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Special Organization of the HLA-G Protein on the Cell Surface

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ABSTRACT: The human leukocyte antigen G (HLA-G) molecule possesses unique properties such as low polymorphism and restricted distribution mainly to the extravillous cytotrophoblast (EVT) cells. The EVT cells vigorously penetrate into the maternal decidual tissues and are found in contact with maternal lymphocytes, mainly with natural killer (NK) cells. The HLA-G molecule inhibits the effector function of maternal NK cells via interaction with the KIR2DL4 and the ILT-2 inhibitory NK receptors. Previously, we have demonstrated that complexes of the HLA-G protein are expressed on the cell surface. We reported that these complexes are formed due to the presence of two unique cysteine residues located at positions 42 and 147. Finally, we demonstrated that efficient binding and function of ILT-2 is dependent on the presence of HLA-G complexes on the cell surface. Here we expand the significance of these observations by revealing that complexes of HLA-G are present on the cell surface using different assays and cell lines and further demonstrate that complexes of HLA-G might be present in a soluble form after interaction with ILT-2. Therefore, the HLA-G molecule has developed a special mechanism to increase the avidity of NK receptors to the HLA-G molecule, which provides better protection for the fetus from maternal NK rejection. *Human Immunology 64, 1011–1016 (2003).* © American Society for Histocompatibility and Immunogenetics, 2003. Published by Elsevier Inc.

KEYWORDS: HLA-G; ILT-2; NK; complexes; cysteine

ABBREVIATIONS

EVT	extravillous cytotrophoblast
ILT-2	Ig-like transcript-2
ITIM	immunoreceptor tyrosine-based inhibitory motif
NK	natural killer cell

INTRODUCTION

The ability of the semiallogeneic fetus to avoid immunologic maternal recognition during pregnancy is a major enigma in reproductive immunology. The EVT cells that invade the endometrium at the site of implantation and that are found in direct contact with maternal immune cells have been intensively investigated [1, 2]. Indeed, it was reported that one of the reasons accounting for the lack of immunologic rejection of the fetus is the fact that EVT cells do not express the classical major histocompatibility complex (MHC) class I molecules (human leukocyte antigens [HLA-A and -B]) and, therefore, are mainly protected from maternal T-cell mediated allorecognition [1]. In contrast, EVT cells do express a unique nonclassical MHC class I molecule, HLA-G [3, 4]. The HLA-G protein was reported to inhibit the function of maternal immune cells, such as T cells and natural killer (NK) cells [5]. The inhibition of NK cells by HLA-G is particularly important because NK cells are found in very high numbers in the decidua and are known to kill MHC class I deficient cells [6]. Several receptors such as KIR2DL4 (CD158d) [7, 8], ILT-4 (LIR-2), and ILT-2 (LIR-1 or CD85j) [9] were reported to bind HLA-G. In the work presented here we focused on the interactions between ILT-2 receptor and HLA-G protein. ILT-2 can bind to a wide range of MHC class I molecules [10, 11], including the UL18 protein, an MHC class I homolog of the cytomegalovirus [12]. The presence of four immunoreceptor tyrosine-based inhibi-

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tory motifs (ITIMs) on its cytoplasmic domain enables its inhibitory function [13, 14].

We have previously exhibited that HLA-G protein is found in complexes on the cell surface using an Epstein-Barr virus (EBV) transformed cell line 721.221 cells (.221) transfected with the HLA-G molecule [15]. These complexes are formed via disulfide bridges between two unique cysteine residues located in positions 42 and 147. Importantly, we demonstrated that these complexes play a major role in the efficient recognition of HLA-G by the ILT-2 receptor.

In the present study we demonstrate that the HLA-G can be probably found in complexes even in a soluble form. We report that the HLA-G complexes can be formed in various cell types and we use confocal microscopy technique to further demonstrate the significance of cysteine 42 and 147 in the generation of HLA-G complexes.

MATERIALS AND METHODS

Cells, Fusion Protein, and Monoclonal Antibodies

The cell lines used in this work are the EBV transformed MHC class I negative human B-cell line 721.221, 721.221 transfectants, the melanoma cell line LB33 mel B1 that expresses HLA-A24 molecule only [16], and LB33 mel B1 transfectants. Point mutations in HLA-G cDNA were performed by polymerase chain reaction (PCR) as previously described [15]. cDNA was cloned into the PcDNA3 vector (Invitrogen, San Diego, CA, USA), and was stably transfected into 721.221 or LB33 mel B1 cells as previously described [17].

The cDNA encoding for ILT-2 Ig fusion protein was kindly provided by Dr. Cosman (Immunex Corporation, Amgen Inc., Cambridge, MA, USA). The production of ILT-2 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 [18, 19]. The ILT-2 Ig protein was characterized by a single protein band on nonreduced SDS-PAGE and was routinely tested for its degradation. The anti-HLA-G monclonal antibody (mAb) used in this work is MEM-G/13B (IgG1) produced and characterized in the Prague laboratory.

Confocal Microscopy Staining Using LIR-1 Ig-Coated Beads

ILT-2 Ig proteins were covalently coupled to 6-m̈m carboxylated microparticles (Polysciences, Inc., Warrington, PA, USA) by using a carbodiimide kit (Polysciences, Inc.). Coated beads were incubated for 20 minutes at 37 °C and 5% CO_2 with the various .221 transfected cells. Cells were centrifuged and resuspended in ~200 μl of RPMI complete medium; 30 μl of cells coated with various beads were loaded on a glass slide and covered with an 18-mm coverslip. Slides were imaged immediately by confocal laser scanning microscopy 410 (Carl Zeiss Inc., Esslingen, Germany).

RESULTS

Visualization of Binding of ILT-2 Ig to HLA-G

We have previously demonstrated that the cysteine residues located in position 42 and 147 of HLA-G are involved in the formation of HLA-G complexes on the cell surface and that these complexes are important for efficient recognition by ILT-2 [15]. To further confirm our results, we used another assay, based on confocal microscopy, which enables direct visualization of the increased binding of ILT-2 Ig protein. Cells (.221) transfected either with the wild-type or mutated HLA-G proteins (HLA-G C42S and HLA-G C147S), were incubated with beads coated with the ILT-2 Ig fusion protein and imaged using a confocal microscope. Figure 1 illustrates an increased number of ILT-2 Ig-coated beads bound to .221/HLA-G transfectants (Figure 1A) compared with .221/HLA-G C42S (Figure 1B) or .221/HLA-G C147S transfectants (Figure 1C). This binding was specific, because no beads were observed bound to untransfected .221 cells (Figure 1D). The reduced binding of the ILT-2-coated beads to the mutated HLA-G transfectants was not due to a shortage of beads in the assay because free beads could be clearly observed (Figure 1).

It was recently reported that MHC class I proteins can be transferred from one cell to another after interaction with NK receptors [20, 21]. Strikingly, clustering of free beads can be clearly observed with .221/HLA-G cells only (Figure 1A). This result confirmed our previous observations and further suggests that complexes of HLA-G can be found on the cell surface and even as soluble proteins after recognition by ILT-2.

Binding of ILT-2 Ig to LB33 mel B1 Cells Expressing Mutated HLA-G Protein Is Decreased

Our previous results [15] were based on the transfection of the HLA-G molecules into 221 cells. In order to strengthen and generalize these results we transfected the wild-type and mutated HLA-G molecules into the melanome cell line LB33 mel B1 (mel B1), which expresses the HLA-A24 molecule only [16]. Staining of the wild-type and mutated HLA-G transfectants with a conformation-dependent mAb MEM-G/13B confirmed that the level of expression of the HLA-G protein in the various transfectants is similar (Figure 2). To directly investigate whether the cysteine mutations would affect the ILT-2 binding to the HLA-G molecule, we incubated the various mel B1 transfectants with ILT-2 Ig

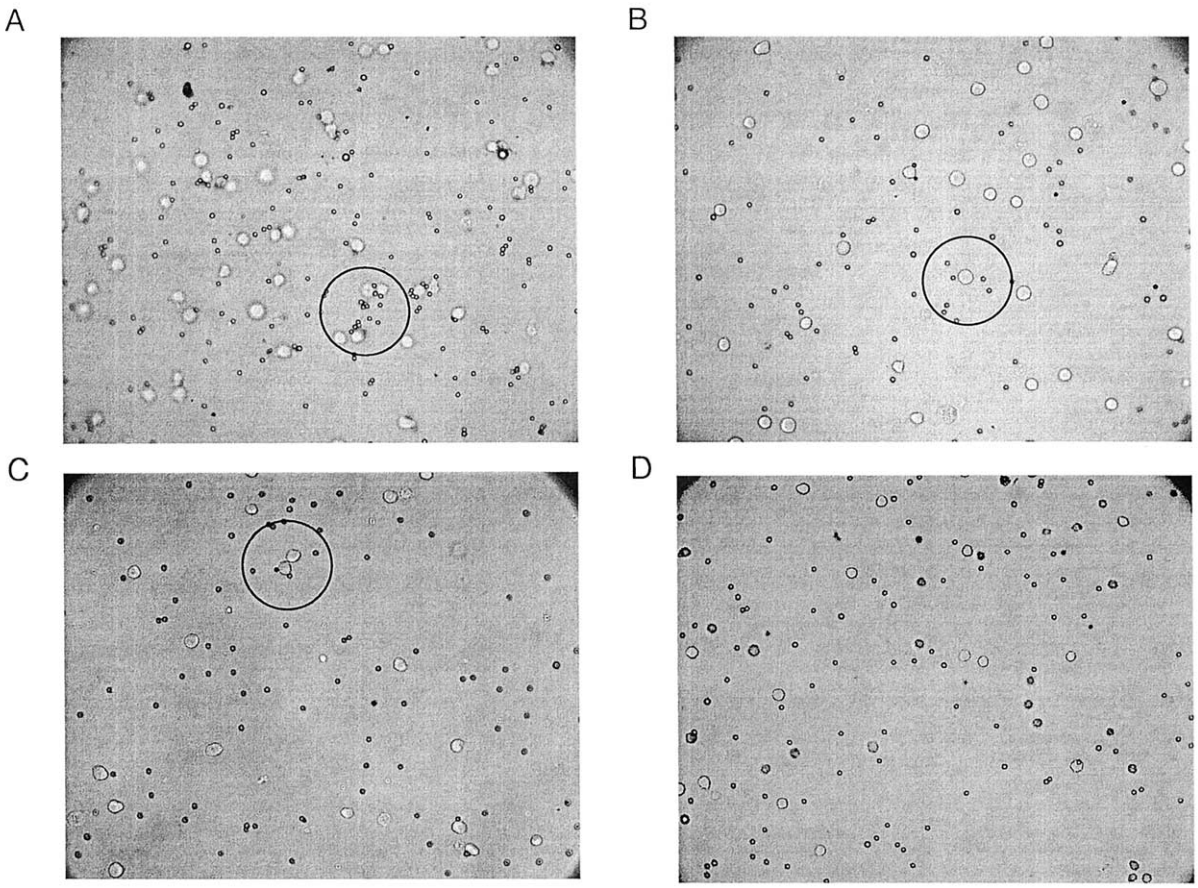


FIGURE 1 Imaging of the binding of Ig-like transcript-2 (ILT-2) Ig-coated beads to the various .221 transfectants. The .221 cells transfected with (A) human leukocyte antigen G (HLA-G), (B) HLA-G C42S, and (C) HLA-G C147S were incubated with beads coated with ILT-2 Ig protein and imaged using a confocal microscope. (D) Controls were untransfected .221 cells.

fusion protein. As illustrated in Figure 3, an effective binding of ILT-2 Ig was observed in the mel B1/HLA-G transfectants. This binding, however, was markedly decreased upon incubation with the mel B1 cells expressing either the mutated cysteine 42 or cysteine 147 HLA-G proteins. No binding of ILT-2 Ig was observed in the untransfected mel B1 cells.

DISCUSSION

A key role in the fetal-maternal tolerance has been attributed to the HLA-G molecule and its interaction with the maternal immune cells. This molecule has several unique characteristics that contribute to its ability to serve as an important immunomodulator protein, including its limited polymorphism [22], the presence of spliced mRNA variants [23], its impaired endocytosis [21], and its restricted distribution mainly to the EVT cells (reviewed in King *et al*. [24]). Recently we have reported that another unique feature of the HLA-G protein is its ability to form high molecular complexes on the cell surface [15]. These complexes exhibited a common phenomenon of increased recognition and inhibition through the ILT-2 receptor. Immunoprecipitation experiments revealed that the wild-type HLA-G molecule is expressed on the cell surface in a unique pattern of homotrimers, homodimers, and monomers. In accordance with these results we proposed a model in which the cysteine residue that is critical for the HLA-G complexes formation is cysteine 42. We also suggested that the presence of high molecular complexes on the cell surface increases the avidity of the ILT-2 receptor to the HLA-G molecule, thus enabling a better interaction between them. In this study we demonstrate, using confocal microscope, that ILT-2 better recognizes the wild-type HLA-G protein compared with the cysteine mutants. The generation of complexes of proteins on the cell surface might be a general phenomenon used to increase avidity and, consequently, the function of various receptors. Indeed, we have recently demonstrated that complexes of MHC class I proteins are formed in influenza virus infected cells and that these complexes,

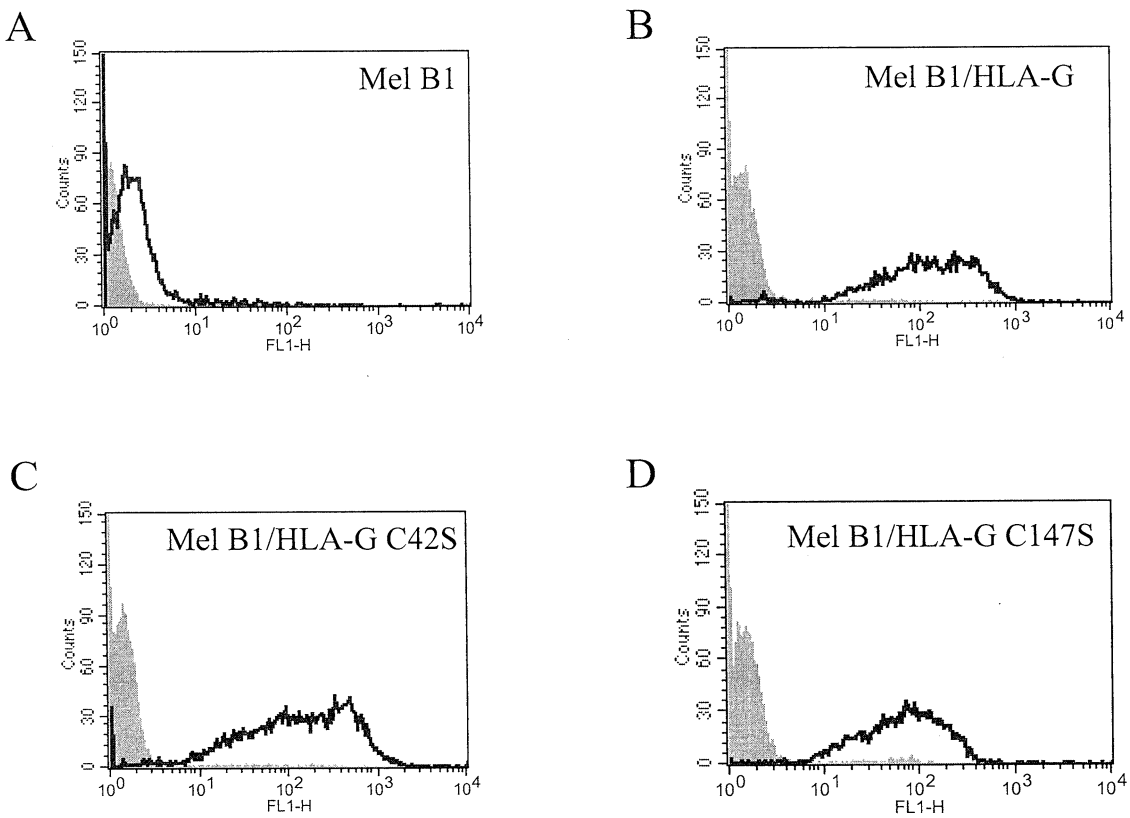
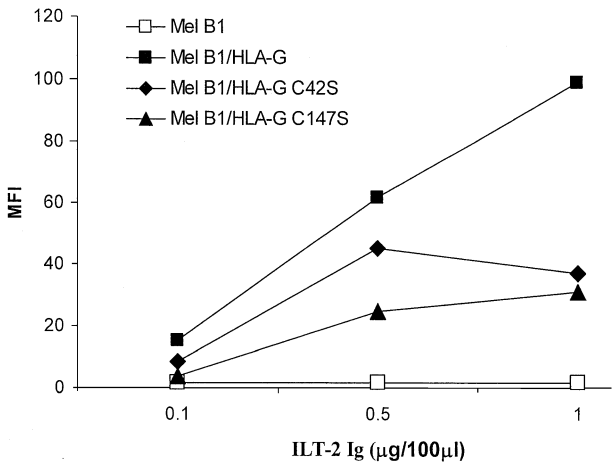


FIGURE 2 Staining of LB33 mel B1 transfectants with antihuman leukocyte antigen G (anti-HLA-G) monoclonal antibodies. LB33 mel B1 cells (A) untransfected and (B) transfected with HLA-G, (C) HLA-G C42S, and (D) HLA-G C147S were stained with anti-HLA-G monoclonal antibody MEM-G/13B, followed by FITC-conjugated goat antimouse Ig. Controls were the same cells incubated with FITC-conjugated goat antimouse Ig.

FIGURE 3 Binding of Ig-like transcript-2 (ILT-2) Ig to the different human leukocyte antigen G (HLA-G) transfectants. LB33 mel B1 cells transfected with the mutated and wild-type HLA-G proteins were incubated with increasing amounts of ILT-2 Ig proteins followed by PE-conjugated antihuman Fc antibodies. Controls were the same cells stained with PE-conjugated antihuman Fc antibodies.



similar to the HLA-G complexes, are important for efficient recognition by NK receptors [20]. The generation of complexes of MHC class I proteins, either through the cysteine interactions of HLA-G or because of an unknown mechanism in the infected cells, provides an effective tool for increasing the binding efficiency without the need for generating new proteins and investing metabolic energy. The generation of such complexes might be extremely important for the interaction between NK receptors and HLA-G molecules, which are expressed in relatively low levels on EVT cells.

Strikingly, cell-free aggregates of coated beads were observed only when ILT-2 Ig was incubated with HLA-G wild-type transfectants, which suggests that the HLA-G proteins maintained their special complex organization even in a solution. Similar cell-free aggregates were previously observed in the influenza virus infected cells [20].

The work presented here, and previously [15], raised some as yet unsolved and intriguing questions. It is clear that cysteine 42 is crucial for the binding and, indeed,

this residue is projecting out of the groove [25]. Yet, it is also clear that the trimers cannot be formed on the cell surface without the participation of residue 147, which is predicted to point into the groove. Therefore, would it be possible that the HLA-G oligomers are expressed on the cell surface in such organization that includes both conformed and β_2-microglobulin free heavy-chain complexes, and that these complexes are significant for the ILT-2 binding? Where are the HLA-G complexes formed, at the cell surface or intracellular? What are the kinetics of the HLA-G complexes formation, arrival, and retention on the cell surface? Are the HLA-G complexes involved in the inhibition of cytotoxic T-lymphocyte mediated killing?

Human leukocyte antigen-G is a fascinating protein expressed, in normal conditions, mostly on EVT cells that are in direct contact with the maternal immune system. A better understanding of the mechanisms controlling the fetus survival in the maternal immune environment might eventually lead to a better treatment of pregnancy-associated abnormalities.

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