Complexes of HLA-G Protein on the Cell Surface Are Important for Leukocyte Ig-Like Receptor-1 Function[1]

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The nonclassical class I MHC molecule HLA-G is selectively expressed on extravillous cytotrophoblast cells at the maternal-fetal interface during pregnancy. HLA-G can inhibit the killing mediated by NK cells via interaction with the inhibitory NK cell receptor, leukocyte Ig-like receptor-1 (LIR-1). Comparison of the sequence of the HLA-G molecule to other class I MHC proteins revealed two unique cysteine residues located in positions 42 and 147. Mutating these cysteine residues resulted in a dramatic decrease in LIR-1 Ig binding. Accordingly, the mutated HLA-G transfectants were less effective in the inhibition of NK killing and RBL/LIR-1 induced serotonin release. Immunoprecipitation experiments demonstrated the involvement of the cysteine residues in the formation of HLA-G protein oligomers on the cell surface. The cysteine residue located at position 42 is shown to be critical for the expression of such complexes. These oligomers, unique among the class I MHC proteins, probably bind to LIR-1 with increased avidity, resulting in an enhanced inhibitory function of LIR-1 and an impaired killing function of NK cells. ***The Journal of Immunology,*** **2003, 171: 1343–1351.**

During mammalian pregnancy, semiallogenic fetal cells invade the uterine structures and survive without immunological rejection. The absence of a harmful maternal immune response against the semiallogeneic fetus remains a major enigma in current biology. Maternal tolerance of the fetus may be primarily achieved by the down-regulation of classical class I MHC protein expression on the fetal extravillous cytotrophoblasts (EVT),[3] which are in direct contact with the maternal immune system in the uterus during pregnancy (1). This down-regulation probably prevents T lymphocyte activation against the semiallogeneic fetus. However, a complete lack of class I MHC molecules on EVT cells would render these cells susceptible to attack by maternal NK cells, a phenomenon known as the missing self (2). It has now become evident that trophoblast cells do express the nonclassical class I MHC molecules HLA-G (3, 4), HLA-E (5), as well as the classical class I MHC molecule HLA-C, which is expressed mainly during the first trimester (6, 7).

The HLA-G protein possesses some unusual characteristics, including restricted expression (mainly on EVT cells), limited polymorphism, and alternatively spliced mRNA variants (8). One of the major functions suggested for the HLA-G protein was the inhibition of NK killing. Indeed, the expression of HLA-G on the cell surface protected susceptible target cells from NK-mediated cytotoxicity (9–13).

Several NK receptors were suggested as being able to recognize HLA-G (14), and recently two such receptors were identified. The first is KIR2DL4 (CD158d), which is expressed either on all NK cells derived from PBLs (15) or only on decidual NK cells (16). The second is leukocyte Ig-like receptor-1 (LIR-1; also designated ILT-2 or CD85j), a member of the LIR/ILT family of proteins, which is expressed on most myelomonocytic cells, B cells, dendritic cells, and subsets of T and NK cells (17, 18).

LIR-1 binds to a wide range of HLA class I molecules, including HLA-G (19), and delivers a negative signal that inhibits killing by NK and T cells (20). It contains four extracellular Ig-like domains and four intracellular immunoreceptor tyrosine-based inhibitory motifs, which transduce the inhibitory signal by binding the Src homology domain-containing tyrosine phosphates (21, 22). In the present study we focused on understanding the molecular basis for the LIR-1 and HLA-G interactions.

Comparison between the sequence of the HLA-G molecule and other class I MHC molecules revealed two unique cysteine residues located in positions 42 and 147 in HLA-G extracellular domains α1 and α2, respectively. The possible influence of these cysteines on NK function was investigated by mutating both cysteine residues to serines. The mutants were transfected into 721.221 cells and tested in binding, cytotoxicity, and serotonin release assays. A dramatic decrease in the binding of the LIR-1 Ig to the mutated HLA-G transfectants was observed compared with the wild-type molecule. Accordingly, the mutated HLA-G transfectants were less effective in inhibition of NK killing or of RBL/LIR-1 FcϵR-induced serotonin release.

Immunoprecipitation experiments with anti-HLA-G Abs and analysis by two-dimensional gel electrophoresis suggested a possible role for the cysteine residues in the expression of high molecular weight complexes of HLA-G protein on the cell surface. The cysteine residue located in position 42 is shown to be critical

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[3] Abbreviations used in this paper: EVT, extravillous cytotrophoblast; LIR-1, leukocyte Ig-like receptor-1; TNP, trinitrophenyl.

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for the expression of HLA-G complexes on the cell surface. Such HLA-G complexes most likely bind with increased avidity to the LIR-1 and consequently efficiently trigger the inhibitory function of LIR-1.

Materials and Methods

Cells, mAbs, and HLA-G mutations

The cell lines used in this work are the EBV-transformed class I MHC-negative human B cell line 721.221 (23), 721.221 transfectants, RBL (rat basophilic leukemia) cells, and RBL cells transfected with LIR-1 (24).

Point mutations in HLA-G cDNA were performed by PCR, using the following primers: 5′ primer (including the *Kpn*I site and Kozak sequence), 5′-GGGGTACCCCGCCGCCACCATGGTGGTCATGGCACCACGA-3; 3′ mut S42C primer, 5′-CATCCTCGGAGACGCCGAGTC-3′; 3′ mut C147S primer, 5′-CATTGGCCGCCTCAGACTTGCGCTTGGAG-3′; 3′ primer (including the *Eco*RI site), 5′-CGGAATTCCGTCAATCTG AGCTCTTCTTTCTCCACAG-3′; 5′ mut S42C primer, 5′-CGACTCG GCGTCTCCGAGGATG-3′; and 5′ mut S147C primer, 5′-CTCCAAGC GCAAGTCTGAGGCGGCCAATG-3′. cDNA was cloned into the PCDNA3 vector (Invitrogen, San Diego, CA) and transfected into 721.221 cells as previously described (25).

NK cells were isolated from PBL using the human NK cell isolation kit and the autoMACS instrument (Miltenyi Biotec, Bergisch-Gladbach, Germany). NK cells were kept in culture as previously described (25).

All mAbs used in this work were generated in mice, including W6/32 mAb (IgG2a), directed against class I MHC molecules, anti-HLA-G mAbs, MEM-G/09, MEM-G/13B, and MEM-G/01 (all of which are IgG1, produced and characterized in the Prague laboratory), anti-trinitrophenyl (anti-TNP) IgE (BD Biosciences, Mountain View, CA), anti-LIR-1 mAb-HPF1 (IgG1), anti-CD99 mAb-12E7 (IgG1), HC10 (IgG2a) directed against class I MHC β_2-microglobulin-free heavy chain, and anti-β_2-microglobulin mAb BBM1 (IgG2b). The W6/32 mAb was also used in the form of $F(ab')_2$. Digestion and purification of the $F(ab')_2$ were performed using the ImmunoPure $F(ab')_2$ preparation kit (Pierce, Rockford, IL) according to the manufacturer's instructions.

Isolation of decidual NK cells

The institutional board of The Hadassah Organization approved obtaining deciduae and placenta from elective pregnancy termination procedures, according to the principles of the Helsinki Declaration. Decidual lymphocytes were isolated as previously described (26–28). Briefly, the tissue was trimmed into 1-mm pieces and enzymatically digested for 20 min, using vigorous shaking, with 1.5 mg of type I DNase and 24 mg of type IV collagenase present in 15 ml of RPMI 1640 medium. This procedure was repeated three times. After an additional 5-min incubation at room temperature without shaking, the supernatants were collected and loaded on Ficoll-density gradient to purify the lymphocyte population. NK cells were purified using the human NK cell isolation kit and the autoMACS instrument (Miltenyi Biotec). Positive (NK and NKT cells) and negative (T cells) fractions were collected and cloned (1 cell/well) in the presence of IL-2.

Cytotoxicity assay

The cytotoxic activity of NK cells against the various target cells was assessed in 5-h ^{35}S release assays as previously described (25). In experiments in which mAb were included, the final mAb concentration was 10 μg/ml for W6/32, $F(ab')_2$ of W6/32, HC10 and 12E7 or 1/1000 ascities dilution for anti-LIR-1 mAb (HPF1). In all the presented cytotoxic assays, spontaneous release was $<25\%$ of maximal release.

Ig fusion proteins

The cDNA encoding for the LIR-1 Ig fusion protein was provided by Dr. Cosman (Immunex, Seattle, WA). The production of 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 previously described (29–31). The LIR-1 Ig protein was characterized by a single protein band on nonreduced SDS-PAGE and was routinely tested for its degradation.

Immunoprecipitation and two-dimensional SDS-PAGE

Cells (10^6/ml) 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 21335) 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 iodoaceteamide, 5 mM EDTA, 1 mM PMSF, 10 μM leupeptin, and pepstatin A). Cell lysates (10×10^6/sample) were precleared overnight at 4°C with protein A-Sepharose beads (Zymed Laboratories, San Francisco, CA) precoated with rabbit anti-mouse IgG. Precleared lysates were then immunoprecipitated overnight at 4°C with MEM-G/09, MEM-G/13B, or BBM1 mAbs. 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 first-dimension SDS-PAGE on 8–10% gels in special tubes (0.5 cm in diameter). The gels, each representing an immunoprecipitate, were pulled out of the tubes and reduced with 32 mM 1,4-DTT (Roche, Mannheim, Germany) at room temperature for 2 h, then placed on top of the second dimension SDS-PAGE on 8% gels, followed by elctroblotting. The blotted biotinylated proteins were visualized by streptavidin-HRP conjugate (Amersham Pharmacia Biotech, Little Chalfont, U.K.), and HLA-G chains were specifically detected by mAb MEM-G/01, followed by goat anti-mouse Ig HRP (Sigma-Aldrich, St. Louis, MO) using ECL detection.

Serotonin release, cell haptenization, and conjugation

RBL cells (10^6/ml) were pulsed for 3 h at 37°C in RPMI 1640/10% FCS containing 2 μCi/ml [^{3}H]serotonin (DuPont-NEN, Boston, MA). Cells were washed, incubated at 37°C for an additional hour, then washed again and resuspended at 4×10^6 cells/ml. For cell haptenization, 721.221 cells (5×10^6 cells/ml) were incubated for 15 min at 37°C in PBS containing 0.5 mM trinitrobenzene sulfonic acid (Sigma-Aldrich), then washed twice in 50 ml of PBS and resuspended in RPMI 1640/10% FCS medium at 8×10^6 cells/ml. To measure stimulation-dependent serotonin release, 4×10^5 haptenized cells were cocultured with 2×10^5 [^{3}H]serotonin-loaded RBL cells in the presence of 1 μg/well anti-TNP IgE (BD Biosciences) for 1 h in round-bottom, 96-well plates in a final volume of 150 μl. After 1 h at 37°C, 50 μl of supernatant was collected from each well, and radioactivity was measured. Serotonin release was calculated as % serotonin release = [(cpm sample − cpm spontaneous release)/(cpm total − cpm spontaneous release)] × 100. Total serotonin release was measured after incubation of the cells with 0.1 M NaOH (22).

Results

The HLA-G protein contains two unique cysteine residues located in positions 42 and 147

The expression of HLA-G is mainly restricted to EVT cells. This is in marked contrast to other classical class I MHC proteins that are expressed on the surface of almost every nucleated cell. Comparison of the sequence of HLA-G to other class I MHC molecules revealed two unique cysteine residues in positions 42 and 147 that are localized in extracellular domains α1 and α2, respectively (Fig. 1).

The role of these cysteines was investigated by mutating them to serine residues using a site-directed mutagenesis approach. Wild-type and mutated HLA-G molecules were transfected into 721.221 (.221) cells, and surface expression was monitored by using three conformationally dependent Abs, including W6/32 (a pan anti-class I MHC mAb that also recognizes HLA-G), and MEM-G/09 and MEM-G/13B (both anti-HLA-G Abs). As expected, the MEM-G/09 and MEM-G/13B mAbs stained all the HLA-G transfectants, including wild-type and mutants (Fig. 2), but did not stain any other .221 transfectant. This confirms that the anti-HLA-G mAbs are specific to the HLA-G protein and that the mutations in the cysteine residues did not alter the epitope on the HLA-G protein recognized by these mAbs. Similarly, the cysteine mutants reacted equally well with the W6/32 mAb as the wild-type HLA-G (Fig. 2). Similar results were obtained when the HLA-G proteins (both wild-type and mutant) were expressed in a different cell line, LB33 Mel B1 (data not shown).

Binding of LIR-1 Ig to .221 cells expressing the cysteine-mutated HLA-G proteins is dramatically decreased

The results shown above demonstrate that the cysteine mutations did not affect mAb binding. One of the receptors recognizing HLA-G is LIR-1 (ILT-2) (17, 18). We next tested whether the generated cysteine mutations would affect LIR-1 Ig binding. Efficient LIR-1 Ig binding was observed to the wild-type HLA-G

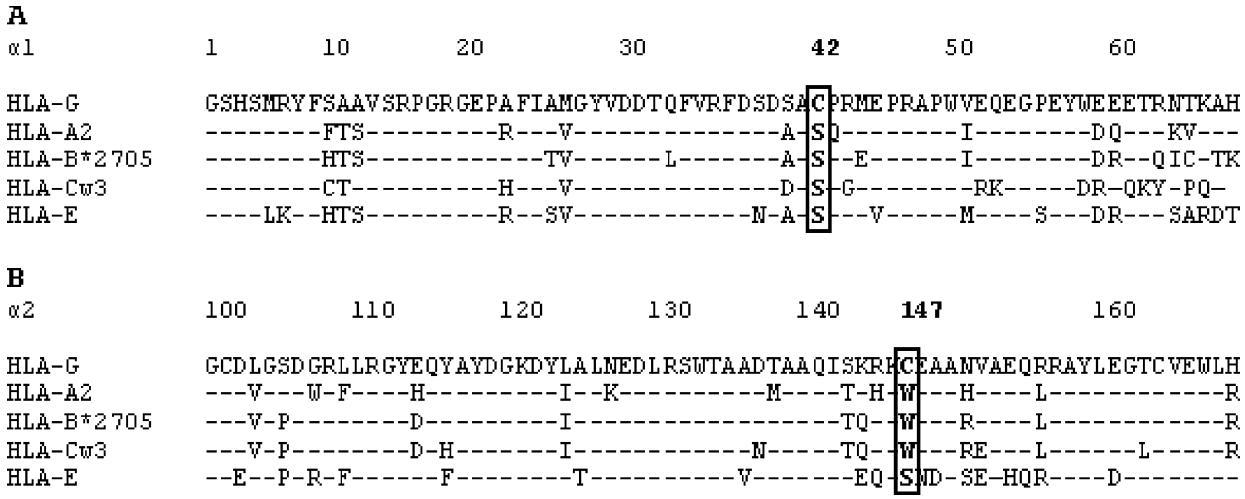

FIGURE 1. Sequence alignment of the HLA-G and several class I MHC proteins. *A*, The sequence is shown for positions 1–68. Bold letters in the rectangle indicate the unique cysteine residue located at position 42 of HLA-G, which is absent in any other class I MHC protein (representative alleles are shown). *B*, The sequence is shown for positions 100–169. Bold letters in the rectangle indicate the unique cysteine residue located in position 147 of HLA-G, which is absent in any other class I MHC protein (representative alleles are shown).

transfectants. In contrast, the LIR-1 Ig binding to both of the mutated HLA-G proteins was significantly decreased (Fig. 3). Importantly, the level of HLA-G protein expression of all HLA-G transfectants was similar, as detected by the W6/32 mAb or the anti-HLA-G mAbs (see Figs. 2 and 3 legend and data not shown). Similar results were obtained when the LB33 Mel B1 cells expressing the various HLA-G proteins were used (data not shown). These results indicate that the cysteine residues are important for efficient binding of LIR-1 to the HLA-G molecule.

The epitope on the class I MHC proteins that is recognized by LIR-1 was located to the conserved α3 domain of the molecule (32). In contrast, the unique cysteine residues of the HLA-G protein are located in the α1 and the α2 domains. Nevertheless, we tested whether the mutations in the cysteine residues altered the HLA-G conformation by using the conformationally dependent W6/32 mAb. W6/32 mAb was reported to bind to a combination of epitopes composed of residues in the β_2-microglobulin, α2, and α3 domains of the class I MHC molecule (33, 34). Preincubation of the targets with W6/32 mAb abolished the binding of LIR-1 Ig to all the HLA-G transfectants (Fig. 4). As described above, the level of HLA-G protein expression detected by W6/32 mAb or the anti-HLA-G mAbs on all the HLA-G transfectants was similar (data not shown). The anti-HLA-G mAbs MEM-G/09 and MEM-G/13B could not block LIR-1 Ig binding (data not shown). The binding site for both the anti-HLA-G mAbs is as yet unknown. These results suggest that the epitopes recognized by the W6/32 mAb were not affected by the cysteine mutations.

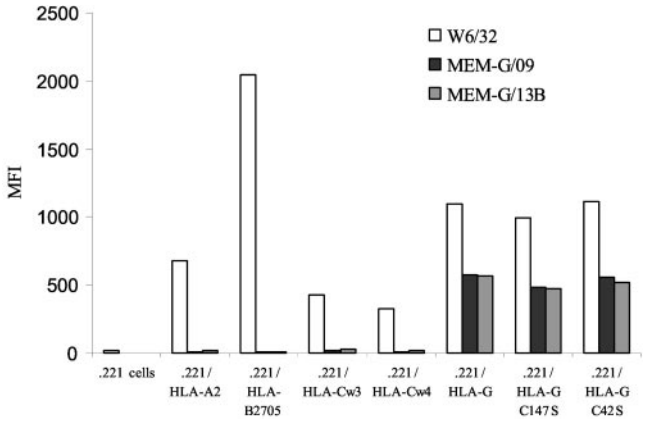

FIGURE 2. Recognition of .221 transfected cells by different conformation-dependent Abs. The .221 transfectants expressing various class I MHC proteins (indicated in the figure) were stained with the anti-class I MHC mAb W6/32 and anti-HLA-G Abs, MEM-G/09 and MEM-G/13B, followed by FITC-conjugated goat anti-mouse Ig. The levels of expression are indicated by the median fluorescence intensity (MFI). Controls were the same cells incubated with FITC-conjugated goat anti-mouse Ig. This figure shows one representative experiment of 10 performed.

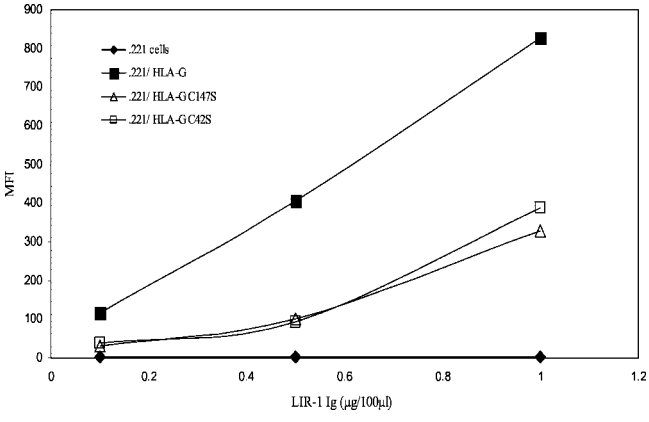

FIGURE 3. Binding of LIR-1 Ig to various HLA-G transfectants. The .221 cells expressing the mutated and the wild-type HLA-G proteins were incubated with increasing concentrations of LIR-1 Ig fusion protein, followed by PE-conjugated anti-human Fc Abs. The levels of expression are indicated by the median fluorescence intensity (MFI). In parallel, all .221 transfectants were stained with the anti-HLA-G mAbs, MEM-G/09 and MEM-G/13B. The MFI values for .221/HLA-G, .221/HLA-G C147S, and .221/HLA-G C42S (stained by MEM-G/09) were 572.5, 486.9, and 504.8, respectively. Controls for the Ab staining were the same cells stained with FITC-conjugated goat anti-mouse Ig. Controls for the fusion protein staining were the same cells stained with PE-conjugated anti-human Fc Abs. This figure shows one representative experiment of 13 performed.

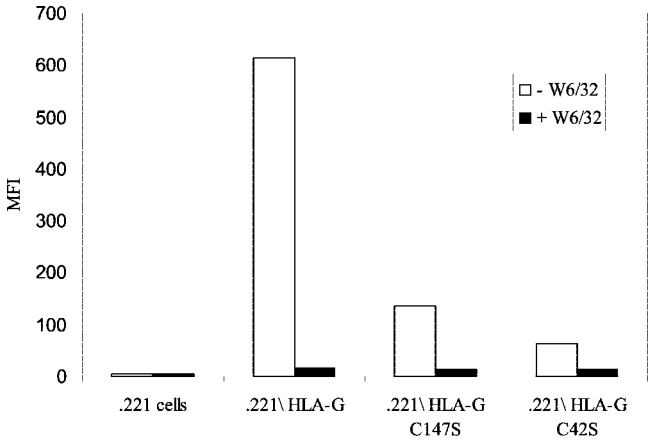

FIGURE 4. Blocking of LIR-1 Ig binding to HLA-G transfectants using W6/32 mAb. The .221 transfectants (5×10^4 cells) were incubated with 0.5 μg W6/32 mAb, washed, and then stained with 1 μg of LIR-1 Ig fusion protein. The levels of expression are indicated by the median fluorescence intensity (MFI). Controls for the fusion protein staining were the same cells stained with PE-conjugated anti-human Fc Abs. The figure shows one representative experiment of three performed.

LIR-1-mediated inhibition of NK killing is decreased when .221 cells expressing the mutated HLA-G proteins are used

The functional relevance of the above observation, suggesting the involvement of the cysteine residues in efficient HLA-G recognition by the LIR-1 receptor, was tested using NK killing assays. NK clones were generated as described in *Materials and Methods*. One hundred and twenty independent NK clones were tested in killing experiments against HLA-G-transfected target cells. About 20% of the NK clones tested were inhibited by HLA-G, and the expression of the LIR-1 receptor was observed on the surface of all of them. One representative clone expressing the LIR-1 receptor is shown in Fig. 5*A*.

As previously reported (16–18), inhibition of lysis was observed when .221/HLA-G cells were tested as target cells for the NK clones expressing LIR-1 (Fig. 5, *B* and *C*). We conclude that the inhibitory effect was due to the interaction between the LIR-1 receptor and the HLA-G protein, since incubation of target cells with either W6/32 mAb or the $F(ab')_2$ of W6/32 mAb resulted in restoration of the killing (Fig. 5*B*). No significant change in lysis of the .221/HLA-G transfectants was observed when the isotype-matched control mAb (HC10) was used (Fig. 5*B*). Abolishment of the inhibitory effect was also observed when the anti LIR-1 mAb

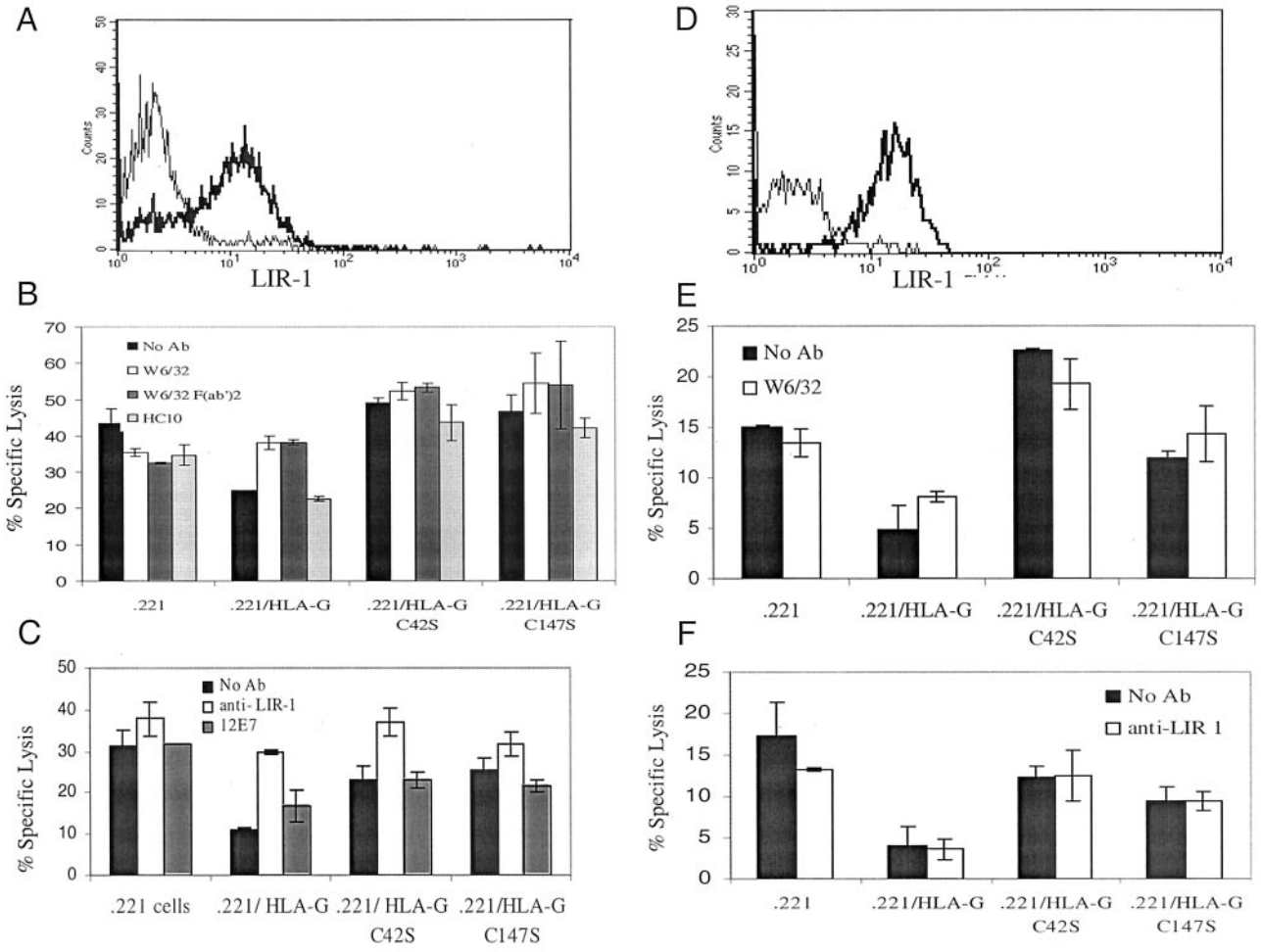

FIGURE 5. Inhibition of NK killing by various HLA-G transfectants. *A* and *D*, FACS analysis of LIR-1 expression on peripheral NK clone 37 (*A*) and decidual NK clone 276 (*D*) using anti-LIR-1 Ab HPF1. *B* and *C*, Killing assays of .221 transfectants (indicated in the figure) by NK clones, with or without various mAbs incubated on either target cells (*B*) or effector cells (*C*). The difference in the level of killing and inhibition presented in *B* and *C* is because independent experiments are presented. Similar results were obtained when other NK clones were used (data not shown). *E* and *F*, Killing assays data not shown of .221 transfectants by decidual NK clones with or without the indicated mAb. The figure shows a representative experiment of four performed.

(HPF1) was used (Fig. 5*C*). The isotype-matched control anti-CD99 mAb (12E7) had no significant effect.

In agreement with the binding experiments, the inhibitory effect of HLA-G was significantly reduced when both the 42 and 147 cysteine mutants were assayed against the NK clones (Fig. 5, *B* and *C*). The still observed minimal inhibition was the result of LIR-1 interaction with the mutated HLA-G proteins, as the anti-class I MHC mAb W6/32, the $F(ab')_2$ of W6/32 mAb, or the anti LIR-1 mAb abolished this modest inhibition (Fig. 5, *B* and *C*).

HLA-G mainly functions in the maternal-fetal interface where the EVT cells are in direct contact with maternal lymphocytes, of which 70% are decidual NK cells. These cells are characterized by the $CD16^{-}$ $CD56^{bright}$ phenotype (35, 36). We therefore subsequently tested the decidual NK clones in killing against the various HLA-G transfectants. One representative decidual NK clone expressing the LIR-1 receptor is shown in Fig. 5*D*. In general, and in agreement with previous results (16), decidual NK cells have a decreased killing activity against class I MHC-negative target cells. In accordance with the killing assays performed with peripheral NK clones, efficient inhibition of lysis mediated by the .221/HLA-G transfectants was observed, while the mutations in both 42 and 147 cysteine residues resulted in an impaired inhibitory effect (Fig. 5, *E* and *F*). Blocking the target cells with W6/32 mAb or the effector cells with anti LIR-1 mAb had little or no effect and did not restore the NK-mediated lysis (Fig. 5, *E* and *F*). This is probably because virtually all decidual NK cells express one or more inhibitory receptors capable of interacting with the HLA-G molecules, including LIR-1 and KIR2DL4 (16), or with the HLA-E molecule, including CD94/NKG2A (24, 37). Among these three receptors, KIR2DL4 was demonstrated to play a predominant role in preventing the NK-mediated lysis of HLA-G by decidual NK cells (16). The W6/32 mAb, which blocks the interaction between LIR-1 and HLA-G, probably does not interact with the binding site of the KIR2DL4 receptor on HLA-G, and therefore restoration of killing by decidual NK clones could not be observed.

To directly test the interaction between LIR-1 and the various HLA-G transfectants, we used the RBL cells transfected with LIR-1 (18, 22, 24). RBL/LIR-1 transfectants were labeled with [^{3}H]serotonin, incubated with anti-TNP mAb of the IgE isotype, and cocultured with the various .221/HLA-G transfectants, previously haptenized with TNP. As shown in Fig. 6, FcϵR-induced serotonin release was inhibited when RBL/LIR-1 cells were incubated with .221/HLA-G cells. The inhibition was less pronounced when HLA-G C147S cells were used, and only a slight inhibition was observed when HLA-G C42S cells were assayed (Fig. 6). Incubation of all HLA-G transfectants with parental RBL cells showed no significant change in serotonin release (Fig. 6).

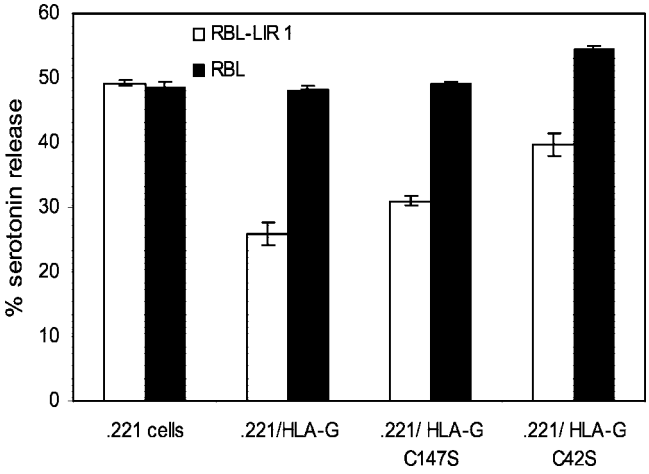

FIGURE 6. Inhibition of IgE-induced serotonin release from LIR-1-transfected RBL cells by various HLA-G transfectants. The .221 transfectants were haptenized with TNP, incubated with purified mouse anti-TNP IgE, and used to challenge RBL-LIR-1 and parental RBL cells loaded with [^{3}H]serotonin. The percentage of serotonin release was determined after 1 h at 37°C. The figure shows a representative experiment of four performed.

The unique cysteine residues found in HLA-G protein are essential for generation of covalent oligomers on the cell surface

Several explanations might account for the decreased LIR-1 recognition of the mutated HLA-G protein. It is unlikely that both cysteine residues are in contact with LIR-1, as the LIR-1 binding site on HLA-G is located in the α3 domain (32). It is also unlikely that the protein conformation was grossly altered due to the mutations, since conformationally dependent mAb recognized all HLA-G transfectants in a similar manner (Fig. 2). It is still possible, however, that the cysteine residues are involved in the formation of HLA-G covalent oligomers present on the cell surface that may exhibit increased avidity for LIR-1.

To test this hypothesis, wild-type and mutated .221/HLA-G transfected cells were cell surface biotinylated, lysed, and then immunoprecipitated as described in *Materials and Methods*. The lysates were immunoprecipitated using conformational anti-HLA-G mAbs, MEM-G/09 or MEM-G/13B (Fig. 7), and then were analyzed by two-dimensional (nonreduced/reduced) SDS-PAGE. To prevent post-lysis disulfide bond formation of HLA-G molecules, iodoacetamide was included in the cell lysis buffer in all immunoprecipitation experiments. As shown in Fig. 7*A*, the wild-type HLA-G is expressed on the cell surface as homotrimers, homodimers, or monomers. Their molecular masses were, as expected, ~120, 80, and 40 kDa, and the ratios of their expression (calculated by densitometric analysis) were 0.1, 0.5, and 1 for the homotrimers, homodimers, and monomers, respectively. The HLA-G oligomers can be found on the cell surface in different ratios, suggesting that the formation of HLA-G oligomers is a dynamic process. For example, when the amount of the monomers on the cell surface decreased, the amount of the dimers increased respectively, and the various HLA-G oligomers can be found on the cell surface in ratios of 0.1, 1, and 0.6 for the homotrimers, homodimers, and monomers, respectively. The HLA-G mutants were also tested in the immunoprecipitation experiments. Mutating the cysteine located in position 147 completely abolished HLA-G trimer formation and expression on the cell surface, leaving dimers and monomers only in ratios of 0.86 and 1, respectively (Fig. 7*B*). Importantly, mutating the cysteine residue located in position 42 abolished both the formation of HLA-G trimers and dimers, leaving the expression of monomers only (Fig. 7*B*). The control immunoprecipitation of .221/HLA-Cw3 proteins showed only the monomeric pattern of expression of HLA-Cw3 on the cell surface (Fig. 7*B*). No protein bands were observed when the control .221 cells were used in the immunoprecipitation experiments (data not shown).

To confirm that all precipitated forms are indeed HLA-G, we performed Western blot analysis on the wild-type HLA-G, HLA-G C147S and HLA-G C42S, presented in Fig. 7*B* using anti-HLA-G mAb MEM G/01. Similar to the surface biotinylation experiments, three forms of HLA-G complexes were obtained in the wild-type HLA-G (Fig. 7*C*, I), two in the HLA-G C147S (Fig. 7*C*, II), and one in the HLA-G C42S (Fig. 7*C*, III). A similar pattern of expression was obtained when the wild-type and mutated HLA-G proteins expressed on LB33 Mel B1 were used (data not shown).

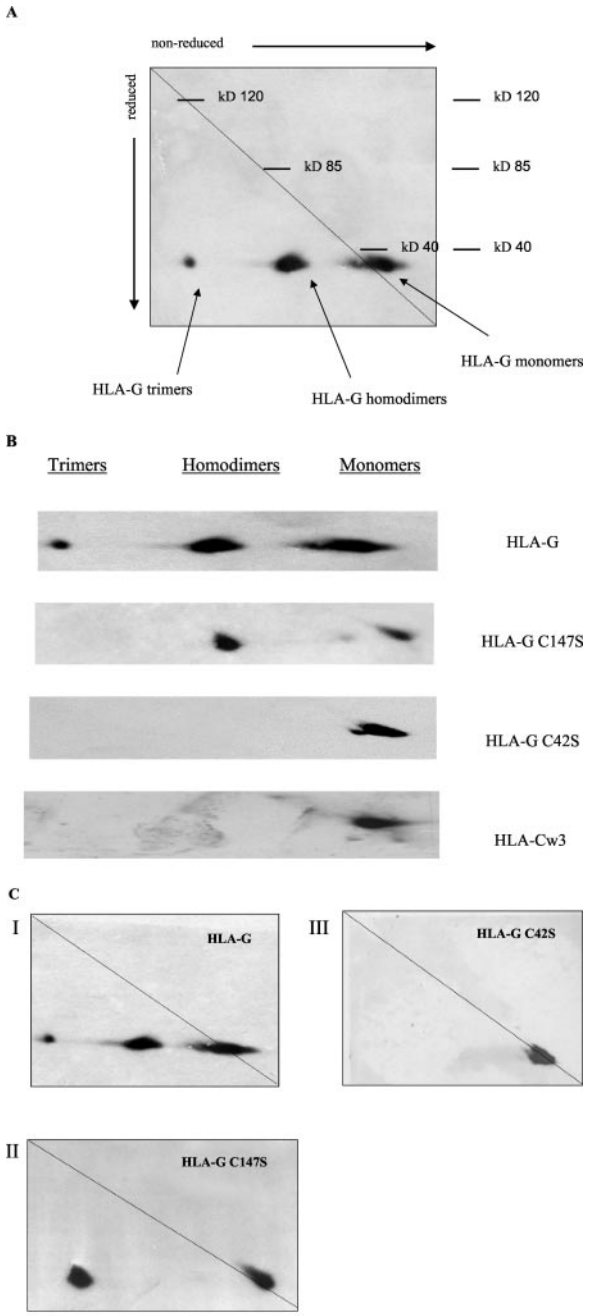

FIGURE 7. Expression of HLA-G complexes on the cell surface. *A*, Surface biotinylation of .221/HLA-G transfectants was performed as described in *Materials and Methods*. Lysates of biotinylated cells were immunoprecipitated using anti-HLA-G mAb MEM-G/09. The immunoprecipitate was analyzed by two-dimensional SDS-PAGE (first dimension, nonreduced; second dimension, reduced conditions), followed by electroblotting and visualization of biotinylated proteins by streptavidin-HRP. Similar results were obtained when MEM-G/13B was used for immunoprecipitation. The figure shows a representative experiment of seven performed. *B*, A comparison of immunoprecipitations between the wild-type and the mutated HLA-G. Surface biotinylation was performed as described in *Materials and Methods*. Lysates of biotinylated cells were immunoprecipitated using anti-HLA-G mAb MEM-G/09 or anti-β_2-microglobulin mAb BBM1 for the control .221/Cw3 immunoprecipitation. The immunoprecipitates were analyzed by two-dimensional SDS-PAGE. Only the region around 40 kDa is shown. The immunoprecipitations from each transfectant were obtained in independent experiments. The figure shows a representative experiment of five performed. *C*, Western blot analysis. Blots containing the immunoprecipitated wild-type HLA-G (I), HLA-G C147S (II), and HLA-G C42S (III) were detected by immunostaining using anti-HLA-G mAb MEM-G/01, followed by goat anti-mouse Ig HRP conjugate. The figure shows a representative experiment of three performed.

These results demonstrate that the cysteine-dependent complexes of HLA-G are expressed on the cell surface and that these complexes may play a crucial role in recognition by the LIR-1 receptor.

Discussion

The nonclassical class I MHC molecule HLA-G has garnered a lot of attention in the past few years due to its unique characteristics. Two receptors were reported to recognize the HLA-G protein, LIR-1 and KIR2DL4 (15–18). In the present study we focused on characterization of the interactions between HLA-G and the LIR-1 receptor. The LIR-1 receptor is expressed on a wide spectrum of cells, including monocytes, dendritic cells, B cells, T cells, and NK cells (reviewed in Ref. 38).

In this study we show that the two unique cysteine residues located in positions 42 and 147 are involved in the formation and expression of HLA-G homodimers and homotrimers on the cell surface. Mutating these cysteine residues to serines dramatically impaired recognition of the HLA-G protein by the LIR-1 receptor. This resulted in impaired binding of the LIR-1 to HLA-G, decreased inhibition of NK lysis by HLA-G, and decreased inhibition of FcϵR-induced serotonin release from RBL/LIR-1 cells. Importantly, the cysteine mutations probably did not alter the conformation of the HLA-G protein (Figs. 2 and 4). The cysteine residues probably also play an important role in the recognition of HLA-G by other receptors, such as KIR2DL4, since LIR-1-independent inhibition was observed when decidual NK clones were assayed against HLA-G, while virtually no inhibition was observed when both cysteine mutants were used (Fig. 5).

The expression of HLA-G oligomers on the cell surface can be schematically diagrammed, as shown in Fig. 8. The Cys residue located in position 42 is critical for the formation of HLA-G oligomers. Mutating this residue abolished the formation of dimers and trimers, whereas mutating residue 147 only partially affected the formation of HLA-G dimers (Figs. 7*B* and 8*B*). The structure of the HLA-G protein is as yet unsolved; however, the critical role of residue 42 is supported by the superimposition of the HLA-G molecule onto the HLA-A2 crystal structure. Such superimposition reveals that the Cys^{42} residue is located on a loop projecting out from the MHC class I molecule (39). The Cys^{147} residue, on the other hand, is predicted to be located on a position pointing into the groove. The concealed position of Cys^{147} residue probably contributes to its limited ability to interact with another Cys^{147}; however, interactions with Cys^{42} can occur (Fig. 7). The trimer formation might occur either because the superimposition does not accurately reflect the HLA-G structure or because the HLA-G on the cell surface might be found in a slightly different conformation, enabling the interaction of Cys^{147} with Cys^{42}. In addition, the disulfide bond that is generated between Cys^{147}, which points into the groove, and Cys^{42} might affect either peptide composition or peptide stability of the HLA-G proteins, resulting in limited amounts of HLA-G trimers on the cell surface. These HLA-G trimers might be stabilized by the two other HLA-G proteins that are connected via Cys^{42}.

In accordance with the biochemical results, we proposed a model in which Cys^{42} is the key residue controlling the disulfide bond formation interacting either with another Cys^{42} residue or with Cys^{147}. Cys^{147}, on the other hand, can only interact with Cys^{42} (Fig. 8). These interactions result in the expression of a relatively small amount of HLA-G trimers on the cell surface (Fig. 7, *A* and *B*, and Fig. 8*A*).

The proposed model does not rule out the existence of smaller amounts of higher HLA-G multimers on the cell surface or exclude

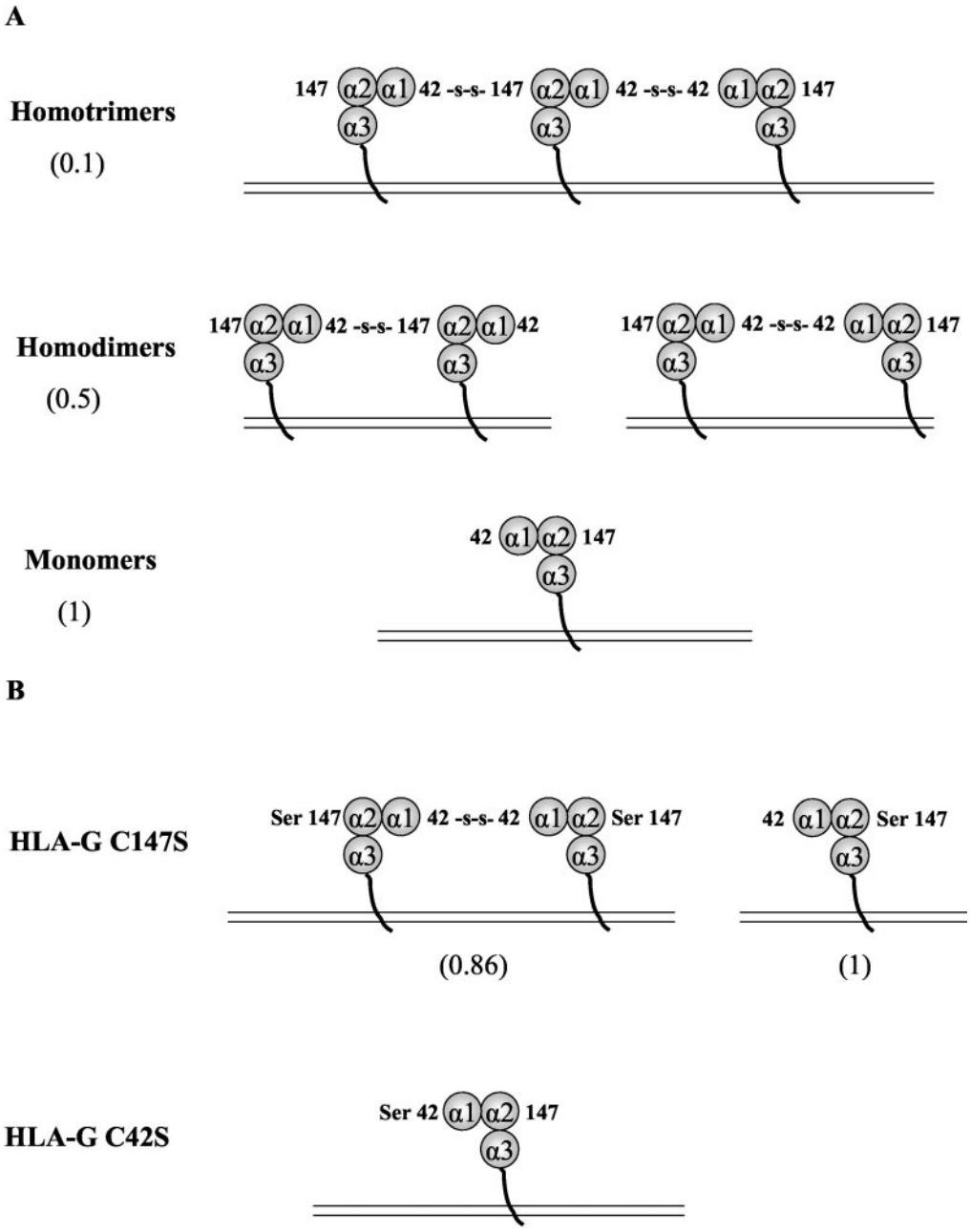

FIGURE 8. A model describing the expression of HLA-G covalent oligomers. *A*, A proposed model demonstrating HLA-G expression on the cell surface. The expression of homotrimers and homodimers is the result of the disulfide bridges between cysteine residues located in positions 42 and 147. The ratios of the different forms of expression are shown (data from Fig. 7). *B*, A proposed model for the pattern of expression of the mutated HLA-G molecules on the cell surface.

the presence of intrachain disulfide bridges formed by these cysteine residues. Although the observed complexes are likely to be HLA-G oligomers (Fig. 7), our results and the proposed model do not preclude the existence of another as yet unknown protein(s) that might bind the HLA-G molecule via disulfide bonds and interact, either directly or indirectly, with the LIR-1 receptor.

Supporting our results, a recent report suggested the presence of disulfide-linked dimers of HLA-G on the cell surface that are formed via the Cys^{42} residue (39). The role of residue 147 or the functional relevance of these dimers was not investigated (39). Importantly, in this study we show that both dimers and trimers of HLA-G are expressed on the cell surface, that both Cys^{42} and Cys^{147} residues are involved in the formation of these high molecular HLA-G complexes on the cell surface, and that these complexes play a crucial role in recognition by the LIR-1 receptor.

Although a relatively small amount of these trimers is found on the cell surface (Fig. 7, *A* and *B*, and Fig. 8*A*), they probably play a critical role in the LIR-1 recognition of HLA-G. This is indicated by the fact that both cysteine mutations give a similar pattern of decreased recognition of the LIR-1 receptor, and that the common phenomenon for both the mutations was the absence of expression of HLA-G homotrimers on the cell surface (Figs. 7 and 8).

HLA-G is the only class I MHC protein reported to date that might be expressed on the cell surface as trimers. A recent report showed that aberrant HLA-B27 disulfide-linked dimers may be present on the cell surface under some circumstances (40); in this

case, the disulfide bond was formed between two extracellular α1 domains (Cys^{67}).

Our findings may reconcile the discrepancies previously reported regarding recognition of HLA-G by NK cells. While it is well established that inhibition of NK lysis can be observed when using intact cells expressing the HLA-G protein on the cell surface (10, 14, 15, 37) (Fig. 5), no recognition of NK cells by HLA-G was observed when HLA-G tetramers were used (41). We suggest that this absence of recognition by HLA-G tetramers might be due to the special organization of the HLA-G proteins on the cell surface (Figs. 7 and 8). As we demonstrate in this study, an efficient recognition of HLA-G by NK cells might depend on the presence of high molecular complexes of HLA-G on the cell surface. It is possible that when the HLA-G proteins are expressed as tetramers, they are not found in the most suitable arrangement to be optimally recognized by the NK cell receptors.

The HLA-G protein is expressed on the placenta when HLA-A and -B class Ia molecules are largely absent (3, 4). This may indicate its important role in the immunological tolerance of the fetal semiallograft facing the maternal immune system. It was therefore surprising to learn that the binding affinities of the inhibitory receptor LIR-1 to HLA-G are low (kilodaltons in the micromolar range) (32). It had also been previously suggested that high local concentrations of class I molecules would have to be presented to trigger the inhibitory function of LIR-1 (32). In this study we show that HLA-G has probably developed a unique mechanism to enhance LIR-1 binding by the expression of disulfide-linked oligomeric structures on the cell surface. This pattern of expression might increase LIR-1 avidity to the HLA-G protein and enable efficient inhibition of NK cells.

Similar effects of increased avidity were observed when including a free cysteine in the KIR2DL1 receptor. This disulfide-linked KIR2DL1 bound its ligand, the HLA-Cw4, at a molar ratio of one dimer to one HLA-Cw4 molecule. In addition, the dimer bound more tightly to HLA-Cw4, suggesting the occurrence of a second binding event that increases the overall avidity of the KIR2DL1 dimer for HLA-C (42).

The HLA-G protein has unique characteristics that distinguish it from other HLA class I molecules, such as tissue-restricted distribution, limited polymorphism, and impaired spontaneous endocytosis (43). Here we present another unique characteristic of HLA-G, which is the presence of high molecular complexes of HLA-G on the cell surface, that is absent in any other class I MHC molecules. This might be especially important in the maternal-fetal interface, where there must be strict control of the interaction between HLA-G and the inhibitory receptors that bind it.

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