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Immunology

Alternative splicing of HLA-G transcripts yields proteins with primary structures resembling both class I and class II antigens

(mRNA/nonclassical class I antigen/placenta)

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ABSTRACT **We have investigated HLA-G mRNA expression in cells and tissues expressing the gene. This analysis has demonstrated that the HLA-G primary transcript is alternatively spliced to yield at least three distinct mature mRNAs. Sequencing of the transcripts has shown that the largest mRNA is essentially that previously characterized, encoding a leader sequence, three external domains, a transmembrane region, and a cytoplasmic sequence. Of the two smaller messages, a 900-base mRNA does not include exon 3, resulting in a predicted protein sequence with the α1 and α3 external domains joined. The smallest mRNA results from splicing out exons 3 and 4, connecting the α1 domain directly to the transmembrane sequence. Alternative splicing of HLA-G mRNA was found in placental tissues and in eye tissue as well as in HLA-G-transfected cell lines. In term placental tissue the smallest mRNA appeared to be more abundant than the full-length form, while in a cell line derived from an earlier developmental stage the larger form predominated. Immunoprecipitation of [^{35}S]methionine-labeled cell lysates showed that three different HLA-G proteins were present in transfected cells, with sizes corresponding to those predicted from the three alternative mRNA sequences. These findings are discussed in terms of potential functions of the alternative HLA-G proteins.**

Class I antigens are heterodimers that consist of a 40- to 45-kDa glycosylated heavy chain anchored in the cell membrane, in association with a 12-kDa light chain, β_2-microglobulin. In humans, these antigens consist of a family of highly polymorphic molecules encoded in the HLA-A, -B, and -C loci of the major histocompatibility complex. The exceptional polymorphism of these molecules is of considerable interest in clinical transplantation because they are able to elicit strong antibody and cytolytic-T-lymphocyte responses upon allogeneic stimulation. In addition, cytolytic T lymphocytes and their precursors are restricted in their capacity for antigen recognition of virus-infected or otherwise modified cells by the associative recognition of self-HLA class I molecules (1).

The human class I gene family has been studied in detail and three new class I genes, HLA-E, -F, and -G, have been discovered (2–5). Perhaps the most significant recent finding about the nonclassical antigens since their discovery is that HLA-G is expressed in cytotrophoblast cells of the placenta (6–8). Not only is HLA-G expressed on the surface of these cells, but a secreted form can be detected in supernatants of cultured cytotrophoblasts and cell lines (6). We have further investigated HLA-G expression by examining the structure of HLA-G mRNA derived from a variety of sources. It was apparent from this analysis that the primary HLA-G transcript is differentially spliced, giving rise to at least three different forms. Potential functions of the proteins derived from these alternatively spliced mRNAs are discussed.

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MATERIALS AND METHODS

Cell Lines and Tissues. Cell lines .221, JEG-3, and HS738 were obtained from the ATCC. Transfectants were constructed by electroporation using the pHEBo vector with the HLA-G gene cloned into the *Hin*dIII site as described (9) and were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 200 μg of hygromycin (Calbiochem) per ml. Term placenta and extravillous membrane tissue were obtained locally from normal deliveries. Two adult eyes were obtained from the Lion's Eye Bank at the University of Washington Medical Center.

RNA Isolation and PCR Amplification. RNA was prepared according to a guanidinium isothiocyanate procedure (10). cDNA was prepared by random hexamer priming (11) from 1–3 μg of total RNA. The reaction mixture was incubated at 37°C for 1 hr and then at 95°C for 5 min. The PCR was carried out with 5 μl of the cDNA reaction mixture in a total volume of 25 μl containing primers Gc1.2 and Gc2 (50 pM each), dNTPs (200 μM each), and PCR buffer (Cetus). Gc1.2 (5′-CCCAAGCTTCCCTGACCCTGACCGAGACCTGGG-3′) contains a *Hin*dIII linker at the 5′ end followed by 24 bases from the 5′ leader sequence of HLA-G. Antisense primer Gc2 (5′-CCGGAATTCGGCTGGTCTCTGCACAAAGAGA-3′) contains an *Eco*RI linker followed by a 22-base sequence from the 3′ untranslated region. The PCR was run at 94°C for 1 min, 65°C for 2 min, and 72°C for 3 min for 35 or 40 cycles depending on the source of RNA. Actin primers were bAC5.1 (5′-TCGTCGTCGACAACGGCTCC-3′) and bAC3.2 (5′-GAAGCATTTGCGGTGGACGA-3′). Reaction products were separated by electrophoresis in a 1% agarose gel and stained with ethidium bromide.

cDNA Cloning and Sequencing. cDNAs amplified in a PCR were excised from a gel by using glassmilk (Bio 101, La Jolla, CA) and digested with *Hin*dIII and *Eco*RI. After phenol/chloroform extraction, the DNA fragments were ligated with digested M13 mp18 and mp19 and sequenced (12). Several clones were analyzed and a representative of each of the three alternative HLA-G cDNAs that contained an unaltered HLA-G DNA sequence was excised and subcloned into vector pT3T7 (BRL).

RNase Protection Assays. RNase protection probes were constructed by using the full-length HLA-G cDNA (referred to in this report as HLA-G1) as a source of DNA subcloned into vector pT3T7 as described (13). Probe rG23 was constructed by cloning the 314-base-pair (bp) *Bgl* II–*Pvu* II fragment from the HLA-G1 cDNA, containing portions of domains α2 and α3, into *Bam*HI/*Sma* I-cut pT3T7. Probe rG3T consists of the 248-bp *Sst* I fragment from HLA-G1 cDNA cloned in *Sst* I-cut pT3T7 and includes the terminal

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portion of α3 and the transmembrane sequence. Both probes were linearized with *Eco*RI, phenol/chloroform-extracted, and ethanol-precipitated. The β-actin probe was constructed by subcloning the 460-bp *Taq* I fragment (positions 25–712 in ref. 14) isolated from PCR amplified DNA into pT3T7. Labeled RNA probes were synthesized by using T7 RNA polymerase (BRL) with [α-^{32}P]CTP (400 Ci/mmol; 1 Ci = 37 GBq) under conditions recommended by BRL. After elongation, the synthesized RNA was purified through a 6% polyacrylamide gel and eluted. Hybridization of the labeled probe (5×10^5 cpm) with 5–80 μg of total RNA was performed overnight in 80% formamide with Pipes buffer at 45°C (13). RNase digestion was initiated by the addition of 300 μl of 300 mM NaCl/10 mM Tris, pH 7.5/5 mM EDTA containing RNase T1 (700 units/ml; BRL) and RNase A (40 μg/ml; BRL). RNase was destroyed by the addition of proteinase K (50 μg/ml; BRL) and SDS (1%) followed by incubation for 10 min at 37°C. Protected fragments were analyzed in 5% polyacrylamide sequencing gels.

Protein Labeling and Immunoprecipitation. Cells were metabolically labeled for 4 hr with [^{35}S]methionine (100 mCi/ml) in methionine-free RPMI medium; the labeling period was followed by a chase with complete medium for 1 hr. The membrane-bound protein was solubilized with Triton X-114 and the lysates were precleared with protein A-Sepharose three times. Samples were incubated with saturating concentrations of antibody for 2 hr and, after washing, were precipitated with protein A-Sepharose. Samples were denatured and then electrophoresed in an SDS/6% polyacrylamide gel. The gels were treated with En^{3}Hance, dried, and exposed to Kodak XAR-5 film at −70°C.

RESULTS

Identification of Alternatively Spliced Forms of HLA-G mRNA. We used the PCR with primers derived from the 5′ leader and 3′ untranslated sequences to amplify cDNA from RNA of placenta, of the choriocarcinoma cell line JEG-3, of HLA-G-transfected B-lymphoblastoid cells, and of the parent B-lymphoblastoid cell line .221 (Fig. 1). Three predominant bands of approximate size 1200, 900, and 600 bp were observed. In addition, two to three fainter bands were apparent. All of these bands were excised from a gel, subcloned into M13 mp18 and mp19, and sequenced by standard methods.

The full-length copy (G1 in Fig. 1) was similar to the previously described cDNA sequence (7). Both the 900-bp and 600-bp cDNAs were found to be altered in a rather surprising and unpredicted way. The 900-bp cDNA, G2, results from exon 3 having been spliced out, leaving a deduced protein with only the α1 and α3 external domains. The 600-bp cDNA has an additional domain removed, leaving only the leader and α1 regions spliced with the transmembrane, cytoplasmic, and untranslated domains. Relevant portions of autoradiograms of the sequences at the points where the three transcripts are differentially joined are presented in Fig. 2. The alternative splicing in HLA-G2 joins an A residue from the α1 domain with AC from α3 to create an AAC (asparagine) codon rather than the GAC (aspartic acid) codon found at the beginning of the α3 domain in HLA-G1. The alternative splicing generating HLA-G3 does not generate a new codon at the splice junction.

The bands between those labeled G1, G2, and G3 in Fig. 1 were also cloned into M13 vectors and sequenced. Several M13 clones derived from each band were analyzed, and all of these sequences were found to be identical to that from one of the G1, G2, or G3 bands. One explanation for the apparent discrepancy of the band size with the sequence results is that these interbands are in fact heterologous paired molecules similar to those described in other systems (15). That is, one strand in a duplex is paired with a smaller molecule, with G1 pairing with G2, G1 with G3, and G2 with G3. It appears then that only three alternative forms of mRNA are amplified by the HLA-G-specific primers used in this study.

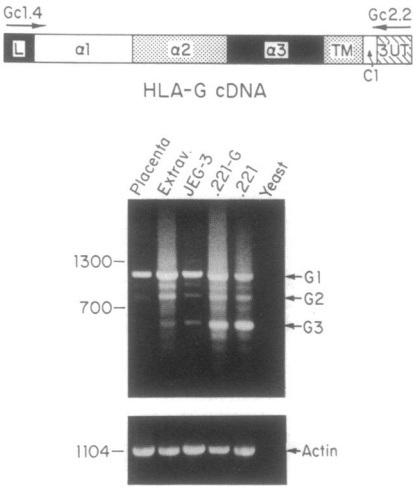

FIG. 1. PCR amplification of HLA-G cDNA from tissues and cell lines. (*Top*) Primers Gc1.4 and Gc2.2 are indicated approximately above the positions in the cDNA sequence from which they were derived. L, leader; α1–α3, external domains; TM, transmembrane; C1, cytoplasmic; 3′UT, 3′ untranslated. (*Middle*) PCR products amplified from various sources of RNA and fractionated in an agarose gel. Yeast RNA served as a negative control. Cell line .221 has one intact copy of the HLA-G gene (9). Line .221-G is .221 transfected with the HLA-G gene cloned into the pHEBo vector. JEG-3 is a choriocarcinoma cell line obtained from the ATCC. Placenta and extrav refer to RNA isolated from term placental tissue and from the extravillous membrane, respectively. Size markers (bp) are indicated at left. Bands G1, G2, and G3 are those DNAs described in the text. (*Bottom*) PCR products amplified by β-actin (14)-specific primers fractionated in an agarose gel. The single β-actin band is indicated.

We have used RNase protection to confirm the existence of the alternative forms and to quantitate each mRNA, as this method is sensitive and allows accurate estimates of relative levels (13). Indicated in Fig. 3 are the regions of the HLA-G1 cDNA used in these experiments. Probe rG23 can be protected by two mRNAs, yielding bands of 314 and 189 bases after RNase digestion corresponding to G1 and G2, respectively. A second probe, rG3T, derived from the α3 and transmembrane region of G1 cDNA, is protected by the G1 and G2 mRNAs, yielding a band of 248 bases, and has a 115-base portion protected by G3 mRNA.

These probes were used to analyze total RNA from term placental tissue, extravillous membrane, and various cell lines. The relative levels of the G1, G2, and G3 mRNAs can be estimated by comparing the intensity of the bands in the autoradiogram from the different-sized products. Placental tissue as well as cell line JEG-3 appeared to have similar levels of G1 and G2 mRNAs. However, the relative amounts of total HLA-G per cell was highest in the .221-G transfectants; 5 μg of total RNA gave signals nearly equal to that seen in 40 μg of JEG-3 and extravillous membrane mRNA. Analysis of 80 μg of .221 RNA did not give detectable bands corresponding to the G1 or G2 mRNAs in this length of exposure. However, PCR using .221 cDNA did amplify all three bands (Fig. 1). A significant difference in relative levels of G1 and G3 is apparent between the term placental tissues and lines .221-G and JEG-3. Placental and extravillous membrane both contained significantly more G3 mRNA than the combined G1 and G2 mRNAs. The opposite relative levels appeared in JEG-3 and .221-G, with more G1 and G2 mRNA than G3 mRNA. In addition, .221 mRNA contained relatively

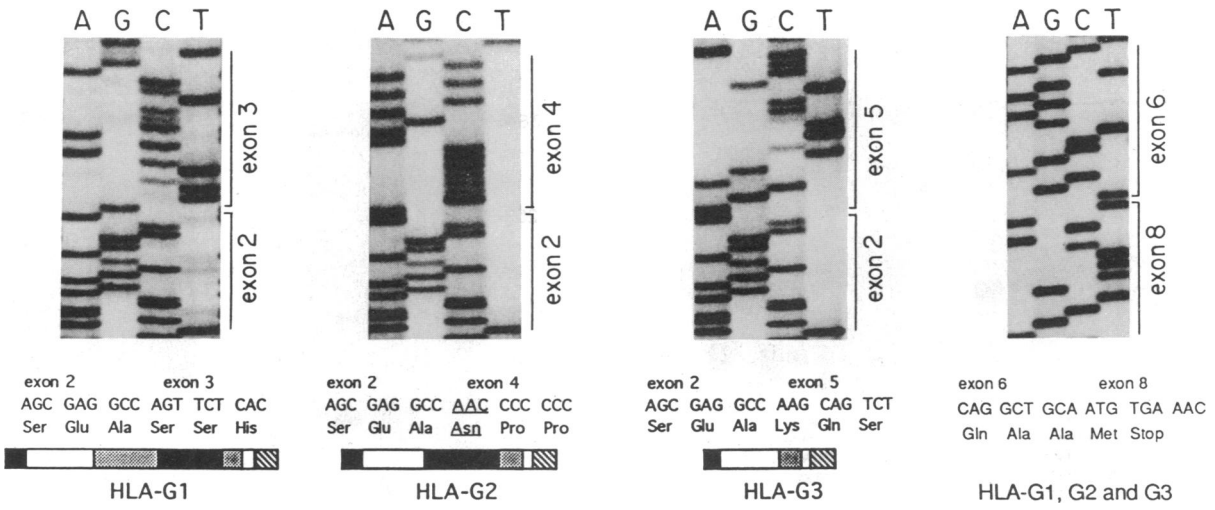

FIG. 2. Sequence analysis of PCR-amplified DNAs derived from placental mRNA. The autoradiograms show the regions within the respective cDNA sequences where alternative splicing has occurred to form the G1, G2, and G3 mRNAs. Below each autoradiogram is a cartoon depicting the complete cDNA sequence with the exons designated as shown in Fig. 1.

low but significant levels of G3 mRNA. Probe rG3T did not detect products corresponding to the presence of an mRNA containing the $\alpha 3$ domain but missing the transmembrane exon.

The RNase protection results differed quantitatively from the results of the PCR. This was not surprising, since PCR is not quantitative when three distinct cDNAs are amplified from the same primers in the same PCR. Our experience has shown that the relative levels of the three amplified G1, G2, and G3 cDNAs varies depending on several variables including the source of reverse transcriptase and the PCR machine used (unpublished data). However, in order to control for the quantity and quality of the RNAs used in both the PCR and RNase protection assays, β-actin (14)-specific primers and a β-actin-specific RNA probe were used. These results indicated that the RNA was largely intact and that the quantity of RNA was within a factor of 2 of that estimated (Figs. 1 and 3).

Expression of HLA-G in Eye Tissue. Although HLA-G expression was first detected in placental tissue, some evidence exists for its expression in eye tissue (16). Cell line HS738 is a normal eye cell line derived from eye tissue (obtained from the ATCC). This line is primary and is comprised of a mixed population of cells and was therefore subcloned in order to examine whether the individual cells had similar patterns of HLA-G expression. In addition, mRNA from adult anterior and posterior eye was isolated and examined. These RNAs were subjected to RNase protection using the rG23 probe detecting the G1 and G2 forms and to PCR using 5′ and 3′ primers (Fig. 4). For comparison, RNA choriocarcinoma line JEG-3 was included. RNA from the original eye cell line HS738 protected a band corresponding to the full-length G1 and G2 mRNA essentially similar in relative levels of G1 and G2 to the results obtained with JEG-3 mRNA, although the percentage of HLA-G mRNA of the total was considerably lower. These results confirmed that HLA-G is expressed in the eye cell line. It was also apparent that the relative levels of the alternative forms present in the HSsub1 subline were different from those found in the parent line HS738. RNase protection showed the G2 band to be much more abundant in line HSsub1 than in the parent line, whereas the G1 band was barely detectable. Consistent with this result, only the G2 band was amplified in a PCR using HSsub1 cDNA (Fig. 4*B*). In total, we analyzed five HS sublines and found one additional line that gave patterns identical with the HSsub1 pattern and three that gave a pattern identical with the pattern of the parent line.

Total RNA was isolated from the anterior and posterior portions of two adult human eyes and used in an RNase protection experiment with probe rG23. Very low but detectable levels of the G1 band were protected by anterior eye mRNA. Lower levels, but still above background, were protected by the posterior eye sample. However, it was not possible to distinguish a G2 band above background in either of the eye mRNA samples. The PCR analysis confirmed the presence of G1 mRNA in the anterior eye mRNA and amplified the G2 band, giving a pattern similar to that of the eye cell line, although the G3 band was not detected. The PCR was unable to amplify visible levels of DNA from the posterior sample. It is apparent that the levels of HLA-G RNA are quite low in eye tissue, probably <1 copy per cell. This level of expression is consistent with HLA-G mRNA being expressed in a subset of cells located in the anterior portion of the eye.

Analysis of Alternative Protein Forms. Previous work (9) analyzing HLA-G protein expression of .221 cells did not detect the G2 and G3 forms even though these cells do express the three alternative mRNAs. To obtain evidence that all three proteins are in fact expressed, we analyzed protein expression in transfected cells by using monoclonal antibodies we had developed that are specifically reactive with cells expressing HLA-G (unpublished data). The class I null cell line .221 was transfected with the HLA-G gene as described (9). Both .221 and .221-G cells were metabolically labeled with [^{35}S]methionine and total protein was incubated with monoclonal antibodies reactive with HLA-G-transfected cells. Results of a representative immunoprecipitation are shown in Fig. 5. While antibody w6/32 bound strongly to the mature protein, no band of the predicted size of the G2 form was apparent. However, a second HLA-G-specific antibody, g30, did immunoprecipitate distinct proteins that were present only in HLA-G-transfected cells. This antibody was isolated by immunizing mice with mouse fibroblasts transfected with the HLA-G gene. The antibody reacts specifically with HLA-G-transfected .221 cells and with the transfected mouse cells. The g30 immunoprecipitation showed a larger protein, which appeared to be the G1 form, while a second band migrated at a size essentially that expected for the G2 protein. The relative amounts of the G1 and G2 proteins were similar, although the relative affinity of this antibody for the two proteins is unknown and thus an accurate quantization is not possible. A third band specific to the .221-G transfectants was also apparent in both the w6/32

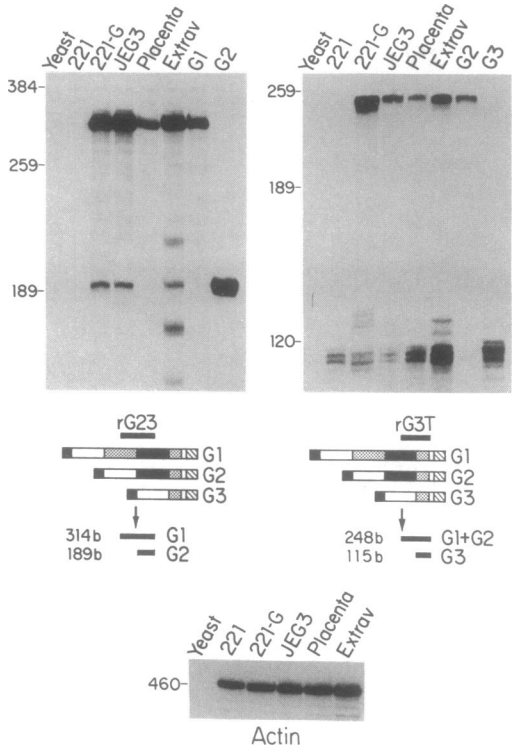

FIG. 3. RNase protection of HLA-G mRNA from transfected cell lines and placental tissue. Positions of RNA size markers are shown to the left of each autoradiogram. Listed above each lane is the source of the RNAs analyzed. Lanes labeled G1, G2, and G3 contained homogeneous preparations of each of the three mRNAs mixed with total .221 mRNA. These RNAs were produced by cloning each of the HLA-G cDNAs into the RNA transcription vector pT3T7 and producing RNA with the T3 RNA polymerase and serve as positive controls of the RNase protection experiments and as additional size markers. The amount of total RNA used in each hybridization was 5 μg for .221-G, 80 μg for .221, and 40 μg for JEG-3 and placental tissues. Both probes were derived from HLA-G1 cDNA as described in *Materials and Methods*. The cartoons below the autoradiograms indicate the regions from which the probes were derived and the sizes [in bases (b)] of the protected fragments resulting from an RNase protection experiment done in the presence of G1, G2, and G3 mRNA. Shown at the bottom are the results of analysis with a β-actin-specific probe, using 20 μg of each of the indicated RNAs.

and g30 immunoprecipitates. The apparent size of 18 kDa and its specific expression in transfected cells indicated this band may be the HLA-G3 protein.

DISCUSSION

We have demonstrated that the HLA-G primary transcript is alternatively spliced, giving rise to at least three distinct mRNAs. Only one of these mRNAs had previously been recognized. The HLA-G1 transcript does not include exon 7, which contains no protein coding information because of the introduction of a stop codon in exon 6. The G2 and G3 mRNAs preserve this feature. In addition, the G2 mRNA excludes exon 3, thereby bringing together the α1 and α3 domains of the protein. Because the class I exons divide codons, this alternative splicing results in an asparagine residue being introduced at the point of joining which is not present in the mature protein. The third form, G3, does not contain exons 3 and 4 and results from the splicing of the α1 domain directly to the transmembrane sequence. All three mRNAs are translated into proteins in the .221-G cell line, and all three mRNAs are expressed in placental tissue, placental-derived cell lines, and HLA-G-transfected cells.

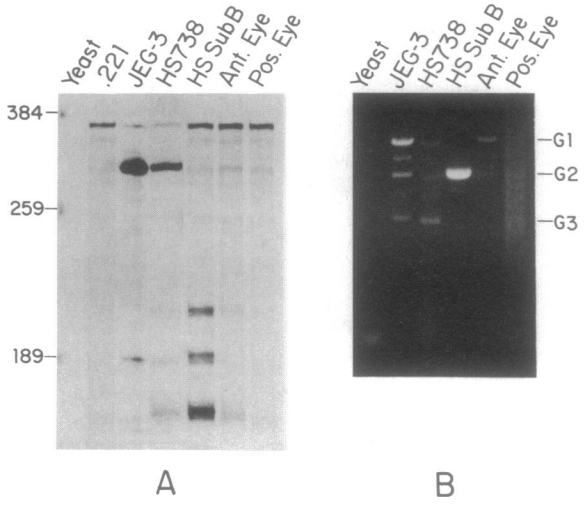

FIG. 4. Detection of HLA-G mRNA in eye tissue. RNA from eye cell line HS738, a subline derived from HS738 (HS SubB), and adult anterior (Ant.) and posterior (Pos.) eye tissue was analyzed in an RNase protection assay (*A*) and in a PCR assay (*B*). Probe rG23 (Fig. 3) was used in the RNase protection experiments and the primers described in Fig. 1 were used in PCR assays.

Alternative splicing of nonclassical and classical class I primary transcripts has been described. Krangel (17) has demonstrated that some HLA-A and -B alleles expressed in cultured cells have alternatively spliced transcripts that exclude exon 5 and thus could produce a class I protein without a transmembrane domain. The HLA-G transcripts share the exclusion of exon 7 with HLA-F transcripts (ref. 3 and unpublished data) and with one of two distinct murine H-2D mRNAs (18). A soluble form of the Qa-2 antigen is encoded by an alternatively spliced mRNA lacking exon 5 (19), and cDNAs from Qa genes lacking exons 4–7 have been described (20). None of these alternative forms, however, is similar to the G2 and G3 mRNAs.

The relative levels of G1 and G3 mRNAs contrast sharply between the choriocarcinoma cell line JEG-3, which is de-

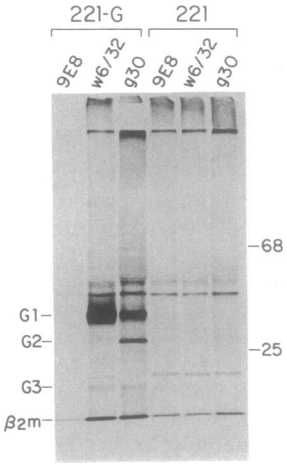

FIG. 5. Immunoprecipitation of the HLA-G isoforms from transfectant .221-G. Positions of size (kDa) markers are indicated at right. The left three lanes show the products of an immunoprecipitation from transfected cells. Antibody 9E8 is an IgG2a monoclonal reacting with a mouse retroviral protein and serves as a negative control. w6/32 is a monomorphic antibody reactive with all HLA class I proteins, including HLA-E, -F, and -G (9). Antibody g30 is a monoclonal specifically reactive with HLA-G isolated as described (unpublished data). The rightmost three lanes show immunoprecipitates from untransfected .221 with the same antibodies. β_2m, β_2-Microglobulin.

rived from an early developmental placental stage, and term placenta and extravillous membrane. These cell types do not appear to have substantially different relative levels of G1 and G2 mRNA. Conversely, the eye cells in line HS738 apparently consist of a mixture of distinct cells some of which express predominantly HLA-G2. While developmental regulation of the alternative forms is only implied by comparing results from cell lines and tissues, comparisons of mouse cell lines have reflected expression patterns *in vivo* (19).

The alternative forms of the HLA-G protein raise interesting speculations about their surface expression. Because it has $\alpha 3$ and transmembrane sequences identical to those of G1, the G2 protein might be expected to react with w6/32 if G2 were associated with β_2-microglobulin. In fact, the results of immunoprecipitation with w6/32 suggest that is not the case (Fig. 5). In addition, the two external domains of the G2 protein bear a striking resemblance to the two external domains of a class II protein. The $\alpha 1$ region in a class I molecule forms one half of the antigen binding site, consisting of one α-helix and half of the β-pleated sheet that forms the floor of the binding site. A similar structure is envisioned for the first external domain of a class II α or β chain (21). The $\alpha 3$ domain lies underneath the antigen binding site and serves as scaffolding holding up the presenting portion of the molecule. In a class II molecule this structure is assigned to the second of the two external domains. With these considerations, it is tempting to speculate that a nonpolymorphic HLA-G2 homodimer or perhaps an HLA-G2 class II α-chain heterodimer acts as a surrogate class II molecule on cytotrophoblasts.

Recent evidence suggests that a homodimeric form of CD8 binds to .221-G-transfectant cells (22). Clearly a question is raised as to which of the HLA-G forms CD8 is binding. Since one of the CD8 binding regions is located in the $\alpha 3$ domain of class I molecules (23), it is unlikely the G3 protein plays a role. However, both G1 and G2 contain $\alpha 3$ and therefore both may bind CD8. The possibility that the G2 protein in a homodimeric form interacts with the α_2 homodimer of CD8 whereas the heterodimeric G1 protein associated with β_2-microglobulin interacts with the $\alpha\beta$ CD8 heterodimer should be considered.

The expression pattern of HLA-G and the absence of allelic polymorphism have led to the hypothesis that HLA-G may play a fundamental role in protecting the placenta from a maternal immune response (6–8). Data described by Redman *et al.* (24) suggest that a class I protein, presumably HLA-G, may be present in cytotrophoblasts found in the placental bed but is either missing or weakly expressed on cytotrophoblasts of the floating chorionic villi. This upregulation is suggestive of a critical role for HLA-G in the normal interactions of maternal and fetal cells in the placental bed. The methods used in that work would not have detected the HLA-G2 and -G3 forms, leaving in question whether or not they are upregulated as well.

The observation that HLA-G is expressed in the anterior eye supports the hypothesis that HLA-G is involved in inducing immune tolerance. Like the placenta, the anterior chamber of the eye is recognized as an immune privileged site. An additional similarity between placental and eye tissue is the presence of cells lacking the expression of the classical HLA antigens. Considering the different needs for immune protection in the two tissues, it is possible that a subset of the alternative HLA-G proteins are required in eye tissue where distinct cell types may differentially express the alternative forms.

Nonclassical antigens are not conserved among different species to the same extent as the classical antigens. One explanation proposed suggests that the lack of conservation reflects different selective pressures and adapted specialized functions in each species (25). The HLA-G gene is certainly a striking example in support of that hypothesis. When the work described here and that described for the Qa-2 antigen in the mouse (19) are considered, it is reasonable to suggest that alternative splicing of the primary transcript may be a mechanism used to provide new forms of the nonclassical molecules. These new forms may then be selected by evolutionary forces unique to the organism in which they arose.

The apparent importance of HLA-G in reproductive immunology emphasizes the importance of understanding the function of all of the nonclassical antigens. Each may indeed have a specialized function underlying some little-understood component of the immune response.

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