAb overnight at 4°C. Cells ($80 \times 10^6$/sample) attached to mAb were detergent solubilized on ice in lysis buffer (as above), and the samples went through the same subsequent procedures as the 721.221-transfected cells.

### *Immunohistochemistry*

Immunohistochemistry was performed on 5-$\mu$m formalin-fixed, paraffin-embedded sections as described in Ref. 34 with few changes. Briefly, sections were dewaxed and rehydrated ending in water. Microwave Ag retrieval was performed in citrate buffer. Endogenous peroxidase was quenched with $H_2O_2$ treatment for 5 min. Blocking was for 15 min with CAS-block buffer (Zymed Laboratories). Sections were then incubated overnight at 4°C with the primary Ab at a concentration of 1:50 for HLA-G (4H84) and 1:25 for HCA2. The sections were then washed in 1× PBS and incubated with the secondary Ab, DakoCytomation envision system, and labeled polymer HRP (DakoCytomation), according to the manufacturer's instructions. Enzymatic color development was with the Zymed 3-amino-9-ethylcarbazole chromogen/substrate single solution for HRP, which produces a red color. Slides were counterstained with hematoxylin and mounted with Aqueous Mounting Solution (Zymed Laboratories).

## Results

### *HLA-G oligomers are naturally present in Jeg3 cells*

We have demonstrated previously that HLA-G is expressed on the cell surface in a unique organization of disulfide-linked high molecular complexes (26, 27). As these findings were generated in transfected cells, we now investigated whether the unique form of HLA-G expression would also be observed in a cell line that endogenously expresses the HLA-G protein, such as in Jeg3 cells (35). We addressed this question by immunoprecipitations of the HLA-G protein from Jeg3 cell line, followed by two-dimensional SDS-PAGE (reduced and nonreduced dimensions). The blotted biotinylated proteins were visualized by streptavidin-HRP conjugate (data not shown), and HLA-G proteins were specifically detected by MEM-G/01 mAb. Fig. 1 demonstrates the expression of the HLA-G protein on the cell surface as monomers, dimers, and trimers (40, 80, and 120 kDa respectively, marked by the arrows). However, there are other complexes of intermediate molecular masses (60 and 90 kDa) that may imply for the presence of other proteins connected to the HLA-G via disulfide bonds or for degradation of the complexes. The complexes observed ~50 and 25 kDa are degradation products of the mAb. Thus, we describe here for the first time the existence of endogenous HLA-G complexes in Jeg3 cells.

### *CD85J/LIR-1 Ig does not bind $\beta_2$m-free HLA-G molecules on the cell surface*

The HLA-G molecule is present on the cell surface in a ternary complex of H chain, associated peptide, and L chain $\beta_2$m. However, there are small amounts of HLA-G $\beta_2$m FHCs on the cell surface believed to be dissociated from the mature fully conformed protein (Fig. 2*A*, *middle panel*, and Refs. 24 and 36). Our first goal was to determine whether CD85J/LIR-1 would interact with HLA-G FHC. Increased amounts of FHC proteins can be observed on the cell surface after acid treatment, which releases the peptide from the binding groove initiating $\beta_2$m dissociation (37). To assess the possible interaction of HLA-G FHC with the CD85J/LIR-1 receptor, we treated 721.221-transfected cells with wild-type or

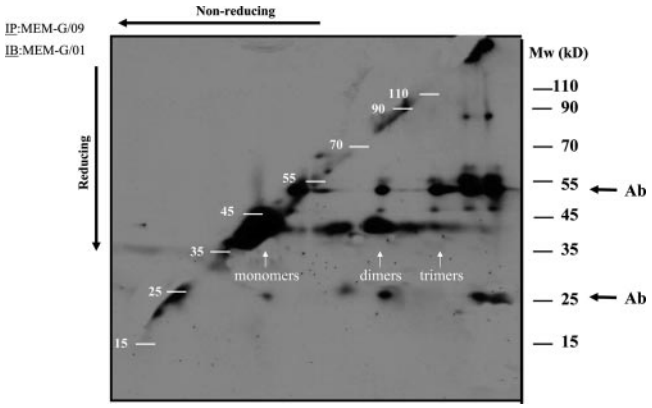


**FIGURE 1.** HLA-G in Jeg3 cells is found in high molecular complexes on the cell surface. Jeg3 cells were surface biotinylated, lysed, and immunoprecipitated using the conformed anti HLA-G mAb, MEM-G/09. The immunoprecipitates were analyzed by two-dimensional SDS-PAGE followed by electroblotting and visualization of biotinylated proteins by streptavidin-HRP (data not shown). To confirm that the precipitates are indeed HLA-G molecules, the blots were then stripped and restained with MEM-G/01 followed by goat anti-mouse Ig HRP conjugate. The HLA-G complexes appear in the region ~40 kDa. The complexes ~50 and 25 kDa are degradation products of the mAb. Figure shows a representative experiment of two performed.

mutated HLA-G and HLA-A2 with glycine-HCl buffer as described in *Materials and Methods*. As Fig. 2*A* demonstrates, the HLA-G protein is expressed on the cell surface of the indicated cell lines mainly as the $\beta_2$m-associated molecule, which is evidenced by the staining with the conformational-dependent anti MHC class I mAb W6/32 (Fig. 2*A*, *left panel*). However, HLA-G FHCs are present only in small amounts as evident from the anti-class I FHC mAb staining HCA2 (Fig. 2*A*, *middle panel*).

Remarkably, acid treatment of the cells resulted in a marked reduction in the expression of the conformed HLA-G molecules and a notable increase in HLA-G FHC expression (Fig. 2*A*, *left* and *middle panel*). Acid treatment of the cells did not affect the expression of HLA-E (Fig. 2*A*, *right panel*). Furthermore, increased expression of HLA-E was observed in the HLA-G C42S mutant. We do not completely understand why we observed increased staining after acid treatment only to this protein.

As was previously reported, strong CD85J/LIR-1 Ig binding to the HLA-G protein was observed, and this strong binding was reduced when the HLA-G mutants were used (Fig. 2*B*). Importantly, acid treatment abolished the CD85J/LIR-1 Ig binding to all cells tested (Fig. 2*B*). These results indicate that HLA-G FHC on the cell surface cannot be recognized by CD85J/LIR-1. To generalize our findings, we tested whether this pattern of CD85J/LIR-1 recognition could be reproduced for other classical MHC class I molecules, which are recognized by CD85J/LIR-1 such as HLA-A2 (38). Indeed, similar to the staining of the HLA-G transfectants, the HLA-A2 molecule demonstrates mainly the expression of conformed molecules on the surface and only small expression of FHC (Fig. 2*A*, *left* and *middle panel*), while acid treatment reverses this pattern of expression. In addition, the small binding of CD85J/LIR-1 Ig to the HLA-A2 molecule was abrogated following acid treatment analogous to the HLA-G molecule (Fig. 2*B*).

The HLA-G oligomers might be present on the cell surface in a complex composed of FHC and conformed HLA-G proteins, and this complex might be the one that is recognized by CD85J/LIR-1. To determine whether only the conformed HLA-G molecule is able to interact with CD85J/LIR-1, we next treated the cells with papain, which was shown previously to strip $\beta_2$m-free MHC class I molecules from the cell surface (39). Fig. 3*A* shows that following papain treatment, W6/32 staining of the 721.221 transfectants is retained (Fig. 3*A*, *left panel*), while surface-HCA2 reactive HLA-G FHC are largely removed (Fig. 3*A*, *middle panel*). Surprisingly, increased binding of the CD85J/LIR-1 Ig to papain-treated, wild-type, and mutated 721.221/HLA-G transfectants was observed (Fig. 3*B*). As above, 721.221/HLA-A2 cells demonstrated a similar behavior. Thus, it is clear that HLA-G FHCs are not recognized by CD85J/LIR-1 either alone (Fig. 2) or in complexes with conformed HLA-G protein. Similarly to the acid treatment, the HLA-E expression was not markedly changed after papain treatment (Fig. 3*A*, *right panel*). As it is apparent from Figs. 2*A* and 3*A*, there are changes in the expression level of HLA-G FHC between different experiments. Because both experiments were conducted on the same cell line, these changes probably result from the cell's condition and from the level of conformed HLA-G expression, which influence the amount of HLA-G FHC.

### *CD85J/LIR-1 mediated inhibition of NK killing through the HLA-G molecule is abolished following acid treatment but increased after papain treatment*

The functional relevance of the above observations, suggesting that HLA-G FHC molecules are not recognized by the CD85J/LIR-1 receptor, was tested using NK cytotoxicity assays. NK clones expressing the CD85J/LIR-1 receptor were tested in killing assays against the 721.221 transfectants with or without the indicated treatments. A representative NK clone is shown in Fig. 4*A*. As was reported previously (26), lysis of 721.221/HLA-G cells is reduced compared with lysis of 721.221 cells due to an inhibitory interaction with the CD85J/LIR-1 receptor (Fig. 4*B*). Preincubation of the target cells with anti-MHC class I mAb W6/32 restored NK killing, indicating for HLA-G involvement in the inhibition (Fig. 4*B*). No significant change in the lysis of the 721.221/HLA-G transfectants was observed when the isotype match control HC10 mAb was used (Fig. 4*A*). As we have demonstrated previously (Ref. 26 and Fig. 4*B*), inhibition of NK killing by HLA-G was markedly reduced when the mutated HLA-G transfectants are used. The lack of inhibition by the HLA-G mutants is probably due to the reduction in the CD85J/LIR-1 binding avidity to the mutated HLA-G protein. Despite the fact that the HLA-G mutants are recognized to a lesser extant than HLA-G by CD85J/LIR-1 (Ref. 26 and Figs. 2*B* and 3*B*), this recognition is not strong enough to confer an efficient protection. Similarly, it is not surprising to see that the 721.221/HLA-A2 transfectant, which is inefficiently recognized by CD85J/LIR-1 (Fig. 4*B*), could not confer protection (Figs. 2*B* and 3*B*). In agreement with the binding experiments, subjecting the various 721.221 transfectants to mild acid treatment significantly reduced the inhibitory effect mediated through the HLA-G protein (Fig. 4*C*). Furthermore and in agreement with the binding results, papain treatment did not abolish the inhibition of the 721.221/HLA-G transfectants but rather increased the HLA-G-mediated inhibition (Fig. 4*C*). This inhibition was the result of HLA-G interaction with CD85J/LIR-1 as killing was partially restored when W6/32 was included in the assay (data not shown). Moreover, increased CD85J/LIR-1-mediated inhibition was also observed with the HLA-G mutants after papain treatment. The inhibition of the HLA-G mutants was less efficient than that of the wild-type HLA-G, and killing was fully restored when W6/32 was included in the assay (data not shown). Although the different cell transfectants express the HLA-E molecule (Figs. 2*A* and 3*A*) and some of the NK clones tested express the CD94/NKG2A complex (data not shown), it seems as if the HLA-E protein and the CD94/

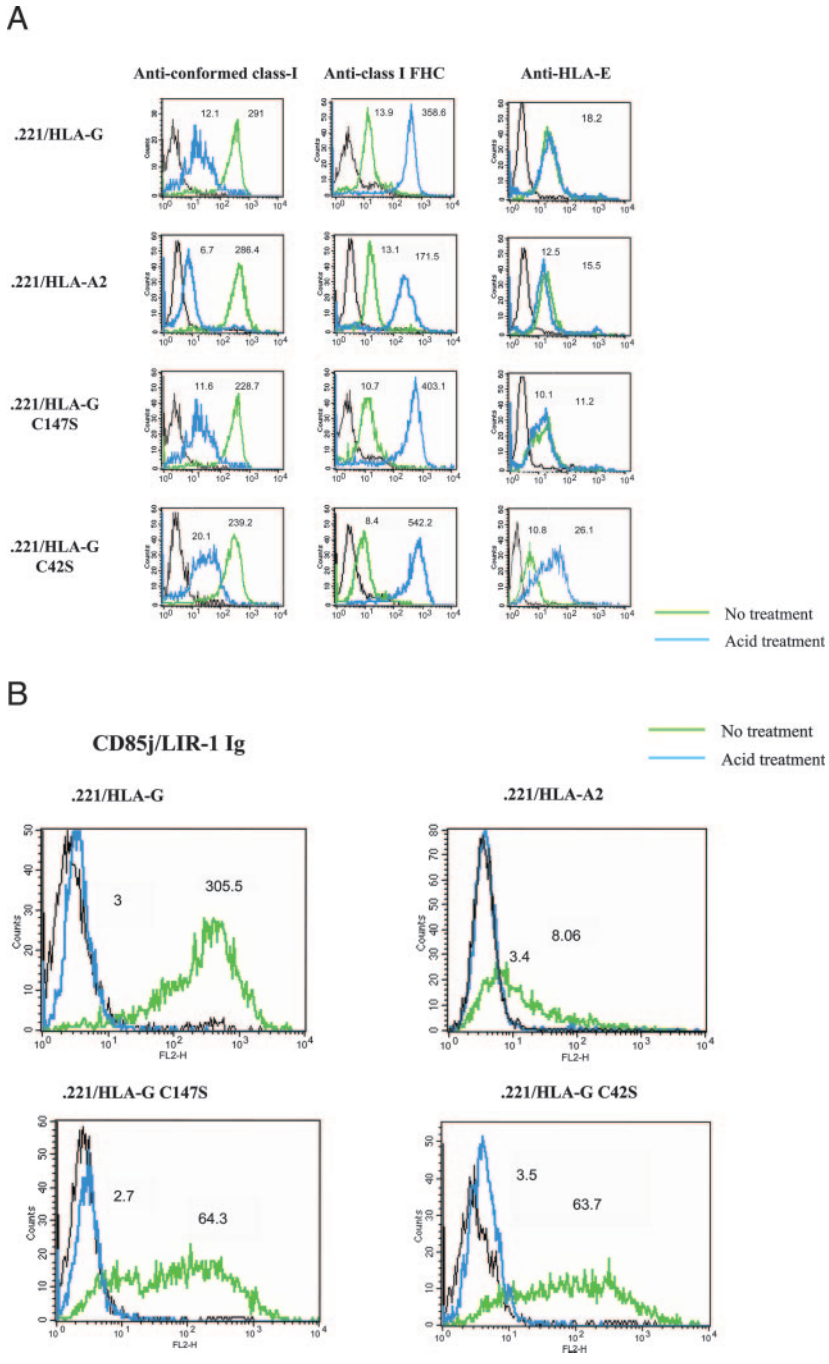


**FIGURE 2.** HLA-G-FHC on the cell surface are not recognized by CD85J/LIR-1. *A*, 721.221 cells expressing the wild-type, mutated HLA-G molecules, and the HLA-A2 were subjected (blue line) or not (green line) to mild acid treatment and stained with the conformed anti-MHC class I mAb (W6/32) (*left panel*), anti-class I-FHC mAb (HCA2) (*middle panel*), and anti-HLA-E mAbs (MEM-E/06 or MEM-E/08) (*right panel*). Figure shows a representative experiment of five performed. *B*, 721.221 cells expressing the wild-type, mutated HLA-G molecules, and the HLA-A2 were subjected (blue line) or not (green line) to mild acid treatment and stained with CD85J/LIR-1 Ig. Figure shows a representative experiment out of five performed. The background staining (black thin line) represents staining of the untreated cells with the second reagent only. This background staining was not changed after the acid treatment.

NKG2A receptor are not involved in inhibition of NK lysis in our experiments. This is probably due to relatively low expression of HLA-E in comparison to the high level of HLA-G expression. Indeed, blocking of the HLA-G molecule with W6/32 mAb reversed the inhibition almost completely (Fig. 4). Importantly, lysis of 721.221 cells was not affected by the papain treatment, indicating that this treatment have a specific effect related to the MHC class I proteins. The elevation in the HLA-G killing after acid treatment and the increase in HLA-G-mediated inhibition after papain treatment were statistically significant ($p < 0.05$).

### *HLA-G FHC complexes are present on the cell surface*

We next tested whether HLA-G oligomers can be found on the cell surface also in a form of $\beta_2$m-free complexes. This question was addressed by immunoprecipitation of the wild-type and mutated 721.221/HLA-G-transfected cells with the anti-class I-FHC mAb HCA2, with or without acid treatment. The cells were surface biotinylated, lysed in the presence of iodoacetamide (to prevent postlysis disulfide bond formation), and then immunoprecipitated as described in *Materials and Methods*. The precipitates were subjected to two-dimensional gel with nonreduced and reduced dimensions followed by electroblotting and visualization of biotinylated proteins by streptavidin-HRP (Fig. 5*A*, *right panel*). To confirm that the precipitates are indeed HLA-G molecules, the blots were then stripped and stained with anti-HLA-G mAb MEM-G/01 (Fig. 5*A*, *left panel*). As shown in Fig. 5*A*, the small amount of native HLA-G FHC molecules present on the cell surface are arranged in a form of monomers, dimers, and trimers (40, 80, and 120 kDa, respectively). The other observed complexes might be degradation products or complexes of HLA-G with other proteins.

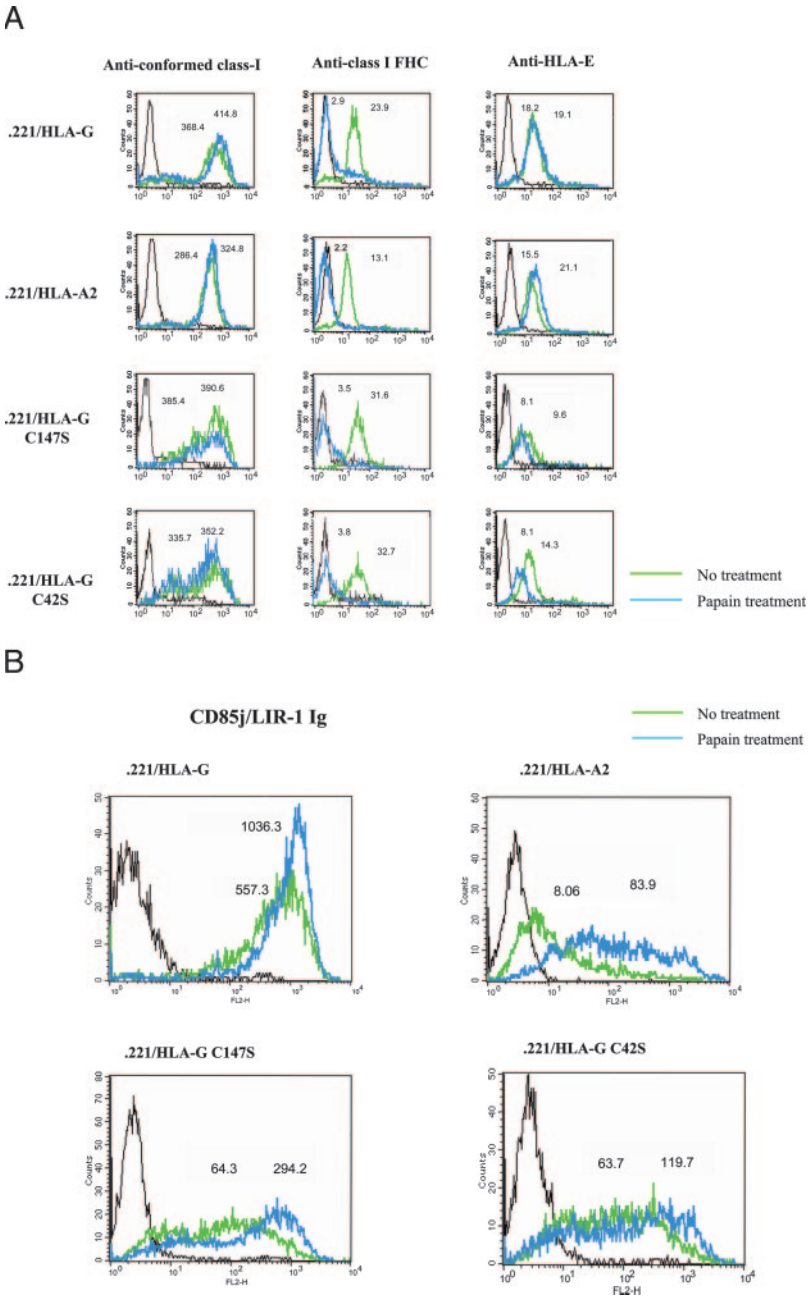


**FIGURE 3.** Removal of HLA-G-FHCs by papain treatment increase CD85J/LIR-1 Ig binding. *A*, 721.221 cells expressing the wild-type, the mutated HLA-G molecules, and HLA-A2 were subjected (blue line) or not (green line) to papain treatment and stained with conformed anti MHC class I mAb (W6/32) (*left panel*), anti-class I-FHC mAb (HCA2) (*middle panel*), and anti HLA-E mAbs (MEM-E/06 or MEM-E/08) (*right panel*). Figure shows a representative experiment of five performed. *B*, 721.221 cells expressing the wild-type, mutated HLA-G molecules, and the HLA-A2 were subjected (blue line) or not (green line) to papain treatment and stained with CD85J/LIR-1 Ig. Figure shows a representative experiment of five performed. The background staining (black thin line) represents the untreated cells stained with the second reagent only. This background staining was not changed after the papain treatment.

Similar to the conformed HLA-G protein (26), $Cys^{42}$ also plays a crucial role in the formation of the HLA-G FHC complexes because no complexes could be observed when $Cys^{42}$ was mutated, whereas monomers and dimers of HLA-G FHC were observed when $Cys^{147}$ was mutated (Fig. 4*A*). To determine whether the HLA-G FHC molecules formed after acid treatment are also arranged in high molecular complexes, we immunoprecipitated the various transfectants after acid treatment. In accordance with the FACS staining (Fig. 2*A*, *middle panel*), the amount of HLA-G FHC molecules increased dramatically after treating the cells with acid as evident from the large quantities of HLA-G proteins precipitated (Fig. 5*B*). As above, the HLA-G FHC proteins are organized in a structure of HLA-G oligomers composed of monomers, dimers, and trimers, even after acid treatment (Fig. 5*B*). Similarly, mutation of $Cys^{147}$ resulted in the formation of HLA-G FHC molecules in a pattern of monomers and dimers (Fig. 5*B*). However, surprisingly, in the acid-treated $Cys^{42}$ mutant, the HLA-G FHC molecules are expressed as monomers and dimers, opposite both to the conformed $Cys^{42}$ mutant HLA-G molecules and to the $Cys^{42}$ mutant FHC proteins, where there is an expression of HLA-G monomers only (Ref. 26 and Fig. 5*A*, respectively). This pattern of expression implies that the $Cys^{147}$ residue, which was not accessible for interacting with another $Cys^{147}$ in the conformed and HLA-G FHC proteins, is now, after acid treatment, free to interact with another $Cys^{147}$ residue for the formation of the dimers. Additional complexes were precipitated after acid treatment (Fig. 5*B*, *right middle panel*). The nature of these complexes is not completely understood because these additional complexes were not recognized by the anti-HLA-G mAb (Fig. 5*B*, *left middle panel*).

It is important to note that although we performed the immunoprecipitations with the anti-HLA-G FHC mAb HCA2, it is still possible that conformed HLA-G molecules are also found in complexes with the HLA-G FHC.

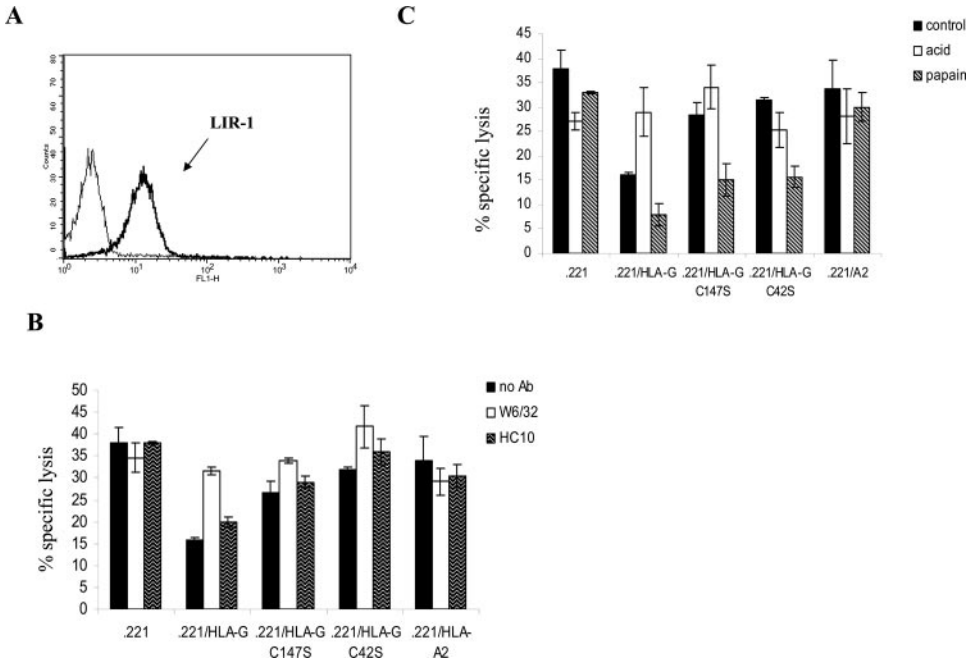


**FIGURE 4.** Inhibition of NK killing mediated through the HLA-G molecule is abolished following acid treatment and increased after papain treatment. *A*, FACS analysis of CD85J/LIR-1 expression on a representative NK clone using anti-CD85J/LIR-1 Ab HPF1. The background staining represents the cells stained with the second reagent only. *B*, Killing assays of various 721.221 transfectants with or without W6/32 mAb and the isotype match control HC10 mAb. *C*, Killing assay of various 721.221 transfectants without (data are obtained from *B*) or after acid or papain treatment. The E:T used was 4:1. Error bars represent mean ± SD of duplicate samples. Value of $p < 0.05$ compared between all controls and all treatments (paired *t* test). Figure shows a representative experiment of four performed.

Therefore, we next determined whether removal of HLA-G FHC molecules from the cell surface by the protease papain would affect the conformed HLA-G complexes present on the cell surface. To address this question, we performed immunoprecipitation assays of the wild-type and mutated 721.221/HLA-G-transfected cells with the conformed anti-HLA-G mAb MEM-G/09, after papain treatment. In agreement with the binding and killing experiments (Figs. 3 and 4), HLA-G is still expressed on the surface of papain-treated cells in oligomers composed of monomers, dimers, and trimers (Fig. 5*C*). Papain treatment of $Cys^{147}$ mutants resulted in the formation of monomers and dimers, whereas treatment of $Cys^{42}$ mutants resulted in the appearance of monomers only (Fig. 5*C*). All of the precipitated proteins are expressed on the surface as demonstrated by the avidin-HRP staining (Fig. 5, *right panel*). It is important to note that in all of the blots shown in Fig. 5, *A–C*, the same amounts of proteins were introduced onto the gels, the same conditions were used, and the same exposure length was applied. Thus, complexes of HLA-G FHC can be found on the cell surface; however, these complexes are not important for interaction with the CD85J/LIR-1 receptor.

Finally, we analyzed whether all of the above results might have any functional consequence in vivo. To solve this question, immunohistochemistry analysis of serial first trimester placental sections were performed. Indeed, expression of HLA-G FHC (Fig. 5*D*) are detected in trophoblast cell columns. Syncytiotrophoblast and Hofbauer cells (fetal macrophages) were not stained. Some staining in the area of the cytotrophoblast was observed with the HCA2 Ab, while no staining was observed in a control experiment without primary Ab (Fig. 5*D*, *III*). The expression of HLA-G FHC on EVT cells raises some intriguing questions as to the balance between the expression of conformed and FHC HLA-G molecules in various pathological conditions during pregnancy. Further investigation is required to understand the special role of the FHC molecules on trophoblast cells and their interaction with the maternal immune system in vivo.

## Discussion

The fetal-maternal interface at the implantation site is comprised of the EVT cells and the decidua. The EVT cells are the front embryonic cells, which are in direct contact with the mother during implantation. These cells display several distinctive features, which are relevant to the interaction with the maternal immune system (4). Remarkably, one of the unique properties of these cells is the expression of the nonclassical MHC class I, HLA-G protein (8, 40–42). This molecule has gained a lot of interest due to its ability to protect the semiallogenic fetus from maternal rejection, especially by maternal NK cells (17, 21, 42–45). HLA-G is a unique class I MHC protein, and among the classical MHC class I proteins, it has the highest resemblance to HLA-A2 (29, 46, 47). We have recently discovered another unique feature of the HLA-G protein, which is its ability to form multimers that increase the binding avidity of its cognate inhibitory NK receptor CD85J/LIR-1 (26). Indeed, the very recently published crystal structure of the HLA-G protein confirmed our results and presented a model of the HLA-G oligomers and the relative position of CD85J/LIR-1 near the complex (48).

Our study provides new insights into the unique features of the HLA-G molecule on the cell surface. Similar to other MHC class I molecules, HLA-G FHCs are found in small amounts on the cell surface ((24) and Fig. 2*A*). In the present study, we show that CD85J/LIR-1 distinguishes between HLA-G FHC and HLA-G/$\beta_2$m molecules. This interesting observation was also demonstrated for HLA-B27 molecule (49, 50). For example, it was reported that HLA-B27 FHC bound LIR-2- transfected but not LIR-1-transfected cells (51). Importantly, the solved crystal structure of a complex between CD85J/LIR-1 D1D2 domains and the MHC class I molecule HLA-A2 reinforces our observations (38). The structure reveals that CD85J/LIR-1 forms two contact surfaces on the side of the HLA-A2 molecule that include residues from the nonpolymorphic HLA-A2 $\alpha$3 domain and the conserved $\beta_2$m molecule. Consequently, the reason for the failure of CD85J/LIR-1 Ig to recognize HLA-G FHC is probably due to the absence of $\beta_2$m. In contrast to the strong diminish in HLA-G FHC recognition by CD85J/LIR-1, cleavage of HLA-G FHC from the cell surface by the protease papain (Fig. 3*A*) resulted in a significant increase of CD85J/LIR-1 Ig binding (Fig. 3*B*). This notable increase can be attributed to the absence of FHC, which facilitate the accessibility

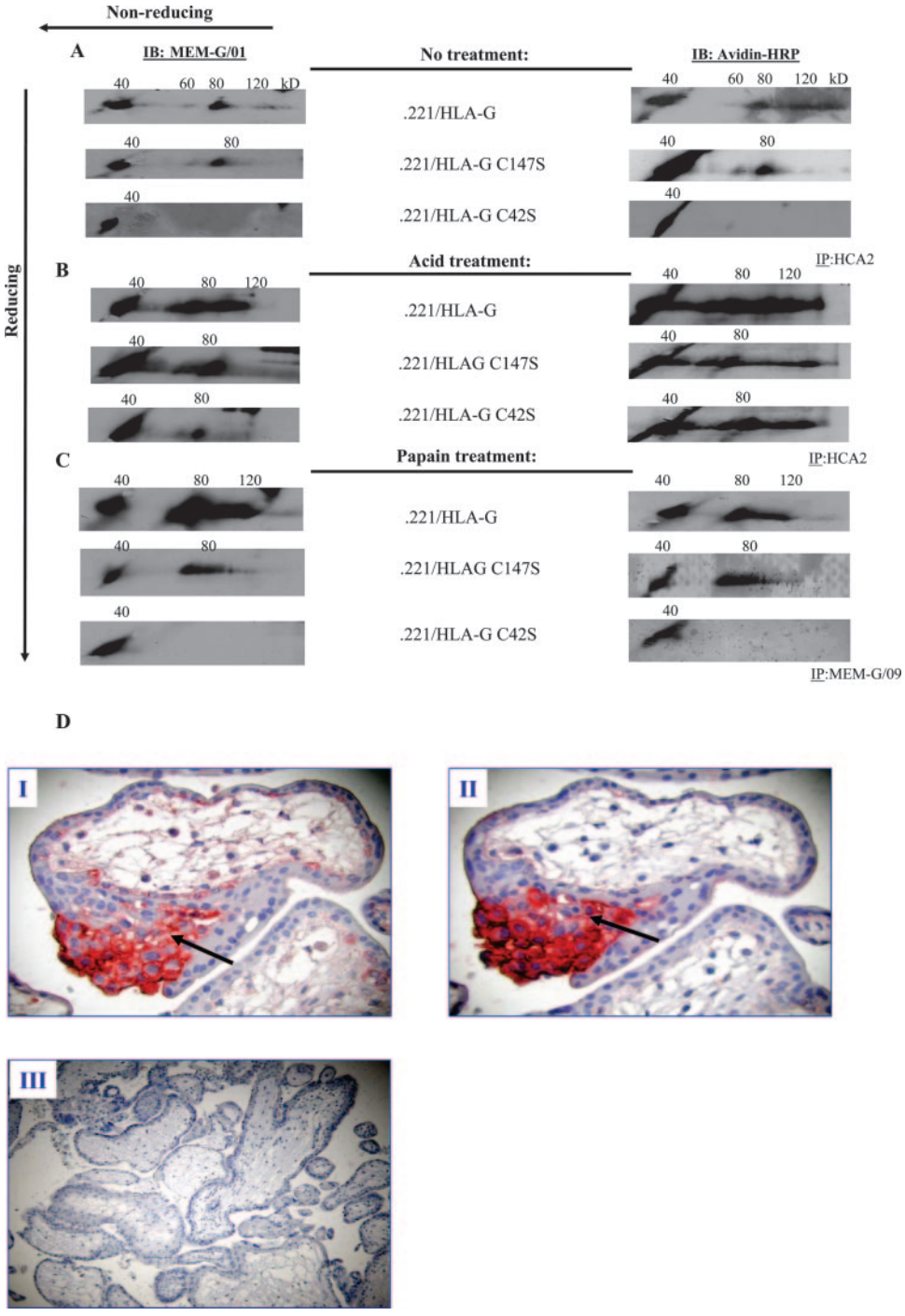


**FIGURE 5.** Expression of HLA-G oligomers on the cell surface subsequent to acid or papain treatment. *A*, A comparison of immunoprecipitations between the wild-type and the mutated $\beta_2$m-free HLA-G complexes. Surface biotinylation was performed as described in *Materials and Methods*. Lysates of biotinylated cells were immunoprecipitated using anti-HLA-G FHC mAb, HCA2. The immunoprecipitates were analyzed by two-dimensional SDS-PAGE (first dimension nonreduced, second dimension reduced conditions) followed by electroblotting and visualization of biotinylated proteins by streptavidin-HRP (*right panel*). To confirm that the precipitates are indeed HLA-G molecules, the blots were then stripped and restained with anti-HLA-G mAb MEM-G/01 followed by goat anti-mouse Ig HRP conjugate (*left panel*). Only the region ~40 kDa is shown. The approximated molecular masses of the complexes are shown above each precipitate. The immunoprecipitations from each transfectant were obtained in independent experiments. Figure shows a representative experiment of three performed. *B*, A comparison of immunoprecipitations between the wild-type and the mutated $\beta_2$m-free HLA-G complexes following acid treatment. Lysates of biotinylated cells were immunoprecipitated using HCA2. Figure shows a representative experiment of three performed. *C*, A comparison of immunoprecipitations between the wild-type and the mutated HLA-G complexes after papain treatment. Lysates of biotinylated cells were immunoprecipitated using anti-conformed HLA-G mAb, MEM-G/09. Figure shows a representative experiment of two performed. *D*, Immunohistochemistry of first trimester placental serial sections using HCA2 mAb (*I*) , 4H84 mAb (*II*), and control without primary Ab (*III*). Arrows indicate positive (red) trophoblast cell column. Magnification, ×400 with ×1.4 zoom (*I* and *II*) and ×100 with ×1.4 zoom (*III*).

of CD85J/LIR-1 to the conformed $\beta_2$m/HLA-G molecules. A possible alternative explanation might be that the presence of complexes composed of FHC and conformed HLA-G molecule, which would be recognized less efficiently by the CD85J/LIR-1 receptor, interfere with the strong binding of CD85J/LIR-1 to complexes composed of conformed HLA-G proteins only. However, this assumption cannot explain the increased binding of CD85J/LIR-1 observed in the $Cys^{42}$ mutant or HLA-A2 transfectant. It may also be referred to the removal of some unknown molecule(s) connected to MHC class I molecules, which interfere with CD85J/LIR-1 Ig binding. These findings were substantiated by NK-mediated assays demonstrating a strong functional affect of the acid and papain treatments on the CD85J/LIR-1 and HLA-G interactions (Fig. 4). Although the different cell transfectants express the HLA-E molecule, which is stabilized on the cell surface by binding the HLA-G leader peptide, it seems not to play a major role in NK inhibition in our system. In that regard, the effect of the treatments on NK susceptibility was probably the direct result of the modifications in the HLA-G molecule and not due to an effect on the HLA-E molecule as the expression of this molecule was not markedly disrupted following the treatments (Figs. 2*A* and 3*A*). There are several studies suggesting that in the absence of HLA-G NK inhibition is predominantly exerted by HLA-E through binding with CD94/NKG2A. However, once HLA-G is expressed, it becomes the major NK inhibitory ligand as blockage of CD94/NKG2A cannot reverse the HLA-G-mediated inhibition (52–54). In contrast, other reports demonstrate that NK recognition of cells expressing HLA-G involves at least two nonoverlapping receptor

ligand systems: the CD94/NKG2A interaction with HLA-E and the engagement of CD85J/LIR-1 by HLA-G (32). These disputing observations may be due to differences in the level of expression of the CD94/NKG2A receptor on the NK cells and the level of HLA-E on the target cells. It seems that when HLA-E is present in low levels on the target cells (such as observed here, Figs. 2*A* and 3*A*), the effect of CD94/NKG2A is not so dominant. Indeed, HLA-E-mediated inhibition of dendritic cell killing by NK cells was observed only with the minor NK subset expressing CD94/NKG2A$^{bright}$ and not with the CD94/NKG2A$^{dim}$ population (55).

The physiological importance of MHC class I FHC molecules in protection against NK killing has been tested previously (56, 57). It has been shown that NK cell inhibitory receptors detect a conformation-dependent epitope. What is then the function of the HLA-G FHC? The expression of HLA-G FHC may serve as an important modulator of other immune cells present in the early pregnant decidua. For example, the HLA-G FHC may bind the LIR-2 (ILT4) receptor similar to HLA-B27 FHC. One may assume a possible scenario in which the balance between the expression of MHC class I molecules (HLA-C, -E, and -G) present on EVT cells as FHC or as conformed proteins (24, 58) would determine the strength of the inhibitory response. These FHCs following dissociation from $\beta_2$m are released from the cell surface through proteolytic cleavage by metalloproteases (24, 58). For example, in various pathological conditions, such as advanced HIV-1 infection or chronic hepatitis C, serum levels of $\beta_2$m-free HLA proteins are increased (59). In the fetal-maternal interface, a possible viral infection of the EVT cells might augment the expression of HLA-G FHC proteins and thereby reduce the inhibition of decidual NK cells killing. On the other hand, the release of these HLA-G FHC into the serum might be advantageous to the virus as these FHCs might induce apoptosis in CD8$^+$ NK and T cells (60). Importantly, HLA-G FHC are present in vivo only on EVT cells as demonstrated here by us (Fig. 5*D*, *I*) and by others (34, 61). Further research is required to assess the physiological role of these FHC molecules and the process of their generation in the decidua.

We have demonstrated previously a unique capability of the HLA-G protein to form disulfide-linked high molecular complexes on the cell surface. We have also demonstrated that Cys$^{42}$ plays a crucial role in the formation of these complexes. As shown here, a similar organization is observed for the HLA-G FHC. However, surprisingly, treatment of Cys$^{42}$ mutant with acid resulted in the formation of monomers and dimers, as opposed to expression of monomers only, without treatment. This pattern of expression implies the ability of Cys$^{147}$ to interact with another Cys$^{147}$ for the formation of the dimers. The exclusion of $\beta_2$m from the conformed HLA-G molecule, which causes the immense up-regulation in HLA-G FHC molecules, possibly enables the exposure of the concealed residue of Cys$^{147}$ and facilitates its interaction with another Cys$^{147}$ residue. Although these FHC dimers may not be physiologically important, understanding the mechanisms of complexes formation is scientifically significant.

The formation of FHC oligomers of various sizes was reported for the HLA-B27 molecule. This molecule is able to form H chain homodimers, which can be detected on the cell surface (62, 63). The homodimer formation is dependent on unpaired cysteine at position 67, as well as on the conserved structural cysteine at position 164 (64). In addition, high molecular mass disulfide-linked multimers were detected, indicating for additional involvement of extracellular and intracellular cysteine residues in the process (64, 65). In this study, we also detected various HLA-G oligomers with intermediate molecular masses (shown in Fig. 5). These complexes might result from postlysis degradation process or because HLA-G protein might interact with other unknown protein(s). These protein(s) are not recognized by the anti-HLA-G mAb MEM-G/01 or by avidin-HRP.

Importantly, we demonstrate, using the Jeg3 cell line, the existence of endogenous complexes of HLA-G. It was suggested that HLA-G might also provide tumor cells with an effective immune escape mechanism (66). It would be interesting to test in the future whether HLA-G is found in complexes in tumors and whether these complexes are important for NK-mediated inhibition.

Our study provides new insights as to the unique properties of HLA-G and its recognition by the CD85J/LIR-1 receptor. Further evaluation of the interplay between the various forms of the HLA-G molecule may yield novel insights into the special relationship between decidual NK cells and EVT during pregnancy.

## Disclosures

The authors have no financial conflict of interest.

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