Efficient Leukocyte Ig-like Receptor Signaling and Crystal Structure of Disulfide-linked HLA-G Dimer*8

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HLA-G is a nonclassical major histocompatibility complex class I (MHC) molecule, which is expressed in trophoblasts and confers immunological tolerance in the maternal-fetal interface by binding to leukocyte Ig-like receptors (LILRs, also called as LIR/ILT/CD85) and CD8. HLA-G is expressed in disulfide-linked dimer form both in solution and at the cell surface. Interestingly, MHCII dimers have been involved in pathogenesis and T cell activation. The structure and receptor binding characteristics of MHCII dimers have never been evaluated. Here we performed binding studies in solution and at the cell surface. Interestingly, MHCII dimer formations have been involved in pathogenesis and T cell activation. The structure and receptor binding characteristics of MHCII dimers have never been evaluated. Here we performed binding studies showing that the HLA-G dimer exhibited higher overall affinity to LILRB1/2 than the monomer by significant avidity effects. Furthermore, the cell reporter assay demonstrated that the dimer formation remarkably enhanced the LILRB1-mediated signaling at the cellular level. We further determined the crystal structure of the wild-type dimer of HLA-G with the intermolecular Cys42–Cys82 disulfide bond. This dimer structure showed the oblique configuration to expose two LILR/CD8-binding sites upward from the membrane easily accessible for receptors, providing plausible 1:2 (HLA-G dimer:receptors) complex models. These results indicated that the HLA-G dimer conferred increased avidity in a proper structural orientation to induce efficient LILRs signaling, resulting in the dominant immunosuppressive effects. Moreover, structural and functional implications for other MHCII dimers observed in activated T cells and the pathogenic allele, HLA-B27, are discussed.

During pregnancy, the fetus can be the allogenic object for the maternal immune system, and thus a special system of immune tolerance is necessary for escaping from maternal immune surveillance to achieve a successful pregnancy. However, knowledge of the molecular mechanism of the maternal-fetal immune tolerance is still limited. In the

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MHC class I molecules, such as HLA-E, which can present a very limited repertoire of peptides, including the MHC class I signal sequence and heat shock and viral proteins. HLA-G shows the slow transport to the cell surface and the long half-life on the cell surface because of its truncated intracytoplasmic domain lacking an endocytosis motif (14, 15). The result is that HLA-G selects a limited set of high affinity peptides for presentation, even though it has the peptide presentation mechanism of the classical MHC class I. The recently reported crystal structure of the HLA-G C42S mutant monomer (16) also showed that the peptide recognition of HLA-G includes an extensive network of contacts, supporting the constrained mode of the peptide binding.

HLA-G has two free cysteine residues (Cys42 and Cys47) unlike most of other MHC class I molecules. Boyson et al. (17) reported that the bacterial recombinant soluble form of HLA-G can form a disulfide-linked dimer with the intermolecular Cys42–Cys42 disulfide bond. Moreover, the soluble HLA-G1 expressed by human embryonic kidney 293 cells also showed the mixture of monomer, disulfide-linked dimer, and oligomer forms, which could reduce the CD8 expression level on cytotoxic T lymphocytes (9). However, it is uncertain how much effect each form has. On the other hand, the membrane-bound form of HLA-G can also form a disulfide-linked dimer on the cell surface of the Jeg3 cell line, which endogenously expresses HLA-G (18) and also HLA-G transfectants (17, 19, 20). The mutagenesis studies suggested that the HLA-G dimer was responsible for efficient LILRB1-mediated inhibition of the killing activity of NK cells (19, 20).

Recently, the β2m-free form of HLA-G also forms disulfide-linked dimers and multimers on the cell surface mainly by Cys42–mediated disulfide bond. Moreover, the soluble HLA-G1 expressed by human embryonic kidney 293 cells also showed the mixture of monomer, disulfide-linked dimer, and oligomer forms, which could reduce the CD8 expression level on cytotoxic T lymphocytes (9). However, it is uncertain how much effect each form has. On the other hand, the membrane-bound form of HLA-G can also form a disulfide-linked dimer on the cell surface of the Jeg3 cell line, which endogenously expresses HLA-G (18) and also HLA-G transfectants (17, 19, 20). The mutagenesis studies suggested that the HLA-G dimer was responsible for efficient LILRB1-mediated inhibition of the killing activity of NK cells (19, 20).

The native-PAGE for characterizing the HLA-G C42S mutant monomer, LILRB1, and LILRB2 were essentially the same as for those without the tag. Site-directed mutagenesis for the C42S mutant HLA-G monomer was performed by the two-step PCR method. The refolded monomer of wild-type HLA-G naturally formed the disulfide-bonded dimer (10–30% of the total protein) for ~20 days at 4°C. For the binding studies, the HLA-G dimer was purified by gel filtration (Superdex 200 10/30, Amersham Biosciences).

Native Gel Electrophoresis—The native-PAGE for characterizing the protein samples and analyzing the binding was performed by the Phast system (Amersham Biosciences). We used the commercially available native-PAGE buffer strip (0.25 M Tris, 0.88 M l-alanine, pH 8.8; Amersham Biosciences), and the experiment was performed at 15°C. In the complex formation experiments, the sample mixture was incubated at 20°C for 1 h before applying to the homogeneous 12.5% polyacrylamide gel (Amersham Biosciences). Less than 4 μL of samples on each lane was applied.

Equilibrium Gel Filtration Studies—The equilibrium gel filtration was performed on a SMART system with Superdex-200 PC 3.2/30 column (Amersham Biosciences) at a flow rate of 60 μL/min. The column was equilibrated with 10 mM Hepes, pH 7.4, 150 mM NaCl, 3.4 mM EDTA, and 0.005% surfactant P20 (HBS-EP buffer) with 10 μM LILRB1 or 15 μM LILRB2. The mixtures of HLA-G dimer (10 μM for LILRB1 and 15 μM for LILRB2) and LILRB1/2 at molar ratios of 1:0 (dimer alone), 1:1, 1:2, and 1:3 were incubated at room temperature for 1 h before injections.

Surface Plasmon Resonance Studies—Surface plasmon resonance experiments were performed using a BIACore2000™ (BIACore AB, St. Albans, UK) following the standard protocol of our previous report (3). Briefly, the biotinylated LILRBs were immobilized on the research grade Sensor Chip CM5 (BIACore AB) on which streptavidin was covalently immobilized. The HLA-G dimer or C42S mutant monomer flowed over at 50 μL/min. Kinetic constants were derived using the curve fitting for the bivalent analyte model or the simple 1:1 binding model by the Bioevaluation version 3.2 (BIACore). For equilibrium binding analyses, the equilibrating binding response at each concentration of analyte was calculated by subtracting the response measured in the control flow cell from the response in the sample flow cells. Affinity constants (Kd) were calculated by nonlinear curve fitting or by Scatchard analysis with the simple 1:1 Langmuir binding model using the program Origin version 5.0 (MicroCal). In the experiments of reverse orientation, the biotinylated HLA-Gs were immobilized. LILRBs flowed over the immobilized HLA-Gs.

6 K. Kuroki, M. Shiroishi, D. Kohda, and K. Maenaka, unpublished data.
The LILRB1 NFAT-GFP Reporter Cell Assay—The chimera molecule that consisted of the extracellular domain of LILRB1 and the transmembrane and cytoplasmic domains of activating PILR (28) was transfected into a mouse T cell hybridoma carrying NFAT-green fluorescence protein (GFP) reporter gene and DAP12 by using retrovirus infection. The chimera molecule (LILRB1-PILR chimera molecule (29)) was transfected into a mouse T cell hybridoma carrying NFAT-GFP reporter gene and DAP12 using retrovirus vector (26, 27). The various concentrations (0–160 ng/ml) of HLA-G were immobilized on the 48-well tissue culture plate (BD Falcon) at 37 °C for 2 h. The reporter cells expressing the LILRB1-PILR chimera molecule (5 × 10^4/well) were stimulated by 0.3% in the disallowed region. Ramachandran plot was calculated using PROCHECK (33). Figures were generated using PyMOL, BOBSCRIPT (34), and Raster 3D (35).

RESULTS

1:2 (HLA-G Dimer:LILRBs) Binding Stoichiometry—To characterize the biochemical properties of the HLA-G dimer, the dimer and C42S mutant monomer were prepared as described under “Experimental Procedures.” The intermolecular disulfide bond in the wild-type HLA-G dimer was confirmed by gel filtration, SDS-PAGE, and native-PAGE using the reducing agents (Fig. 1, A–C). As in the previous report by Boyson et al. (17), the C42S mutation kept HLA-G as the monomer form (hereafter designated as the HLA-G monomer or the monomer), positional and overall B-factor refinement with the program Refmac5 (29), the Fobs − Fcalc clearly showed electron density for the disulfide bond between Cys^12 of the crystallographic 2-fold monomer (Fig. 4B), indicating that two disulfide-bonded dimers existed in this crystal, and each monomer in each dimer complex was placed along the crystallographic 2-fold axis. Further refinement with the grouped (2 groups per chain, 2m and peptide) and show R_{free} 23.5% (R_{free} = 29.8%) between 50 and 3.2 Å. Detailed crystallographic statistics are shown in Table 1. A Ramachandran plot of the backbone angles gave 85.3, 13.4, and 0.9% in most favored, additionally, and generously allowed regions, respectively, and 0.3% in the disallowed region. Ramachandran plot was calculated by PROCHECK (33). Figures were generated using PyMOL, BOBSCRIPT (34), and Raster 3D (35).

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Crystallization, Data Collection, Structure Determination, and Refinement—The detailed description for crystallization of the disulfide-linked HLA-G dimer was published (51). Briefly, 0.2 μl of protein solution was mixed in a 1:1 ratio with the crystallization reservoir solution. The crystals were obtained in the condition of Wizard II-39 (100 mM CHAPS, pH 10.5, 20% (w/v) PEG8000, 200 mM NaCl) at 20 °C. A 3.2-Å diffraction data set was collected at 100 K, at beamline BL38B1 of Spring8 (Harima, Japan) (l = 1.0000 Å). The diffraction data were processed and scaled with HKL2000 program package. The detail crystallographic statistics are shown in Table 1. The HLA-G structure was phased by the molecular replacement procedure using Molrep in CCP4 package (29) with the search probe, the crystal structure of the HLA-E-peptide complex (Protein Data Bank code 1MHE) (30). Two HLA-G monomers (chains A and B) were found by using the HLA-E model in the range 20 to 3.5 Å. After the rigid-body adjustment, one cycle of positional and overall B-factor refinement with the program Refmac5 (29), the Fobs − Fcalc clearly showed electron density for the disulfide bond between Cys^12 of the crystallographic 2-fold monomer (Fig. 4B), indicating that two disulfide-bonded dimers existed in this crystal, and each monomer in each dimer complex was placed along the crystallographic 2-fold axis. Further refinement with the grouped (2 groups per chain, 2m and peptide) and show R_{free} 23.5% (R_{free} = 29.8%) between 50 and 3.2 Å. Detailed crystallographic statistics are shown in Table 1. A Ramachandran plot of the backbone angles gave 85.3, 13.4, and 0.9% in most favored, additionally, and generously allowed regions, respectively, and 0.3% in the disallowed region. Ramachandran plot was calculated by PROCHECK (33). Figures were generated using PyMOL, BOBSCRIPT (34), and Raster 3D (35).
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Figure 2. Surface plasmon resonance analysis of HLA-G dimer and monomer (C425 mutant) binding to immobilized LILRB1 and LILRB2. A, kinetic analysis of the HLA-G dimer for the immobilized LILRB1. The dimer of the indicated concentration was injected through LILRB1-immobilized flow cells (200 response units (RU)). Response curves were fitted globally with the 1:1 model for all concentrations because of the low affinities (native-PAGE, but not all of the monomer formed the LILRB1 complex). B, kinetic analysis of the HLA-G monomer injected through LILRB1-immobilized flow cells. C, kinetic analysis of the HLA-G dimer to the immobilized LILRB2 (270 response units). Response curves were fitted globally with the 1:1 model for all concentrations (left panel, black line) and for low concentrations (63–250 nM, right panel, black line). D, kinetic analysis of the HLA-G monomer injected through LILRB2-immobilized flow cells. E, equilibrium binding analysis of the HLA-G monomer to immobilized LILRB1 (black square) and LILRB2 (red circle). The solid lines represent nonlinear fits of the 1:1 Langmuir binding isofom. Inset, Scatchard plots of the same data. Solid lines are linear fits.

Clearly indicating that the HLA-G dimer was formed dominantly via the Cys42-Cys42 disulfide bond.

The LILRB1 binding to the HLA-G monomer was observed on native-PAGE, but not all of the monomer formed the LILRB1 complex because of the low affinities (K_d ~2–5 μM) as described in our previous report (3) (data not shown). To determine the stoichiometry of the complex between LILRB1 and HLA-G dimer, a series of the mixture (1:1, 1:2, and 1:4 (HLA-G dimer:LILRB1)) was analyzed on native-PAGE (Fig. 1D). It showed that the full complex was composed of one HLA-G dimer and two LILRB1s (1:2 complex). On the other hand, the LILRB2 binding study by native-PAGE (Fig. 1F) demonstrated that the LILRB2 bound to the dimer but with very low affinity. To determine the binding stoichiometry unambiguously, equilibrium gel filtration was performed (Fig. 1, E and G). When protein concentrations in the running solution are above the dissociation constant, the elution profile will depend on the stoichiometry of the injected mixture and the true equilibrium stoichiometry of the complex. The stoichiometry of the HLA-G dimer-LILRB1 and -LILRB2 complexes was determined by applying samples, including either 1:0, 1:1, 1:2, or 1:3 (HLA-G dimer:LILRB1) molar ratios over a column equilibrated with 10 μM LILRB1 or 15 μM LILRB2. The injection of the 1:0 and 1:1 (HLA-G dimer:LILRB) showed a peak corresponding the dimer-LILRB complex and a trough where the free LILRBs migrated, indicating that the LILRBs of the running solution were consumed for the complex formation. On the other hand, the 1:3 molar ratio sample for both LILRBs generated two peaks corresponding to the HLA-G dimer-LILRB complex and the free LILRB, indicating the presence of excess free LILRBs. The 1:2 molar ratio sample showed only one complex peak and thus represented the true equilibrium complex stoichiometry. The 1:2 binding stoichiometry for LILRB1/2 reasonably conferred the avidity effect, which was observed in the later section of the surface plasmon resonance analysis and can be accounted for by the LILRB1 complex model structure of the HLA-G dimer (Fig. 6B).

High Affinity LILRB Binding of HLA-G Dimer—The binding of the HLA-G dimer to LILRBs was further characterized by surface plasmon resonance. The HLA-G dimer and monomer were injected over sensor surfaces on which biotinylated LILRBs had been immobilized. Representative data for binding of HLA-G monomer and dimer to LILRBs were shown in Fig. 2. The monomer showed 1:1 binding with very fast dissociation rates (3.5–5 s^{-1}) (Fig. 2, B and D). Affinity constants (K_d) of the monomer for LILRB1 and LILRB2 were 3.5 and 15 μM, respectively, derived from equilibrium analysis (Fig. 2E), which were similar to those from the opposite orientation studies of the HLA-G monomer (supplemental Fig. S1 and Table S1) (3). In contrast, kinetic analyses of the binding of the dimer to the immobilized LILRBs showed that the global fitting with the simple 1:1 (Langmuir) binding model was not successful at high concentrations of the dimer but was well fitted with a bivalent analytic model (Fig. 2, A and C, left panels). Therefore, the dimer showed the bivalent binding, which is consistent with the 1:2 (dimer:LILRB) complex models built on the basis of the present crystal structure of HLA-G dimer as described below (Fig. 6B). This avidity effect significantly contributed to the high affinity binding clearly observed in Fig. 2. At low concentrations of the dimer, where the dimer can simultaneously bind to two immobilized receptors, the responses could be fitted to the simple 1:1 binding model and gave a high apparent affinity constant (K_d = 6.7 nM for LILRB1 and ~750 nM for LILRB2, Fig. 2, A and C, right panels). The dissociation rates for the LILRB binding to the HLA-G dimer were much slower (k_{off} = 0.28 s^{-1} (LILRB1) or 1.0 s^{-1} (LILRB2)) in a bivalent model, and k_{off} = 0.11 s^{-1} (LILRB1) or 0.96 s^{-1} (LILRB2) in a simple 1:1 binding model) than the monomer. These results clearly indicated that the avidity effects resulted in much higher apparent affinity with slow dissociation rates.
Efficient LILRB1-mediated Signaling of HLA-G Dimer—To examine such avidity effect of the dimer on the receptor-mediated signaling at the cellular level, a mouse T cell hybridoma with the NFAT-GFP reporter system (26, 27) was used. The hybridoma cells were transfected with the LILRB1 chimera gene consisting of the extracellular domain of LILRB1 and of the transmembrane and intracellular domains of the activating PILRβ (28), which can induce the GFP expression upon the proper receptor binding. Thus the LILRB1-mediated signaling could be easily detected by the GFP expression. The various amounts of HLA-G monomer or dimer were immobilized on the wells, and LILRB1 reporter cells were incubated on the wells. After 12 h, GFP expression in LILRB1 reporter cells was analyzed by flow cytometry. Fig. 3 clearly showed that the monomer could merely stimulate the reporter cells even at the highest concentration of 160 ng/ml (mean fluorescence intensity (MFI) = 35.9, and GFP-positive cells are only 13% of total). Surprisingly, the dimer significantly activated the cells at a much lower concentration of 1.6 ng/ml, and GFP-positive cells are 61% of total (MFI = 266) at the concentration of 160 ng/ml. Therefore, the dimer formation remarkably enhanced the LILRB1-mediated signaling. Our data evidenced the high affinity binding and slow dissociation rates of the dimer.

Overall Structure of HLA-G Dimer—The HLA-G (HLA-G*0101, residues 1–274)-peptide (RIIPRHLQL) complex was refolded and purified as a monomer form and was crystallized with small amount of dithiothreitol, which facilitated the dimer formation (51). The crystals diffracted to 3.2 Å resolution. The structure was determined by molecular replacement, using HLA-E structure (30) as a search model (see Table 1).

Two molecules of wild-type HLA-G exist in the asymmetric unit. Each monomer was covalently attached with its symmetrical partner via the Cys52–Cys175 disulfide bond along with 2-fold crystallographic axis, even though the monomer form was used for the crystallization (Fig. 4A

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**TABLE 1**

Data collection and refinement statistics

|                         | HLA-G dimer                  |
|-------------------------|------------------------------|
| **Data collection**     | Spring8 (BL38B1)             |
| Space group             | P2_1 2_1_2                   |
| Cell dimensions         | 94.70, 127.85, 72.60          |
| a, b, c (Å)             | 50–3.20 (3.31–3.20)          |
| Resolution (Å)          | 0.090 (0.325)                |
| l/d                      | 6.2 (2.1)                    |
| Completeness (%)        | 83.8 (77.7)                  |
| Redundancy              | 4.9 (4.0)                    |

**Refinement**

| Resolution (Å) | 50–3.20 (3.31–3.20) |
|----------------|---------------------|
| No. reflections | 12,897 (1,162)      |
| Rwork/Rfree    | 0.235 (0.272)/0.279 (0.376) |
| No. protein atoms | 6,126              |
| B-factor       | 47.3                |
| r.m.s.d.       | 0.010               |
| Bond angles (°)| 1.363               |
and supplemental Fig. S2). This is consistent with the result that the wild-type HLA-G monomer had the tendency to form a dimer (17). The unambiguous electron density for this intermolecular disulfide bond was clearly observed (Fig. 4B). The two HLA-G molecules in the asymmetric unit were almost identical with 0.49 Å of the root mean square distances (r.m.s.d.) for 381 C-\(^\alpha\) positions, although some differences of the peptide-protein interactions were observed in the center region of the peptide. The overall structure of the HLA-G-peptide complex in the dimer form was very similar to the monomer form of HLA-G recently determined (16) (0.63 Å of r.m.s.d for 345 C-\(^\alpha\) atoms of chain A and 0.72 Å of r.m.s.d for 344 C-\(^\alpha\) atoms of chain B) and other MHC class I structures reported previously (Figs. 4, C and D, and 6A). No significant structural changes caused by the Cys\(^{42}\)–Cys\(^{42}\) disulfide bond formation were observed.

**Peptide-HLA-G Interactions**—As observed in the C42S HLA-G monomer structure of the previous report (16), the main chain conformation of the 9-mer peptide bound to HLA-G was similar to that of the fully extended and deeply buried HLA-E-9-mer complex (30) (Fig. 5B and supplemental Figs. S3 and S4). Pool sequencing of eluted peptides and the binding analysis of individual peptides (12, 13) have shown that HLA-G has a peptide-binding motif with primary anchor residues, P2, P3, and PC (C-terminal position) (the detailed structural characteristics for these sites are described in the legend to supplemental Figs. S3 and S4, and the peptide-HLA-G interactions are summarized in supplemental Table S2). The major parts of the peptide–HLA-G interactions, N- and C-terminal sites, were similar to classical MHCs.

On the other hand, there were some conformational differences of the peptide-HLA-G binding interface among the monomer (cyan and pink) and two chains (A (green and yellow) and B (dark blue and orange)) of the dimer (Fig. 5A). First, at the P1 site, the Arg residue made electrostatic interactions with Glu\(^{62}\) and Glu\(^{63}\) in the monomer, however, only with Glu\(^{63}\) in chains A and B (the side chain of Arg in the chain B was largely shifted; Fig. 5C). Therefore, this conformationally flexible recognition of P1 site supported the idea that the P1 site is not a strict anchor but prefers the positively charged residue.

Next, at P6 site, the deeply protruded His residue interacted with Asp\(^{74}\), Trp\(^{97}\), and Tyr\(^{116}\) in all complexes, but the side chain of P6 His could be shifted and/or rotated, coordinating with the rotation of side chains of the above three amino acids (Fig. 5D). In contrast with HLA-E, HLA-G had relatively hydrophilic amino acids (Ser\(^{9}\), Asp\(^{74}\), and Tyr\(^{116}\)) compared with HLA-E (His\(^{9}\), Phe\(^{74}\), and Phe\(^{116}\)) (Fig. 5E). Thus, the P6 amino acid was deeply buried into the pocket but still variable and/or mobile upon the complex formation. This may partly explain that HLA-G can display a limited but still varied repertoire of peptides, more similar to classical MHCs than HLA-E, which showed very tight peptide specificity.

**Dimer Formation and LILR/CD8 Binding Models**—The wild-type HLA-G formed the disulfide-linked dimer with the intermolecular disulfide bond depicted in Fig. 4B.
Cys42–Cys42 disulfide bond in the crystals (hereafter designated as the HLA-G dimer or the dimer). Two HLA-G dimers were found in the crystals as described above, and the Cys42–Cys42 disulfide bond formation did not induce any significant structural changes on the main frames of the monomers. Except for the Cys42–Cys42 disulfide bond, the dimer interface was composed of some electrostatic and hydrogen-bonding interactions (Glu61(O–H9280)–Lys68(N–H9256), 41(CO)–Arg44(Nh1), Glu58(O–H92801,O–H92802)–Gln72(N–H92802,O–H92801)), which are well conserved among MHCs (Fig. 4B). The binding areas were 676 Å² (chain A) and 568 Å² (chain B), which were relatively small in comparison with 1600 Å² of the average protein-protein interactions (36), suggesting that these dimer conformations have some structural flexibility. However, the angle between monomers of two dimers was surprisingly conserved (Fig. 4E), and the intermolecular interactions at the dimer interface were maintained with little structural adjustment. With the maintenance of the Cys42–Cys42 disulfide bond, limited degrees and directions of the angle are allowed because of the structural hindrance and loss of the interface area. Moreover, the N-glycosylation site at position 86 (37) was located outside of the dimer interface, and no structural interference was caused by the bound sugars in the close location (Fig. 6B, green dotted circle). These support the notion that this dimer conformation seems major in both membrane-bound and -soluble forms of the HLA-G dimer. Especially in the membrane-bound form of HLA-G dimer, both of C-terminal sites of the dimer tethering the transmembrane domain should be close to the membrane. Furthermore, the structural characteristics of the LILRB- and CD8-binding sites were maintained in the dimer form of HLA-G and similar to other MHC-I molecules (Fig. 6D). Therefore, this orientation is feasible and rendered two LILR/CD8αα-binding sites fully accessible for the receptors, providing the plausible 1:2 (HLA-G dimer:receptors) complex (Fig. 6, B and C). These complex models further suggested that two bound receptors are properly oriented and close enough to assemble on the cell surface for the appropriate signaling, which were consistent with the biochemical and cellular studies described above.

**FIGURE 5. Peptide-HLA-G Interactions.** A, comparison of peptide-HLA-G interactions in the A–F pockets for peptide binding between HLA-G dimer and monomer forms. The peptide is shown in stick representation primarily with green (chain A), dark blue (chain B), and cyan (monomer) bonds, although the amino acid residues of the MHC class I molecules are shown primarily in yellow (chain A), orange (chain B), and pink (monomer). Structural representation and colorings are applied to C and D, B, comparison of peptide conformations bound in the various MHC class I structures. These superpositions are based on the α1–α2 domains of the MHC class I molecules. C, α traces of 9-mer peptides: red (HLA-G), pink (HLA-E), light gray/A2, Protein Data Bank codes 1hbg, 1hhj, and 1hj(47), and gray (B35 (48)). C, detail interactions in the P1 binding pocket. D, detail interactions in the P7 pocket in the center region. E, comparison of the center region of the peptide binding groove between HLA-G (yellow) and HLA-E (magenta). The side chains of the key amino acids of the MHC class I molecules are shown in stick model.

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**DISCUSSION**

Our structural analysis reveals the first example of a disulfide-linked dimer form of MHC-I, although there are many previous reports showing that the disulfide-linked dimers exist in some MHC-I alleles, for example HLA-B27 (38), and are expressed on the activated but not resting T cells. The HLA-G dimer structure showed that the dimerization does not induce any significant conformational changes on the monomer part, and the dimer interface is not so large and includes some electrostatic and hydrogen-bonding interactions (Glu61(O–H9280)–Lys68(N–H9256), 41(CO)–Arg44(Nh1), Glu58(O–H92801,O–H92802)–Gln72(N–H92802,O–H92801)), which are well conserved among MHCs (Fig. 4B). The binding areas were 676 Å² (chain A) and 568 Å² (chain B), which were relatively small in comparison with 1600 ± 400 Å² of the average protein-protein interactions (36), suggesting that these dimer conformations have some structural flexibility. However, the angle between monomers of two dimers was surprisingly conserved (Fig. 4E), and the intermolecular interactions at the dimer interface were maintained with little structural adjustment. With the maintenance of the Cys42–Cys42 disulfide bond, limited degrees and directions of the angle are allowed because of the structural hindrance and loss of the interface area. Moreover, the N-glycosylation site at position 86 (37) was located outside of the dimer interface, and no structural interference was caused by the bound sugars in the close location (Fig. 6B, green dotted circle). These support the notion that this dimer conformation seems major in both membrane-bound and -soluble forms of the HLA-G dimer. Especially in the membrane-bound form of HLA-G dimer, both of C-terminal sites of the dimer tethering the transmembrane domain should be close to the membrane. Furthermore, the structural characteristics of the LILRB- and CD8-binding sites were maintained in the dimer form of HLA-G and similar to other MHC-I molecules (Fig. 6D). Therefore, this orientation is feasible and rendered two LILR/CD8αα-binding sites fully accessible for the receptors, providing the plausible 1:2 (HLA-G dimer:receptors) complex (Fig. 6, B and C). These complex models further suggested that two bound receptors are properly oriented and close enough to assemble on the cell surface for the appropriate signaling, which were consistent with the biochemical and cellular studies described above.
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The soluble form is probably more flexible, but similar conformations are likely to be dominant.

Based on the monomer structure of the HLA-G C42S mutant, Clements et al. (16) proposed the head-to-tail model of the Cys42-mediated dimer configuration with close contact of β2ms, although other possible conformations could not be ruled out. The current dimer structure clearly evidenced that the dimer had the oblique orientation with the extended interface interactions around the Cys42–Cys42 bond but not between β2ms, exposing the receptor-binding sites upward and more accessible. It provided the plausible 1:2 (HLA-G dimer:LILRB or CD8 receptors) complex (Fig. 6, B and C), explaining that two receptors can assemble on the proper orientation at the cell surface for the efficient signaling. Consistently, the biochemical studies demonstrated much higher overall affinity of the HLA-G dimer toward LILRBs than the monomer by increasing the avidity, similar to the Fce/FcεR interaction (39, 40) and other cell-surface receptor interactions (41, 42). Furthermore, the NFAT-GFP reporter assay with the activating chimera protein of LILRB1 demonstrated that the dimer significantly induced LILRB1-mediated signaling at the cellular level. These results are also in good agreement with the report of Gonen-Gross et al. (20) showing that the HLA-G dimer on the cell surface facilitates the inhibitory signaling of natural killer cells through LILRB1 binding. On the other hand, the previous reports (9–11, 43) showed that the soluble forms of HLA-G impaired the function of CD8+ T cells, NK cells, and dendritic cells by binding to CD8, LILRB1, and LILRB2, respectively. The soluble HLA-G protein was found to have monomer, dimer, and oligomer forms. Based on the remarkable avidity effect demonstrated by the surface plasmon resonance binding studies, the soluble form of HLA-G dimer is likely to have the dominant effect on the LILRB signaling. Furthermore, our preliminary data7 indicated that CD8 also showed similar avidity effects, which can also enhance the CD8-mediated signaling.

Taken together, these suggested that both the membrane-bound and soluble forms of the HLA-G dimer play potential roles to have the appropriate structural conformation to augment a wide range of immunosuppressive effects in immunologically relevant events, including pregnancy and organ transplantation (1). Furthermore, the HLA-G dimer is naturally expressed and will thus be a potential anti-inflammatory reagent for medical treatments.

HLA-B27, which is strongly associated with spondyloarthropathies, has been shown to form β2m-free disulfide-bonded dimers via Cys67–Cys67 bonding (21, 22). The β2m-free B27 dimer can bind to LILRB2 but not to LILRB1 (3, 44).7 Because Cys67 of HLA-B27 is located in the same α1-helix side of Cys62 on HLA-G (Fig. 1D), Cys67–Cys67 disulfide bond formation of HLA-B27 could force the LILRB2-binding site to be exposed to solvent, analogous to the HLA-G dimer. The recent report of Gonen-Gross et al. (18) showed that HLA-G is expressed partly as β2m-free dimers and multimers with Cys42-mediated disulfide bonds, which cannot bind to LILRB1. In the future it will be of great interest to elucidate the avidity effect for the binding of the β2m-free HLA-B27 dimer to LILRB2. Furthermore, the expression of the β2m-free MHC II dimers by activated T cells may have important regulatory function (25). The β2m-free MHC II dimers would be partially misfolded and associated with other molecules, such as CD8αβ and the chaperone calreticulin/ERp57, and thus there exist some different conformations of the MHC II dimers. Moreover, in the MHC dimers the intermolecular disulfide bond was formed at least partially via Cys154, located in the α2-helix, which is the opposite site of Cys62 and Cys67. Therefore, the MHC
dimers of the activated T cells may also have different conformations from those of the HLA-G and HLA-B27 dimers. It is thus important to reveal the structural and functional basis for the receptor binding characteristics of the β2m-free MHC class I dimers in order to understand the regulation of T cell function.

Together with the previous investigations (12, 13, 45), our present structure showed that HLA-G can present a limited but still varied repertoire of peptides, which can be theoretically recognized by T cell receptors as classical MHC-I. Lenfant et al. (46) pointed out that HLA-G in vivo displayed the peptides derived from human cytomegalovirus UL83 protein in HLA-G transgenic mice, and their specific T cells were induced but not so efficient. The HLA-G monomer and dimer forms could exist on the cell surface; however, in the case of the HLA-G dimer, the peptide-bound surfaces of each monomer of the HLA-G dimer seem too close to be fully accessible for T cell receptors. Therefore, HLA-G dimer may not function as the antigen-presenting molecules for T cell responses but can present the LLR- and CD8-binding sites. These characteristics may partly explain the less efficient cytotoxic T cell induction, and would also be important for the receptor recognition of the β2m-free MHC class I dimers, regulating the T cell function.

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