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Placental Cell Expression of HLA-G2 Isoforms Is Limited to the Invasive Trophoblast Phenotype

Pedro J. Morales,* Judith L. Pace,† Jeralyn Sue Platt,* Teresa A. Phillips,* Kim Morgan,2* Asgi T. Fazleabas,§ and Joan S. Hunt3*‡

The HLA-G message is alternatively spliced into multiple transcripts, two of which encode soluble isoforms. To initiate studies on the specific functions of the soluble isoforms, we produced soluble rHLA-G1 (rsG1) and rsG2 in human embryonic kidney 293 cells and characterized the proteins. Both isoforms were glycosylated and formed disulfide-bonded oligomers. Recombinant sG1 associated with β2-microglobulin, whereas rsG2 did not. Mouse mAb generated to rsG1 (1-2C3), which identified exclusively sG1, and mAb generated to rsG2 (26-H11), which identified both soluble and membrane G2 (m/sG2), were used for immunohistochemical isomorph mapping studies on placental tissue sections. Soluble G1 protein was abundant in many subpopulations of trophoblast cells, whereas m/sG2 protein was present exclusively in extravillous cytotrophoblast cells. Although both isolated placental villous cytotrophoblast cells and chorion membrane extravillous cytotrophoblast cells contained mRNAs encoding sG1 and sG2, protein expression was as predicted from the immunostains with m/sG2 present only in the invasive trophoblast subpopulation. Analysis of function by Northern and Western blotting demonstrated that both rsG1 and rsG2 inhibit CD8 activation on PBMC without changing CD3 expression or causing apoptotic cell death. Collectively, the studies indicate that: 1) both sG1 and m/sG2 are produced in placentas; 2) transcription and translation are linked for sG1, but not G2; 3) expression of G2 is exclusively associated with the invasive phenotype; and 4) the two isoforms of sG may promote semiallogeneic pregnancy by reducing expression of CD8, a molecule required for functional activation of CTL. The Journal of Immunology, 2003, 171: 6215–6224.

Considerable information has been acquired on the class lb HLA-G gene first identified by Geraghty et al. in 1987 (1). HLA-G is characterized by low polymorphism, a 16-bp deletion within the enhancer A/IFN-γ-activated site (GAS)4 sequence (2–6). Multiple transcripts encoding both membrane-bound and soluble isoforms are generated by alternative splicing of the mRNA (7). Less is known of the proteins encoded by the transcripts, although it has been determined that at least three of the isoforms of HLA-G are membrane bound; HLA-G1 is the full-length isoform, whereas HLA-G2 and HLA-G3 lack the ΐ and ΐ domains, respectively. The two soluble isoforms, soluble HLA-G1 (sG1) and sG2, also known as HLA-G5 and HLA-G6, appear to resemble membrane HLA-G1 and HLA-G2, except for being truncated by a stop codon in the intron 4 region, which prevents translation of the transmembrane region (8). The secondary and tertiary characteristics of the proteins remain poorly understood, although it has been proposed that HLA-G2 proteins form homodimers and have HLA class II-like rather than class I-like characteristics (7).

HLA-G is reportedly characterized by a degree of tissue-specific expression, with the placenta identified as a major site of production of HLA-G messages and proteins (9–11). In the placental bed, where genetically different maternal and fetal cells reside in apparent accord, the unusual characteristics of the HLA-G gene may be of importance. For example, low polymorphism could ensure that most mothers will fail to recognize trophoblast-presented HLA-G as foreign (12). The deletion in the promoter region and substitution in the GAS element (1, 5), which preclude vigorous responses to endogenous and exogenous IFNs, could stabilize HLA-G expression through implantation and parturition. The spectrum of messages encoding different isoforms could encode proteins with distinctly different structures and functions, which would perhaps permit the polypeptides to perform different functions, as is the case with CD45 (13).

Many studies have now addressed functional aspects of HLA-G1 (14–16), but none has explored HLA-G2, the soluble isoform that appears to circulate in maternal blood throughout pregnancy (17) and is believed to compensate when HLA-G1 is absent (18). The studies reported in this work were therefore designed to investigate and compare structural aspects of sG1 and sG2 and to initiate functional experiments by determining: 1) whether either isoform is positioned appropriately at the maternal-fetal interface for influencing maternal immune cells, and 2) whether either isoform exerts such an influence.

To achieve these goals, we first produced rsG1 and rsG2 in eukaryotic cells so as to compare their biochemical features, then generated mAb to the two recombinant proteins. The new mAb were characterized and used to identify HLA-G soluble isoforms in tissues. In a final group of experiments designed to investigate the functional capacity of the rsG1 and rsG2 proteins, particularly their ability to modulate maternal immune cells, we studied the...
effects of rsG1 and rsG2 on expression of a major CTL Ag, CD8, in activated PBMC.

Materials and Methods

Isolation and cloning of HLA-G transcripts
cDNAs encoding HLA-G transcripts were isolated from Si4/8 cells, a mouse fibroblast cell line stably transfected with a 6.0-kb HindIII fragment of genomic DNA containing the full-length HLA-G gene (gift from B. Koller, University of North Carolina, Chapel Hill, NC, and H. Orr, University of Minnesota, Minneapolis, MN), JEG-3 (HTB-36), and Jar (HTB-144) choriocarcinoma cells obtained from American Type Culture Collection (ATCC, Manassas, VA), as well as villous trophoblast cells and extravillous cytotrophoblast cells isolated and purified, as described below. Total RNA was prepared using TRIzol (Invitrogen, Carlsbad, CA) and treated with DNase I (Sigma-Aldrich, St. Louis, MO). Transcripts encoding membrane HLA-G1 (mG1) and HLA-G2 (mG2) were isolated from villous and extravillous cytotrophoblast cells, respectively, by RT-PCR using G4S-65 (5'-GCC TTC ACC CGT CTG AAC GAG ACG-3') as the forward primer and G1225 as the reverse primer (19), then were TA cloned into pGEM-T Easy Vector System I (Promega, Madison, WI). To develop expression constructs for mG1 and mG2, the insertions were released by EcoRI digestion, purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA). Restriction sites for cloning into the modified expression vector pRC/CMV (20) (Invitrogen), a gift from B. Hudson (University of Kansas Medical Center, Kansas City, KS), were used to clone mG1 and mG2 from Si4/8 cell RNA; two PCR were performed. Messages obtained by RT-PCR using the G4S-65 primer and G1225 were purified as above, then subjected to a second PCR using the following cloning primers: A-NheIMTW, 5'-CTA GCT AGC TAG AGG CTC CCA CTC CA-3'; Xba I: B-XbaIMTW, 5'-CTA GCT TAG ACT AGA TCA TCA TGA CTC TCT-3'. To obtain transcripts encoding sG1 and sG2 from Si4/8 cell RNA, two PCR products were obtained, divergent messages obtained by RT-PCR using the G4S-65 primer and G1225 were purified as above, then subjected to a second PCR using the following cloning primers: A-NheIMTW, 5'-CTA GCT AGC TAG AGG CTC CCA CTC CA-3'; Xba I: B-XbaIMTW, 5'-CTA GCT TAG ACT AGA TTA AAG GTC TTC AGA-3'. Plasmids containing XbaI and XhoI sites were inserted into the XhoI and XbaI sites of the modified vector pRC/CMV (20) (Invitrogen). Plasmid constructs were sequenced by using the ABI PRISM XL BIG DYE sequencing system in the Biotechnology Support Facility at the University of Kansas Medical Center.

Transfections

Human embryonic kidney cells (HEK293, ATCC, CRL-1573) were stably transfected with the constructs encoding sG1 and sG2. HEK293 cells were maintained at 37°C in 5% CO2 in DMEM supplemented with 10% FBS and penicillin (100 U/ml) with streptomycin (0.1 mg/ml). Transfection was performed using LipofectAMINE Plus (Invitrogen), according to the manufacturer’s specifications. Stable transfectants were selected using G418 (Invitrogen) and cloned by limiting dilution. Selection of rsG1- and rsG2-producing clones (patents pending) was achieved by using an ELISA to test for the proteins. HEK293 cells were transiently transfected to obtain mG1 and mG2 proteins using essentially the same system, and were tested for cell surface expression of mG1 and mG2 by flow cytometry after 24 h in culture.

Preparation of purified rsG1 and rsG2

Recombinant sG1 and sG2 proteins were purified from supernatant culture medium by immunoaffinity chromatography using an anti-FLAG-M2 affinity resin (Sigma-Aldrich). Each lot was tested for LPS using the Pyroell detection gel-clot formulation Limulus amebocyte lysate assay (Associates of Cape Cod, Falmouth, MA). Only LPS-free lots were used in functional experiments.

Immunoblotting

Immunoblotting was used to identify rsG1 and rsG2 purified from culture supernatants as well as to determine association of β2-microglobulin (β2-m) with these proteins. The technique was also used to identify cell surface markers on PBMC and to detect cleavage products in an apoptosis assay. In brief, proteins were resuspended in Laemmli buffer (21) under reducing conditions, separated by electrophoresis in acrylamide gels (10 or 15% SDS-PAGE), and electrotransferred onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Recombinant sG1 and sG2 were detected using the mAb 16G1, which is specific for HLA-G intron 4 amino acids (a gift of D. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA) (0.2 μg/ml) and anti-FLAG M1 Ab (Sigma-Aldrich) (1 μg/ml). β2-m was identified using a mAb from Amac (Westbrook, ME), at 1 μg/ml. To detect cell surface markers on PBMC, goat anti-human CD80 and mouse anti-human CD36, both from Santa Cruz Biotechnology (Santa Cruz, CA), and rabbit anti-human β-actin (Sigma-Aldrich) were used. In these experiments, each blot was hybridized sequentially for CD80, CD36, and β-actin. Apoptosis assays were done by investigating poly(ADP-ribose) polymerase (NAD+ ADP ribosyltransferase, EC 2.4.2.30) (PARP) cleavage (Oncogene Research Products, Boston, MA). For all immunoblots, signal was detected using appropriate secondary Ab and SuperSignal West Pico (Pierce, Rockford, IL). Thereafter, the blots were exposed to Hyperfilm ECL (Amersham Pharmacia Biotech, Piscataway, NJ).

Detection of glycosylation

Detection of glycosylation patterns, purified rsG1 and rsG2 were digested under native and denaturing conditions using an enzymatic deglycosylation kit that cleaves N-linked and sialic acid-substituted Gal β1–3 GalNAC α1–2 (D-linked) complex carbohydrates from glycoproteins (Bio-Rad Laboratories, CA). After enzymatic digestion, the samples were denatured in reducing Laemmli buffer, separated on SDS-PAGE, transferred onto nitrocellulose membranes, and detected with the mAb 16G1, as described above.

Detection of oligomerization

Disulfide-based self dimerization was tested by running 4 μg of each purified protein on a 4–20% gradient SDS-PAGE under reducing and non-reducing conditions, transferring and detecting using 16G1, as before.

ELISA

Recombinant protein production was monitored by ELISA, which were constructed and performed essentially as previously described (22), with the exception that the microwells (replicates of three) were coated with supernatant culture media using an appropriate HLA-G clone. The coated plates were incubated with either 16G1, anti-β2-m (Amac), W6/32 (ATCC, HB-95), normal mouse IgG (Vector Laboratories, Burlingame, CA), or isotype controls IgG1 and IgG2a (BD PharMingen, San Diego, CA). ELISA were also constructed and used to characterize the new mAbs described below. The new mAbs were tested for reactivity to pooled HLA class I Ags purified from human platelets (a gift from K. Kao, University of Florida, Gainesville, FL) (23), FLAG-purified rsG1 and rsG2, purified β2-m (Sigma-Aldrich), and intron 4 peptide KEDGGMVSRESRSL (Biotechnology Support Facility, University of Kansas Medical Center). For both ELISA, bound Ab was detected by the addition of HRP-conjugated anti-mouse IgG (Vector Laboratories), followed by tetramethylbenzidine substrate (Kirkegaard & Perry, Gaithersburg, MD). After stopping the reaction with 1 M H3PO4, the plates were read at 450 nm with an ELX808 microplate reader (Bio-Tek Instruments, Winooski, VT).

mAb generation and characterization

mAbs to rsG1 and rsG2 were generated in the facility of the University of Illinois (Urbana-Champaign, IL) using standard techniques (24, 25). Ten-week-old female BALB/c mice were immunized by i.p. injection using 25 μg of rsG1 or rsG2 for each injection. Mouse sera that screened positive for Ab reactive to rsG1 or rsG2 were sacrificed, and standard hybridoma technology was applied. After 2–3 wk, hypoxanthine/aminopterin/thymidine-resistant cultures were isolated, and supernatant culture medium was screened for reactivity to rsG1 and rsG2 by ELISA. Hybridomas producing Ab exclusively reactive with either rsG1 or rsG2 were cloned by limiting dilution, expanded, and frozen.

Flow cytometry assays

Flow cytometry experiments were performed according to standard methods. Briefly, HEK293 cells transiently transfected with vector, mG1, or mG2 were tested using the following mAb: W6/32 (2.5 μg/ml), 1-2C3 (10 μg/ml, 26-2H11 (10 μg/ml), or isotype control (IgG1 or IgG2a, 10 and 2.5 μg/ml), respectively). Untransfected (721.221) and HLA-G1-transfected (721.221-G1) lymphoblastoid cells were tested using anti-HLA-G1 (87G, 5 μg/ml), 1-2C3 (2.5 μg/ml), and isotype controls, as before. Cells were incubated with Ab on ice for 30 min, then were washed four times with buffer (PBS containing 0.1% sodium azide and 1% BSA). Bound Ab was detected by staining with the F(ab')2 of R-PE-conjugated sheep anti-mouse IgG (Sigma-Aldrich). Cells were fixed in PBS containing 1% paraformaldehyde and analyzed by flow cytometry using a FACSCalibur and CellQuest software (BD Biosciences, San Diego, CA).

Tissue collection and purification of villous and extravillous cytotrophoblast cells

First trimester and term placenta as well as extraaplacental membranes were obtained from elective pregnancy terminations and normal term deliveries, respectively, in accordance with a protocol approved by the Human Subjects Committee of the University of Kansas Medical Center. For
immunohistochemical experiments, random areas of the tissues were manually dissected into 1-cm² sections, embedded in TBS (tissue-freezing medium; Triangle Biomedical Sciences, Durham, NC), and stored at −80°C until sectioned for immunohistology. The protein products are represented below the transcripts. FLAG peptide is located in the N terminus, as is the enterokinase (Ek) site used to release the FLAG tag. The 21 aa from intron 4 are located in the C terminus. a, Schematic depiction of the exon-intron organization of the gene encoding HLA-G, the soluble HLA-G-encoding splice variants generated by intron 4-retaining transcripts, and the protein products generated by the constructs. Upper panel. The arrows indicate the positions of the second pair of primers used for isolation of the transcripts encoding the soluble isoforms. The protein products are represented below the transcripts. Lower panel. The protein products are represented below the transcripts. Lower panel. b, Identification of rsG1 and rsG2 by immunoblotting with anti-FLAG and 16G1, a mAb directed toward HLA-G intron 4 amino acids.

Binding of 16G1 to the recombinant proteins in ELISA. No binding was detected when normal mouse IgG was substituted for 16G1. c, Immunoblot demonstrating failure of binding of anti-β2m to rsG2 (lower panel) with loading identified by anti-FLAG (upper panel). d, Expression demonstrating that under both native and denaturing conditions, deglycosylation causes faster migration patterns of the recombinant proteins. e, Evidence for disulfide-based dimers in purified rsG1 and rsG2. M = monomers, D = dimers.

**FIGURE 1.** Expression and characterization of rsG1 and rsG2 in HEK293 cells. a, Schematic depiction of the exon-intron organization of the gene encoding HLA-G, the soluble HLA-G-encoding splice variants generated by intron 4-retaining transcripts, and the protein products generated by the constructs. b, Identification of rsG1 and rsG2 by immunoblotting with anti-FLAG and 16G1, a Fab directed toward HLA-G intron 4 amino acids. c, Binding of 16G1 to the recombinant proteins in ELISA. No binding was detected when normal mouse IgG was substituted for 16G1. d, Immunoblot demonstrating failure of binding of anti-β2m to rsG2 (lower panel) with loading identified by anti-FLAG (upper panel). e, Expression demonstrating that under both native and denaturing conditions, deglycosylation causes faster migration patterns of the recombinant proteins. f, Evidence for disulfide-based dimers in purified rsG1 and rsG2. M = monomers, D = dimers.
μg/ml was used as a negative control. Binding of Ab was detected using 3-amino-9-ethylcarbazole (Histostain-SP; Zymed Laboratories), which yields a red signal in positive cells. All immunostained tissue sections and cells were counterstained with Mayer’s hematoxylin (Sigma-Aldrich).

Assessment of biological activity of rsG1 and rsG2
PBMC were cultured in the absence or presence of human rIFN-γ (100 U/ml; Genzyme Diagnostics, Cambridge MA) and were simultaneously treated with PBS, rsG1, or rsG2 (50 nM) for 6 h at 37°C for Northern analyses or 12 and 24 h for immunoblots. The cells were then lysed in TRIzol reagent for RNA extraction and tested by Northern blotting or were solubilized in radioimmunoprecipitation buffer (PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, PMSF, aprotinin, leupeptin, and EDTA) and tested by immunoblots. These same lysates were used for apoptosis assays.

Northern blot analysis
PBMC were incubated in serum-free lymphocyte medium (AIM-V; Invitrogen) alone (control), or with 100 U/ml of human rIFN-γ (Genzyme Diagnostics), with or without rsG1 or rsG2 (50 nM) for 6 h. The RNA was extracted by TRIzol reagent (Invitrogen) and quantified as before. Northern blots were performed, as previously described, with 5 μg of total RNA loaded into each lane of a 1% agarose-formaldehyde gel. Human CD8α, CD3δ, and β-actin DNA fragments used as probes were generated by RT-PCR using PBMC total RNA. The specific primers for CD8α were: upper, 5'-CCG CCG CCA GTC CCA CCT TCC TAT-3'; lower, 5'-ATG GCC GCC GTT GGT GTG CTC-3'. Primers for CD3δ were: upper, 5'-GTG GCC GGC ACC CTC ATG AGA TGG-3'; lower, 5'-ATG TGA AGG GCC TCG TAG GTG TCC-3'. Both sets were selected using Primer Select Laser Gene (DNASTAR, Madison, WI). To identify β-actin mRNA, previously reported primers were used (28). The amplicons were gel purified, cloned into pGEM-T Easy Vector System I (Promega), and sequenced for authenticity. Each blot was probed first with CD8α, stripped and reprobed for CD3δ, then stripped and reprobed for β-actin. The membranes were hybridized according to the procedures for QuiikHyb (Stratagene, Cedar Creek, TX). The blots were exposed to Hyperfilm (Amersham Pharmacia Biotech), and the OD of each treatment band and corresponding OD were calculated. Northern blots were hybridized according to the procedures for QuikHyb (Stratagene). Each reaction was repeated at least twice and a representative Northern blot from one experiment is shown (Fig. 1a).

Results
Generation and biochemical characterization of eukaryotic rsG1 and rsG2
To study the structure and function of sG isoforms, we generated rsG1 and rsG2 in HEK293 cells, then compared the two proteins in biochemical assays. The intron-exon structure of the HLA-G gene is shown in Fig. 1a, as are the proteins derived from transcripts encoding the two soluble isoforms. Recombinant sG1 and rsG2 were produced by subcloning PCR-generated amplicons from S14/8 cells, a mouse fibroblast cell line stably transfected with 6.0 kb of the HLA-G gene (29), into a modified pRC/CMV vector (20) (Fig. 1a), then transfecting these into HEK293 cells. In initiated experiments to establish the cellular distribution of sG1 and sG2 at the maternal-fetal interface, which might assist in predicting function(s) during pregnancy.

To achieve this goal, we used rsG1 and rsG2 to stimulate mAb in mice. Following generation and cloning by standard techniques, two high-producing clones, 1-2C3 (IgG1, κ-chain) generated to rsG1 and 26-2H11 (IgG1, κ-chain) generated to rsG2, were selected for final characterization studies. The results of ELISA used to test for the specificity of the two mAb are shown in Fig. 2 and summarized in Table I. In Fig. 2, 1-2C3 recognized only rsG1, and 26-2H11 strongly bound rsG2. Neither mAb recognized pooled HLA class I Ag. As a positive control, we used the mAb 16G1 (generated to intron 4-encoded sequences present only in soluble isoforms), which reacted strongly with both rsG1 and rsG2, as expected, but not with pooled HLA class I Ag (Fig. 2). Additional ELISA (data not shown) excluded the possibility that either mAb

<table>
<thead>
<tr>
<th>Table I. Ab reactivities in ELISA</th>
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<tr>
<td>Ag</td>
</tr>
<tr>
<td>-------</td>
</tr>
<tr>
<td>Intron 4</td>
</tr>
<tr>
<td>β₂m</td>
</tr>
<tr>
<td>HLA mix</td>
</tr>
<tr>
<td>rsHLA-G1</td>
</tr>
<tr>
<td>rsHLA-G2</td>
</tr>
</tbody>
</table>

* Mouse IgG isotype controls: IgG1 and IgG2a.
* +, Positive; 0, negative.

Production and characterization of mouse mAb to rsG1 and rsG2
Because earlier experiments in our laboratory indicated that production of sG isoforms is a feature of normal pregnancy (17), we initiated experiments to establish the cellular distribution of sG1 and sG2 at the maternal-fetal interface, which might assist in predicting function(s) during pregnancy.

Acknowledgments
The authors thank Drs. Margaret K. Mahoney and Catherine B. Leibovic for expert advice, Dr. Mary H. Lee for help with antibody generation and Dr. Robert A. Chau for helpful discussion and assistance with cell transfections. We also thank Dr. Gary D. Crabtree for helpful suggestions and encouragement throughout the course of this work, and Drs. John H. Driscoll and John H. McKeating for their support and support of this work. These studies were supported by grants from the National Institutes of Health (AI-12210 and AI-20142) and the National Science Foundation (BIR-9000726).
HLA class I proteins, that were not held in common with the other soluble isoform, other epitopes on properly glycosylated and folded rsG1 and rsG2 identified in tissue sections. These include villous cytotrophoblast cells, syncytiotrophoblast, and extravillous cytotrophoblast cells.

Collectively, therefore, the data indicated that 26-2H11 identified rsG2 in culture supernatants, these results indicated that 26-2H11 identified both soluble and membrane isoforms of G2. Therefore, 26-2H11 is subsequently referred to as anti-m/sG2.

Flow cytometry experiments to detect membrane-bound HLA-G

The ELISA presented above showed definitively that the two new mAb identified the soluble isoforms. To ascertain whether or not the mAb identified the membrane-bound isoforms as well as the soluble isoforms, HEK293 cells were transiently transfected with constructs encoding vector alone, mG1 (obtained from term villous cytotrophoblast cells), and mG2 (obtained from term chorion membrane extravillous cytotrophoblast cells), then tested by flow cytometry.

Table II shows that essentially 100% of the cells in all three transfected cell lines bound W6/32, which was expected because the HEK293 cells express HLA class I Ag. Neither cells transfected with vector alone (negative control) nor cells transfected with mG1 bound 1-2C3 or 26-2H11. Taken together with the ELISA results in which 1-2C3 readily identified rsG1, these results showed that 1-2C3 exclusively identifies the soluble isoform. To verify this interpretation, additional flow cytometry experiments were done with this mAb and 721.221 cells transfected with vector or mG1 (721.221-G1) (data not shown). The 87G, which identifies both mG1 and sG1, was strongly positive with the mG1-transfected cells, but not with vector-transfected cells. The 1-2C3 was negative with both cell lines. Thus, 1-2C3 is subsequently referred to as anti-sG1.

Although cells transfected with vector and mG1 were negative with 26-2H11, 32% of the cells transfected with mG2 were positive (Table II). Taken together with the ELISA results in which 26-2H11 readily identified rsG2 in culture supernatants, these results indicated that 26-2H11 identified both soluble and membrane isoforms of G2. Therefore, 26-2H11 is subsequently referred to as anti-m/sG2.

Structural features of the placenta

In early gestation, distinct subpopulations of trophoblast cells are readily identified in tissue sections. These include villous cytotrophoblast cells, syncytiotrophoblast, and extravillous cytotrophoblast cells. In late gestation, the villous cytotrophoblast cell supply is nearly exhausted and only isolated cells lying directly beneath the synctium are present in the villous placenta (Fig. 3, c and f). The multinucleated syncytiotrophoblast layer is prominent. The extravillous cytotrophoblast cells have ceased migrating and have formed the defined chorion membrane, which lies between the amnion epithelial membrane enclosing the fetus and amniotic fluid, and the modified maternal endometrium termed the decidua (Fig. 3, c and f).

Table II. Binding of 1-2C3 and 26-2H11 to HEK293 cells transiently transfected with vector alone (293-v), with mG1 (293-mG1), or with mG2 (293-mG2) constructs evaluated by flow cytometry

<table>
<thead>
<tr>
<th>Ab</th>
<th>293-V</th>
<th>293-mG1</th>
<th>293-mG2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MFI^a</td>
<td>% Positive^b</td>
<td>MFI^a</td>
</tr>
<tr>
<td>PE anti-mouse IgG</td>
<td>44</td>
<td>&lt;0.5</td>
<td>49</td>
</tr>
<tr>
<td>IgG1, 10 μg/ml</td>
<td>45</td>
<td>&lt;0.5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>IgG2a, 2.5 μg/ml</td>
<td>41</td>
<td>&lt;0.5</td>
<td>&lt;10</td>
</tr>
<tr>
<td>1-2C3, 10 μg/ml</td>
<td>48</td>
<td>&lt;0.5</td>
<td>51</td>
</tr>
<tr>
<td>26-2H11, 10 μg/ml</td>
<td>46</td>
<td>&lt;0.5</td>
<td>42</td>
</tr>
<tr>
<td>W6/32, 10 μg/ml</td>
<td>239</td>
<td>96</td>
<td>210</td>
</tr>
</tbody>
</table>

^a MFI, median fluorescence intensity of positive cells as determined from histograms obtained by plotting log fluorescence vs cell number.

^b Cells having a MFI greater than 10 were considered positive. The MFIs of 293-V, 293-mG1, and 293-mG2 cells stained with control IgG1 or IgG2a, followed by PE anti-mouse IgG were similar (mean = 2.1, range low to high).
Localization of sG1 and m/sG2 at the maternal-fetal interface

The next set of experiments was designed to learn whether HLA-G2 was expressed in cells at the maternal-fetal interface, where it could influence immune cell function(s), and, if so, in which specific cells the protein is found. To achieve this goal, immunohistochemical experiments were done using the new mAbs. In each experiment, a matching tissue section was taken onto the same slide and tested with IgG1 as a substitute for the primary mAb. These controls were negative in all experiments (insets to Fig. 4, a–d).

Fig. 4a shows that anti-sG1 bound to villous cytotrophoblast cells in first trimester placentas and was also present in blood.
Staining was weaker in second trimester placentas (Fig. 4b), but remained detectable in term tissues (Fig. 4c). Soluble G1 was extensive in the maternal blood spaces, where aggregates of protein stained strongly for sG1. Anti-sG1 detected abundant sG1 in the amniochorion membrane (Fig. 4d). Staining with 1-2C3 was granular, suggesting that the protein was located in membrane vesicles suited to export of the protein.

In contrast to the staining pattern exhibited by anti-sG1, antim/sG2 reacted exclusively with extravillous cytotrophoblasts. In early placentas (Fig. 4e), particularly in cells distal to the villi, reactivity was strong. No staining was observed in villous cytotrophoblasts, syncytiotrophoblast, or villous stromal cells. The same pattern was observed in wk 16 placentas (Fig. 4f). Anti-m/sG2 did not bind to any cells in term villous placentas (Fig. 4g), but stained some chorionic cytotrophoblasts in the amniochorion (Fig. 4h). For both new mAb, the same results were obtained on an additional two samples each of first trimester and term placentas and amniochorion membranes.

The new mAb did not detect Ag in paraformaldehyde-fixed, paraffin-embedded tissues.

Transcription and translation are linked in sG1, but not in m/sG2

Having observed in placentas and extraplacental membranes that in situ sG1 was abundant in many trophoblast cell subpopulations, but that m/sG2 proteins were found only in cells that had emerged from villi, we sought to verify this by testing isolated cells obtained from within the villi (term placental cytotrophoblast cells) and those that had migrated from the villi (term chorion membrane cytotrophoblast cells).

Identification of mRNAs encoding sG proteins. We tested for sG1 and sG2 mRNAs using RT-PCR. Total cell mRNA was amplified from the isolated cells using primers G45–65 (signal peptide sequence) and G1225 (3′ untranslated region), after which the transcripts that were obtained were reamplified using nested primers (intron 4-specific primer and G1225). These experiments identified a single band of ∼450 bp in purified villous and chorionic extravillous cytotrophoblast cells, as expected (data not shown). Controls for the experiment consisted of testing RNA from the trophoblast-derived choriocarcinoma cell line, Jar (negative control), and the trophoblast-derived choriocarcinoma cell line, JEG-3 (positive control). The results were as expected. The 450-bp amplicon was shown by sequence analysis to comprise sG mRNA.

To investigate which isoform(s) was present, a second PCR from the same sample was performed using A-Blunt/B-XbaI primers. Two amplicons were obtained from villous and extravillous cytotrophoblast cells as well as from JEG-3 cells. The two amplicons of ∼900 and 700 bp were gel extracted and purified (Fig. 5). Sequence analysis revealed that the 900-bp amplicon was specific for sG1 (containing intact α1, α2, α3, and intron 4 coding regions), and the 700-bp amplicon was specific for sG2 transcripts (α1 coding region joined directly to α3 plus coding portion from intron 4).

Identification of sG1 and m/sG2 proteins. The experiments reported above therefore predicted that both villous and extravillous cytotrophoblast cells would contain sG1 and sG2. To test the prediction, purified villous and extravillous cytotrophoblast cells were cytopun onto glass slides and evaluated by immunocytochemistry using anti-sG1 and anti-m/sG2. As shown in Fig. 6a, the morphologically homogenous, purified villous cytotrophoblast cells were >95% positive with cytokeratin-7. As expected from the immunohistochemical stains, but not from the RT-PCR results, these cells bound anti-sG1 (Fig. 6b), but not anti-m/sG2 (Fig. 6c). The purified extravillous cytotrophoblast cells from chorion membranes, which were morphologically diverse as expected (35), comprised >95% trophoblast cells, as determined by testing for cytokeratin-7 (Fig. 6d). The cells were positive with anti-sG1 (Fig. 6e). Furthermore, ~15% of these cells exhibited reactivity with anti-G2 (Fig. 6f), as predicted by the immunohistochemical stains on chorion membranes in situ as well as by the RT-PCR. Staining appeared to be both cytoplasmic and membrane (Fig. 6f). Isootype-specific and normal mouse Ig controls for these experiments were negative.

IFN-γ-mediated up-regulation of CD8α mRNA and protein is abrogated in blood mononuclear cells by rsG1 and rsG2

The experiments reported above showed that sG1 is present in many trophoblast subpopulations as well as in blood, and that m/sG2 proteins are prominently expressed in invading trophoblast cells, which ultimately contact the maternal immune cell-containing decidua. To evaluate the postulate that either isoform might affect the ability of maternal CTL to attack the placenta, we investigated the ability of the rsG1 and rsG2 proteins to influence CD8 expression.

Northern blot analyses were done, the results were quantified by scanning densitometer, then CD8α and CD3δ signals were normalized to β-actin, as shown in Fig. 7a. In two separate experiments, steady state levels of CD8α mRNA were elevated ~2-fold when PBMC were incubated with IFN-γ (100 U/ml). This increase was completely abrogated by cotreatment with either rsG1 or rsG2. By contrast, levels of CD3δ mRNA in stripped and rehybridized blots remained unchanged (data not shown).

To test for changes in the proteins, immunoblots were done. The results are shown in Fig. 7b. Again, rsG1 and rsG2 decreased CD8α proteins, while having no effect on CD3δ proteins. β-actin was unchanged throughout. Thus, the decreases in CD8α accomplished by rsG1 and rsG2 appeared not to be due to diminished numbers of T lymphocytes. Immunoblots were repeated on a second harvest of PBMC with the same results.

rsG1 and rsG2 do not induce apoptosis in PBMC

Because of a previous study in which partially purified, soluble HLA-G1 from tumor cells reportedly killed PHA-activated T cells (14), we investigated the possibility that the reduction we observed in CD8α mRNA and protein might result from cell death via the apoptotic pathway. However, as shown in Fig. 7c, cleavage products of caspase-3 (CPP32, CASP3) were not observed in either rsG1- or rsG2-treated PBMC. By contrast, HeLa cells used as a positive control and treated with etoposide demonstrated apoptosis-associated activation of caspase-3.

Discussion

This study is the first to report: 1) the generation of rsG1 and rsG2 in eukaryotic cells, 2) biochemical characteristics of the proteins,
3) differential expression of sG1 and m/sG2 in placentas with exclusive expression of m/sG2 by invasive cytotrophoblast cells, and 4) evidence that both soluble isoforms of HLA-G decrease CD8 expression on lymphocytes without stimulating cell death.

Comparison of the structural features of the two recombinant proteins showed clearly that rsG1 associates with β2m, whereas rsG2 does not, which has been the subject of debate (7, 36). The sG1 isoform thus appeared to have the expected L chain, H chain configuration that would facilitate normal peptide binding. By contrast, rsG2 gave evidence of existing naturally as a homodimeric glycoprotein that resembles HLA class II more than HLA class I (7); dimers, but not monomers of rsG2 were identified under non-reducing conditions. However, both isoforms demonstrated oligomerization related to disulfide bonding, as predicted from a recent study that did not distinguish among HLA-G isoforms (37). Whether such oligomerization impairs or facilitates activity remains to be determined, and differential binding of peptides remains unexplored.

Because both sG1 and sG2 were abundant in the culture supernatant medium of sG1- and sG2-transfected HEK293 cells, our results disagree with reports (30, 31) that the smaller isoforms, including sG2, are not released from producing cells. These disparate results are likely to be due to the nature of the constructs used for transfections. The Bainbridge construct (30), which is not described in detail, is artificial rather than natural and might not contain all of the elements needed for intracellular transport and secretion.

Although mAb generated to rsG1 (1-2C3) and rsG2 (26-2H11) identified their specific soluble isoform in culture supernatants of transfected HEK293 cells, flow cytometry experiments with mG1- and mG2-transfected HEK293 cells demonstrated that only 1-2C3 was entirely specific for the soluble isoform. The mAb to rsG2 identified mG2 as well as sG2. Epitopes recognized by the two mAb did not survive fixation of tissues with paraformaldehyde, and did not yield signals in sensitive immunoblots, which limits their usefulness to some extent. A number of other mAb to HLA-G have not been previously identified exclusively with the invasive trophoblast phenotype. By testing isolated villous and extravillous cytotrophoblast cells, we demonstrated definitively that both subpopulations of trophoblast cells transcribe the messages and translate the specific mRNAs into protein identified by anti-sG1. Identification of sG1 mRNA and protein in villous cytotrophoblast cells was not unexpected; Solier et al. (41) have reported that the sG1 mRNA is present in extravillous cytotrophoblast cells. Taken together, our results support the idea that cytotrophoblast cells are the major source of sG1 in placental membranes.

The observation that membrane and/or soluble HLA-G is associated exclusively with the invasive trophoblast phenotype was entirely unexpected, as there are no data in the scientific literature on the expression of these isoforms. In immunohistochemical experiments, the mAb to m/sG2 bound only to cytotrophoblast cells.
in the leading edge of trophoblast columns in first trimester placentas and to some chorionic cytotrophoblast cells, which are derived from the invasive trophoblast cells. This latter finding was confirmed by identifying m/sG2 protein in isolated chorion membrane cells by using 26-2H11. We established that m/sG2 protein is very likely to be produced in the cells, not synthesized in other cells, and internalized by the membrane cells by showing that the cells contain mRNAs encoding both mG2 and sG2. Whether some of the smaller isoforms of HLA-G are able to reach the cell surface and be secreted has been a matter of debate (30, 42). Our results are consistent with those of Riteau et al. (42), who reported membrane insertion of the small isoforms, but not those of Bainbridge et al. (30), who failed to identify membrane insertion. Other results indicate that appropriate processing does occur; soluble isoforms that appear to be mainly sG2 (or free H chains) circulate in maternal blood throughout pregnancy (17). The studies reported in this work show clearly that invasive trophoblast cells are the logical source of this protein.

This highly unexpected finding may have profound biological implications. It will be important to learn, for example, whether or not m/sG2 is aberrantly expressed in diseases of pregnancy that involve reduced cytotrophoblast cell migration such as pre-eclampsia. It is known from studies on women with a genetic alteration resulting in an inability to produce the two isoforms of HLA-G that lack of HLA-G1 does not prevent mothers from concluding successful pregnancies (18). We suggested in that report that G2 may substitute when G1 is missing. The probability that G2 can substitute when G1 is missing is supported by the studies of Lila et al. (43), who demonstrated that successful heart transplantation is related to the presence of circulating sG, but is unrelated to the specific isoform.

Yet, expression of G2 may not be critical only to pregnancy; these isoforms might well have a role in migration/invasion or other cellular functions that are not limited to trophoblast. As with HLA-G, the single transcript derived from the lymphocyte CD45 gene is also differentially spliced into messages encoding multiple (nine) isoforms (13). Expression of cell-specific CD45 isoform(s) is related to lymphocyte differentiation, growth, viability, and specific function. Even more interestingly, isoform switching occurs in CD45. For example, stimulation of thymocytes expressing CD45R epitopes results in their switching to CD45R(0) expression and apoptosis. These parallels suggest that switching into expression of m/sG2 might result from an environmental encounter and might influence multiple cytotrophoblast cell activities.

The third major finding in this study was that both rsG1 and rsG2 are biologically active and are fully capable of influencing expression of a critical Ag in CTL, i.e., CD8. Northern blot analysis showed that in a relatively short time period, 6 h, IFN-γ enhanced steady state levels of CD8α in PBMC. This has not, to our knowledge, been previously reported. CD8α is a subunit of the coreceptor CD8 molecule, which is expressed on the surface of CD8+ T cells and has an important role in CTL activation and, subsequently, graft rejection (44, 45). Concurrent treatment with either rsG1 or rsG2 abrogated the IFN-γ-induced increase in steady state levels of CD8α mRNA and protein in IFN-γ-activated PBMC. This protocol had no observable effect on CD3 δ mRNA or protein, and stimulated no apoptosis in treated cells.

Our findings dispute those of Fournel et al. (14), who reported that partially purified sG stimulated Fas/FasL-mediated apoptosis in PHA-stimulated CTL function, but are in accord with those of Wiendl et al. (46) and Le Fric et al. (47), who also failed to identify death of HLA-G-treated cells. Possibly, different conditions of testing are the reason for the disparate results, or perhaps the preparations used in the earlier report (14) contained apoptosis-inducing contaminants. In this study, we show that sG treatment of IFN-γ-activated PBMC simply decreased levels of the coreceptor molecule, CD8α, without any apparent killing. The biological implications of HLA-G-induced cell death and simple reduction of a cell surface molecule are entirely different. Our observations suggest that CTL might well remain in the pregnant uterus, but be undetected with the usual anti-CD8 mAb and functionally impaired because of low CD8 (48, 49).

In summary, in this work, we present the first report of cell-specific expression of HLA-G isoforms in human placentas as well as critical information on the biological functions of the two structurally different isoforms. It will be of the utmost importance to establish how each isoform functions in pregnancy as well as in tumors and other types of transplantation (50–52).

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**Figure 7.** IFN-γ up-regulation of CD8α is abrogated by cotreatment with rsG1 and rsG2. a. Shows the results of two Northern blot experiments in which PBMC were cultured for 6 h in the absence (control) or presence of IFN-γ, with or without 50 nM of rsG1 or rsG2. Data are shown as fold increase following analysis by scanning densitometer. b. Shows an immunoblot to detect changes in relative protein levels of expression at 12 and 24 h posttreatment. Note that at both 12 and 24 h posttreatment with IFN-γ, CD8α is up-regulated and that this up-regulation is abrogated by the simultaneous addition of either rsG1 or rsG2. CD3δ remained unchanged at both time points. c. An apoptosis assay (PARP cleavage) demonstrates that IFN-γ with or without the addition of rsG1 or rsG2 did not induce cleavage. HeLa cells treated with ectodiposide as a positive control demonstrated PARP cleavage.
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