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The Membrane-Bound and Soluble Forms of HLA-G Bind Identical Sets of Endogenous Peptides but Differ with Respect to TAP Association

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Summary

The class Ib antigen HLA-G is expressed as a membrane-bound protein like classical class Ia molecules (M•HLA-G) but, unlike typical class I, is also expressed as a soluble protein (S•HLA-G) with a unique C terminus. Our results show that, similar to classical class I proteins, the membrane-bound form of HLA-G associated with TAP, as evidenced by the ability to immunoprecipitate HLA-G class I heavy chain with TAP antisera. In contrast, the soluble G protein did not appear to associate with TAP in the same manner, since similar immunoprecipitation experiments failed to detect soluble G complex. A detailed analysis of peptides bound to the soluble and membrane HLA-G proteins expressed in the B lymphoblastoid cell line 721.221 showed that, like class Ia complexes, both HLA-G proteins consist of heavy and light chains complexed with nonameric peptides in a 1:1:1 ratio. The two proteins bind essentially the same set of peptides, which are derived from a variety of intracellular proteins and define a peptide motif for HLA-G. The peptides contain Leu at the C terminus and Pro or small hydrophobic amino acids in position 3 followed by Pro or Gly in position 4. The complexity of the bound peptides is lower than that found for some class Ia complexes, but is more similar to class Ia than to the limited repertoire of some murine class Ib molecules.

Introduction

Major histocompatibility complex (MHC) class I molecules bind a diverse array of peptides for presentation to CD8⁺ T cells as part of the mechanism of recognizing virally infected cells. In humans, this role is carried out by the highly diverse classical transplantation antigens, which are encoded by the HLA-A, HLA-B, and HLA-C genes (Bjorkman and Parham, 1990). Peptides that associate with class I molecules are usually generated from proteins synthesized in the cytoplasm, and their transport into the endoplasmic reticulum (ER) depends upon a heterodimer

composed of one subunit each of the TAP1 and TAP2 proteins (Spies et al., 1992; Kelly et al., 1992; Spies and DeMars, 1991; Attaya et al., 1992). Peptide loading can also occur independently of TAP, as evidenced by the expression of HLA-A2 molecules loaded with peptides derived from signal sequences on the surface of TAP-deficient cells (Wei and Cresswell, 1992; Henderson et al., 1992). Other TAP-independent sources of peptides also exist (Schirmbeck and Reimann, 1994). While minor relative to the TAP-dependent pathway, TAP-independent peptides can be presented to T cells and thus may contribute to the in vivo immune response (Hammond et al., 1993; Gueguen et al., 1994; Sijts et al., 1992; Hosken and Bevan, 1992).

Nonclassical or class Ib molecules, found in most or all mammals, are distinguished from the classical class Ia molecules by their low polymorphism and their tissue-specific expression, and in general their role in the immune response is yet to be determined (Stroynowski, 1990; Shawar et al., 1994). The murine class Ib molecule H-2M3 is highly conserved and peptide binding may be restricted to a limited subset of peptides. H-2M3 is specialized in presenting only N-formylated peptides to T cells, possibly owing to specific substitutions in its antigen binding site (Fischer-Lindahl et al., 1991). The determined crystal structure of a soluble form of M3 combined with a formylated nonamer peptide has revealed a novel mode of peptide binding (Wang et al., 1995). Qa-2 molecules may also have a stringent peptide ligand specificity, possibly presenting only a few different peptides (Rötzschke et al., 1993). However, an independent study of Qa-2-bound peptides has suggested that their diversity is similar to that found in classical H-2 molecules (Joyce et al., 1994). Thus, in the mouse at least, class Ib molecules may be very restricted in the peptides they bind or, alternatively, as promiscuous as are the classical antigens.

The human class I gene family has been studied in detail in a heterozygous individual from which the B lymphoblastoid cell line (LCL) 721 is derived (Koller et al., 1987; Geraghty et al., 1992; Geraghty, 1993). In addition to several pseudogenes, there are three class Ib genes referred to as HLA-E, HLA-F, and HLA-G, all highly homologous to the class Ia antigen genes and all of which associate with β 2-microglobulin (β ₂m). HLA-G is particularly interesting as its expression is restricted to trophoblast cells and there is limited functional polymorphism at this locus. In addition, the protein appears to be expressed in at least two forms in the placenta, a membrane-bound form and a soluble form (Kovats et al., 1990). These findings together suggest that HLA-G plays a significant role in the maternal-placental immune interaction (reviewed by Sargent, 1993; Loke and King, 1991; Hunt, 1992; Schmidt and Orr, 1993).

Further complexity of HLA-G expression was demonstrated by the finding that HLA-G mRNA was alternatively spliced, yielding not only the full-length form with three external domains, but also two smaller messages (Ishitani

and Geraghty, 1992). One of the smaller messages, named HLA-G2, excludes exon 3, resulting in a predicted protein with the $\alpha 1$ and $\alpha 3$ domains joined, while the smallest message has the $\alpha 1$ domain connected directly to the transmembrane region. This alternative splicing was found in placental tissues, a placental-derived cell line, and also in HLA-G-transfected cell lines. However, none of these alternative transcripts encoded a soluble HLA-G protein. Further analysis showed that an unusual transcript encoded a soluble HLA-G with identical leader, $\alpha 1$, $\alpha 2$, and $\alpha 3$ sequences, but which included intron 4, yielding an open reading frame that terminates 21 aa after the $\alpha 3$ domain (Fujii et al., 1994). This mRNA was shown to encode the soluble HLA-G protein by transfection of the intron 4 containing cDNA, inserted into a retroviral expression vector, into LCL .221 cells followed by two-dimensional IEF/SDS gel analysis of the class I protein. A similar intron containing message derived from the HLA-G2 mRNA was found, suggesting the existence of a soluble form of this alternative HLA-G protein (Fujii et al., 1994). All of the alternative HLA-G mRNAs were found in placental tissue.

As a necessary step towards understanding the potential functions of the soluble and membrane forms of HLA-G, we have analyzed and compared the biochemical features of both proteins expressed in HLA-G transfected .221 cells. Both molecules consist of heavy and light chains complexed with nonameric peptides in a 1:1:1 ratio. The peptides bound to these proteins are derived from a variety of intracellular proteins and define a unique peptide motif for HLA-G. Although the majority of peptides are derived from TAP-dependent sources, significant levels of protein are expressed in TAP-negative cells, and peptide sequencing showed that like some classical class I molecules, HLA-G can bind signal peptide-derived sequences. The membrane-bound form of HLA-G associates with TAP, as demonstrated by the ability to immunoprecipitate HLA-G class I heavy chain with TAP antisera. In contrast, the soluble G protein does not appear to associate with TAP in the same manner, since similar immunoprecipitation experiments failed to recognize soluble G complex. This is true despite the fact that both proteins bind essentially the same set of peptides. It appears that both the soluble and membrane HLA-G proteins bind a diverse array of peptides, in a manner similar to that found for classical class I molecules, and that the soluble form of HLA-G may provide some insights into class I processing and association with TAP in general.

Results

HLA-G Membrane and Soluble Proteins Are Expressed at Reduced Levels in TAP-Deficient Cells

To gain insight into the processing of HLA-G and its ability to bind peptide, we undertook an examination of the expression of HLA-G in the TAP-negative cell line .134 (Spies et al., 1992) and compared it with the expression in wild-type cells. Retroviral constructs pLNC-G1 and pLNC-Gs, directing the expression of the membrane and soluble HLA-G1 molecules, respectively, were assembled as de-

scribed previously (Fujii et al., 1994) and used to transfect LCL .221, which expresses a functional TAP complex, and .134, which is TAP1-negative. Using HLA-G-specific antibodies, it was possible to measure the levels of HLA-G present on the surface of the .134 cells despite the presence of classical class I molecules. Flow cytometry was used to compare directly the relative levels of membrane HLA-G in the two cell lines. FACS analysis (Figure 1A) shows a representative experiment demonstrating that HLA-G surface expression is reduced to about 20% relative to .221 cells. Previous studies had shown that in .134 cells, HLA-A2 is expressed at about 40% of wild-type levels, while HLA-B5 was not detectable (Spies and DeMars, 1991). Thus, like HLA-A2, it appears that HLA-G can bind peptide from TAP-independent sources.

Since the soluble HLA-G molecule is secreted from cells expressing it, FACS analysis cannot be used to assess soluble G dependence on TAP for peptide binding. Instead, we designed a two-step enzyme-linked immunosorbent assay (ELISA) for the detection of soluble G complex using two different antibodies, one immobilized and the second biotinylated and used for detection. The results shown in Figure 1B summarize several experiments as outlined in Experimental Procedures. All of the measurements gave similar results, which indicated that the relative amount of soluble G secreted by .134-Gs is about 5% of that secreted by .221-Gs. To confirm that the ELISA results accurately reflect the levels of secreted HLA-G, Western blot analysis was performed using conformation-independent antibody 6Gs, which reacts uniquely with the soluble form of HLA-G. While S*HLA-G was detectable from supernatants of .221-Gs cells, no protein could be detected in supernatants from .134-Gs transfectants (Figure 2C). This was true despite the fact that similar levels of S*HLA-G were detectable from cell lysates of .134-Gs and .221-Gs transfectants, indicating that transfected cells were expressing similar amounts of protein (Figure 2C). These results, combined with those measuring membrane G expression in .134-G1, indicate that the majority of peptides are from TAP-dependent sources, although HLA-G also binds a minor amount of peptide from TAP-independent sources, similar to some classical class I molecules (Engelhard et al., 1993). Indeed, HLA-G may be somewhat more versatile in its ability to bind peptide from TAP-independent sources than at least several other classical class I alleles (Rammensee et al., 1993).

Membrane-Bound HLA-G, but Not the Soluble HLA-G Complex, Can Be Immunoprecipitated with TAP Antisera

It was apparent from the above studies that soluble HLA-G secretion was dependent on an intact TAP complex. To examine further the interaction of both forms of HLA-G with TAP, we carried out experiments based upon those carried out by Ortmann et al. (1994) and Suh et al. (1994), which detected an interaction of MHC class I molecules with TAP. Two immunoprecipitations from .221-G1 and .221-Gs cells were carried out using TAP antisera, in a comparative analysis of transfected and untransfected

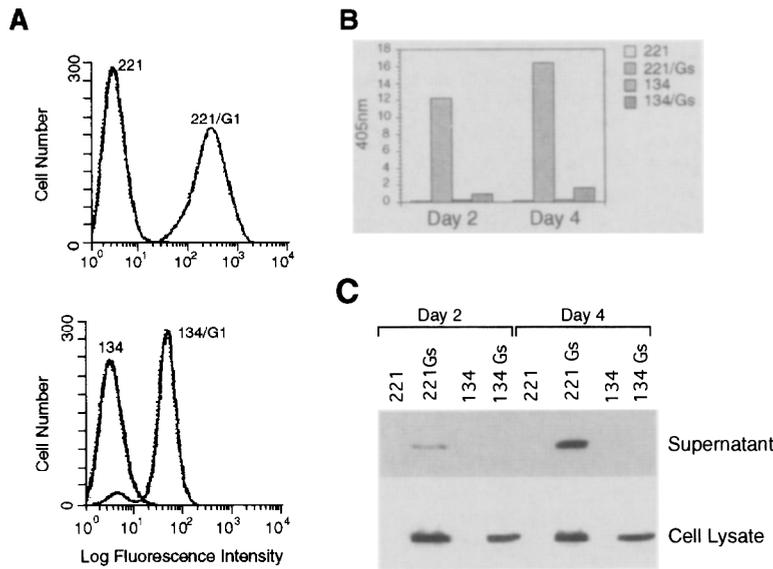


Figure 1. Both Soluble and Membrane HLA-G Are Expressed at Reduced Levels in TAP1-Negative .134 Cells

(A) FACS analysis of wild-type .221-G1 transfectants and TAP-deficient .134-G1 transfectants using HLA-G-specific MAb 87G. Cells were stained and analyzed as described in Experimental Procedures, and relative fluorescence intensity was measured. The transfectant and untransfected counterpart are indicated above each profile.

(B) Relative levels of soluble G protein secreted by .221-Gs and .134-Gs transfectants. An ELISA using plate-bound W6/32 and biotinylated 87G followed by avidin-alkaline phosphatase for detection was used to test supernatants (see Experimental Procedures). Supernatants from 2×10^5 cells incubated for 2 or 4 days in 2 ml of media were analyzed in at least three independent experiments. Average results are shown in absorbency units (405 nm) on the ordinate. A key to the bar code is shown on the right.

(C) Western blot analysis of cell lysates and supernatants from .221-Gs and .134-Gs cells.

Cell lysates (bottom) or cell supernatants from days 2 and 4 (top) were run on SDS-PAGE, transferred to nitrocellulose, and analyzed with MAb as described in Experimental Procedures. The sources of materials analyzed are indicated above each lane.

.221 cells. Membrane-bound HLA-G was observed in immunoprecipitates with TAP antisera alone or with TAP antisera followed by immunoprecipitation with W6/32 (Figure 2). In striking contrast, S•HLA-G was not coprecipitated with TAP antisera, and was not detectable by sequential precipitation with TAP followed by W6/32, either from .221-Gs cells or from .221-G transfectants. The .221-G

transfectant expresses all of the alternative forms of HLA-G, including both the full-length soluble and membrane proteins (Ishitani and Geraghty, 1992; Fujii et al., 1994). Whether this lack of coprecipitation with TAP indicates a weaker association of TAP with soluble G than with membrane-bound class I or a lack of association cannot be conclusively determined from this experiment. However,

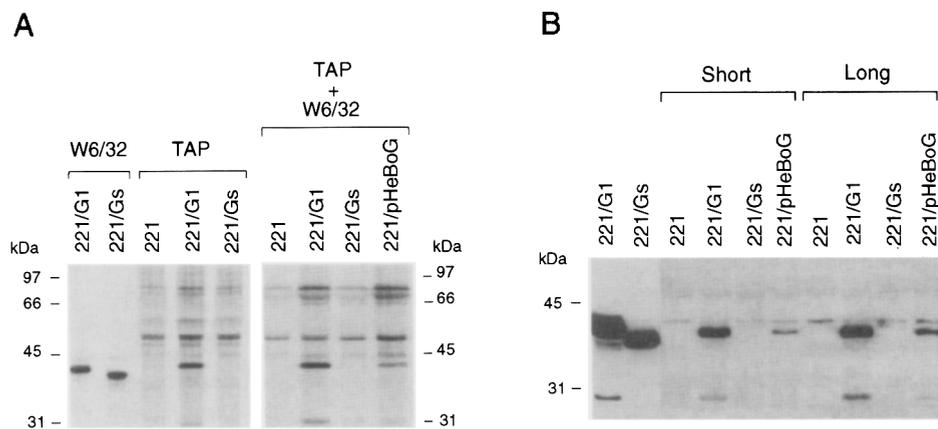


Figure 2. The Membrane HLA-G, but Not the Soluble Form, Associates with TAP Molecules in Digitonin-Solubilized Human B-LCLs

Cells were pulsed with [³⁵S]methionine for 15 min. The cell line analyzed is indicated above each lane and the reagent used is indicated above. Labeling times and the number of cells analyzed were essentially identical for all samples.

(A) Left, cells were lysed with NP-40 and precipitated with W6/32 as indicated above the leftmost two lanes in order to verify that the transfectants are expressing the respective membrane or soluble HLA-G protein and to compare relative amounts of class I protein expressed. For the TAP experiments, cells were lysed with digitonin followed by immunoprecipitation with TAP antisera. Right, sequential immunoprecipitations with TAP antisera followed by W6/32. This experiment included .221-G transfectants that express both the soluble and membrane HLA-G proteins. Only the membrane form is immunoprecipitated in this experiment. The relative positions of molecular weight markers are indicated to the left and right.

(B) Western blot analysis of immunoprecipitates with TAP antisera. The leftmost two lanes contain protein from .221-G1 and .221-Gs lysates to serve as standards. Two TAP immunoprecipitation times were used. The lanes labeled short were precleaned for 1 hr followed by incubation with antisera for 1 hr. The lanes labeled long were precleaned overnight and incubated with antisera for 4 hr. Western blot analysis was performed as described in Experimental Procedures.

it is clear that soluble G is dependent on TAP to supply most of the bound peptides and for subsequent transport out of the cell, as evidenced by the lower levels of S•HLA-G released from .134-Gs cells (Figure 2A). These findings were confirmed in Western blot analysis of TAP immunoprecipitates using monoclonal antibody (MAb) HCA2. Only the M•HLA-G protein was detected after TAP immunoprecipitation, in experiments including immunoprecipitates from the .221-G1 and .221-Gs cells, and from the .221-G transfectant, which expresses both HLA-G forms (Figure 2B). These results suggest that while TAP association with class I may be sufficient for peptide binding, it is not necessary.

Structure of Peptides Associated with HLA-G Membrane and Soluble Proteins

By using the .221-Gs and .221-G1 transfectants, expressing only the soluble and membrane HLA-G proteins, respectively, we purified the two forms separately by immunoaffinity chromatography and determined the structure of the most abundant peptides associated with HLA-G. The naturally processed peptides were isolated from the immunoaffinity-purified complexes by acid extraction and purified by reversed-phase high pressure liquid chromatography (HPLC) (Figure 3). HPLC fractions (numbered in Figure 3) were directly sequenced or rechromatographed (indicated by letters in Table 1) and subsequently analyzed by Edman degradation. The sequences were confirmed by mass spectrometric analyses, and the results are summarized in Table 1.

Sequences have been determined for 13 peptides extracted from both M•HLA-G and S•HLA-G. The majority of these peptides are nine residues long and one is an 11-mer, indicating that nine residues is the optimum length for peptides associated with HLA-G. Comparison of the peptide sequences revealed several conserved residues implicated in peptide binding. All but two of the peptides contained Pro at position 3. The two exceptions were Ala and Ile, followed by Gly and Pro, respectively. Conservation at position 3 appears to be unique for HLA-G peptides and HLA-B14 peptides, which show a preference for Tyr at this position, whereas conservation at position 2 has been found previously in peptides associated with human classical class I molecules (Rammensee et al., 1993). Position 9 is dominated by a single amino acid side chain in HLA-G-associated peptides. For 13 peptides, 12 terminate in Leu and one 11-residue peptide contains Ile at position 9. A comparison of the sequences in Table 1 showed conservation at positions in addition to positions 3 and 9. Positively charged amino acids (Arg, Lys, His) in position 1 and small hydrophobic amino acids (Val, Ile, Leu, Ala) in positions 2 and 7 may also interact with the HLA-G binding site. Variability in the type of amino acids, however, was found at positions 4, 5, 6, and 8.

For the sequences listed in Table 1, gene and protein sequence databases were searched and homologies to different human proteins were found for 11 peptides. The proteins comprise a diverse group with respect to their function and cellular location. Nine HLA-G-restricted peptides are processed in the cytosol and require transport

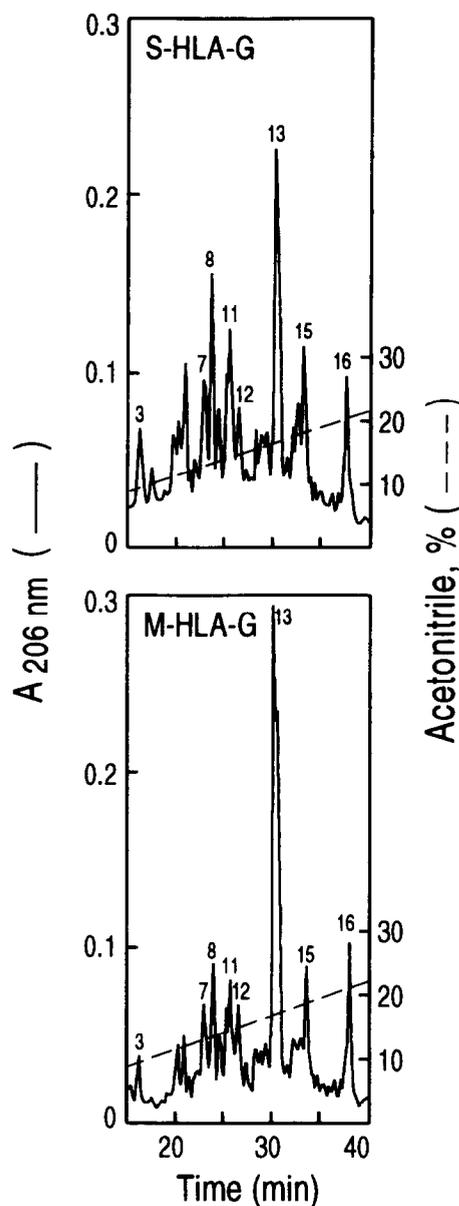


Figure 3. The Membrane and Soluble Forms of HLA-G Bind Essentially the Same Peptides

HPLC profiles of peptides eluted from the soluble (S•HLA-G) and membrane (M•HLA-G) HLA-G proteins are shown. A linear elution gradient with acetonitrile is indicated by a dashed line and the percentages are presented on the right ordinate. UV absorbency is indicated on the left ordinate. Individual peaks, which were sequenced and whose sequences are summarized in Table 1, are indicated by numbers immediately above the peak. Some of these peaks were rechromatographed before sequencing as indicated in Table 1 by letters.

from the cytosol to the ER. One of the identified peptides is derived from the signal sequence of ERP72. Peptides of this origin are derived from proteins localized in the endoplasmic reticulum during cotranslational protein translocation, where they are cleaved by a signal peptidase and become associated with newly synthesized class I molecules. Others had previously identified signal peptides associated with HLA-A2.1, HLA-B7, and H2-D^b (En-

Table 1. Sequences of the Most Abundant Peptides Associated with HLA-G

(M + H) ⁺ calculated	Sequence	Sol-HLA-G		Mem-HLA-G		Protein source*
		HPLC fraction	(M + H) ⁺ observed	HPLC fraction	(M + H) ⁺ observed	
1172.4	RHPKYKTEL	3b	1172.6	3a	1172.9	Nuclear protein; P26651
1001.2	HVPEHAVVL	7e	1001.3	7d	1001.1	Rat fatty acid synthase; P12785
1093.3 + 16	MQPTHP IRL	8	1109.3	8	1109.0	HS1 protein; X57346
1002.2	KIAGYVTHL	11b	1002.0	11a	1001.6	Ribosomal protein S17; JT0405
994.2	GVPKTHLEL	11c	994.4	11b	995.0	HLA class III gene; Z15025
868.1	KGPPAALTL	11d	ND	11c	868.2	Cytokine receptor; L08187
1008.2	SYPTRIASL	12b	1008.3	12b	1008.5	Unknown
1025.2	RLPDGRVVL	12c	1025.4	12c	1025.1	Interferon binding protein; Q02556
1146.4	RI IPRHLQL	13a	1146.9	13a	1146.5	Histone; P02262
1128.4	KSPASDTY I VF	13b	1128.6	13c	1128.1	Nascent polypeptide complex α chain; X80909
1132.5 + 16	MRPRKAFLL	13c	1148.6	13e	1148.6	ERP 72; P13667
1103.3	RLPKDFVDL	15	1102.5	15	1103.7	Unknown
1073.4	VLPKLYVKL	16	1073.1	16	1073.0	Ribosomal protein 40S; Q06722

* The databases used were the following: Genbank (release 87), EMBL (release 41), PIR-Protein (release 43), and Swiss-Prot (release 30). Protein sources for peptides that bind to HLA-G are referenced by their accession number.

(M + H)⁺ is the apparent mass of the singly charged peptide. The (M + H)⁺ of peptides 8 and 13c were observed as oxidized forms. ND, not determined.

gelhard et al., 1993). The identification of a signal sequence-derived peptide in association with HLA-G shows that this pathway can be utilized by nonclassical class I molecules as well.

Experiments were performed to demonstrate that the peptides selected for sequencing are representative of the whole extract. Pool sequencing of G-restricted peptides from both membrane-bound and secreted HLA-G indicated that residues representing >50 mol percent were Arg and Lys in position 1; Ile, Leu, and Val in position 2; Pro in position 3; Val, Leu, Ile in position 7; and Leu in position 9.

Some differences in the architecture and chemical nature of specificity pockets in HLA-G compared with HLA-A2 can be rationalized on the basis of a molecular model of HLA-G deduced from the crystal structure of HLA-A2 (Bjorkman et al., 1987). Table 2 lists peptide side chains that participate in the formation of pockets in class I molecules (Matsumura et al., 1992; Young et al., 1995) and summarizes changes seen in HLA-G when compared with HLA-A2. The A and F pockets in HLA-G and HLA-A2, which accommodate the side chains of residues 1 and 9, respectively, are essentially conserved, suggesting the conservation of critical hydrogen bonds at the peptide termini. However, the side chains found at position 2 in peptides bound to HLA-G show considerable diversity, whereas position 3 displays a striking preference for Pro. The HLA-G model suggests that two changes of binding site residues 9 and 99 are major determinants for the observed differences in specificity. Residues Phe9 and Tyr99 are common to the B and C pockets in HLA-A2 (Matsumura et al., 1992) and form a ridge that separates these pockets. In HLA-G, the change Phe9/Ser enlarges the B pocket and increases its hydrophilicity. Thus, it permits the presence of a variety of residues at position 2, which is seen in the isolated HLA-G-bound peptides. This change does not affect position 3. Model building suggests that the sub-

stitution Tyr99/Leu enlarges the size of the B/C pocket and increases its hydrophobic character. This is thought to result in favorable van der Waals interactions between a Pro at position 3 and residues Ile99 and Tyr149 in HLA-G. The latter residue is conserved in HLA-A2. The carboxy-

Table 2. Peptide-MHC Contacts in HLA-A2 and HLA-G

Position	Peptide Side Chain		MHC Contact Residues
	A2	G1	(A2/G1)
P1	A, V, L, M	V, M	7-Y/Y, 59-Y/Y, 63-E/E, 66-K/N,
	Y		159-Y/Y, 171-Y/Y
	S, T	S	
	K	K, R, H	
P2	V, L, M	V, L, I	9-F/S , 24-A/A, 45-M/M, 67-V/T,
		Y	99-Y/I
		S, Q	
		R, H	
P3	V, L, I F, W N D P	A, I	9-F/S , 70-H/H, 97-R/W , 99-Y/I , 114-H/E , 156-L/R , 159-Y/Y
		P	
P9	V, L, I	L, I	84-Y/Y, 118-Y/Y, 143-T/S , 146-K/N, 147-W/C

The listed MHC contact residues participate in the formation of pockets in class I molecules that accommodate the respective P1-P9 residues of the bound peptides. Residues at corresponding positions in HLA-A2 and HLA-G are given. For example, 7-Y/Y means that a Tyr is conserved at position 7 in A2 and G. Accordingly, 143-T/S means that Thr at position 143 in A2 is replaced by Ser in G. Residue changes shown in bold are thought to be determinants of differences in peptide specificity in G compared with A2. Residues were grouped by chemical type as follows: small hydrophobic: A, V, L, I, M; large hydrophobic: Y, F, W; polar: S, T, N; positively charged: K, R, H; negatively charged: E, D; G and P were each considered separately (Huczko et al., 1993).

terminal residue of the HLA-G bound nonamers is consistently a Leu, compared with the smaller hydrophobic Val seen at the corresponding position in HLA-A2. This may be due to the substitution at position 143 in HLA-G where Ser replaces Thr, increasing the size of the F pocket slightly to accommodate the longer side chain of Leu.

Discussion

We have carried out a detailed biochemical analysis of the HLA-G soluble and membrane-bound proteins, contrasting the findings between the two HLA-G proteins and with those of classical class I. Both the soluble and membrane heavy chains bind peptide and β_2m in a 1:1:1 ratio (data not shown). Both protein forms bind essentially the same set of peptides as shown by the following experiments: first, sequencing of the total peptide pool extracted from either protein form provided essentially qualitatively identical results with small quantitative differences; second, HPLC profiles of the eluted peptides were almost entirely overlapping and all differences are quantitative; third, sequence analysis and mass spectrometric analysis of individual HPLC peptide peaks showed peptides isolated from corresponding peptide peaks were identical.

To examine the mechanisms used in HLA-G peptide binding, we used the HLA-G1-transfected TAP1-mutant cell line .134 and the TAP1-positive .221-G1 transfectant to examine G surface expression. Soluble G complex expression was monitored by comparing the levels secreted by .134-Gs and .221-Gs. Our results showed that both membrane and soluble G are dependent on TAP for efficient binding of cytosolic peptides. However, significant levels of M•HLA-G are expressed on the surface of .134-G1, a pattern reminiscent of HLA-A2 expression on these cells. Quantitatively, HLA-G expression on .134-G1, at about 20% of .221-G1 expression, appears to be intermediate between HLA-A2 and HLA-B5 levels. The soluble G complex appears to be secreted at relatively lower levels in .134 cells (about 5% of .221-Gs). However, the relative levels are about 10-fold higher than that seen for the soluble Qa-2 molecule in TAP-negative cells (Tabaczewski and Stroynowski, 1994). Consistent with these observations, at least one of the endogenous peptides we found presented by HLA-G is derived from a signal peptide (Table 1, A11/4).

The lack of detectable TAP association with S•HLA-G, despite the fact that both soluble and membrane proteins bind identical sets of peptides, is intriguing. This difference in TAP binding between the two HLA-G proteins implies that the residues on M•HLA-G interacting with TAP are located in the transmembrane domain of HLA-G. Alternatively, the charged carboxyl terminus unique to S•HLA-G may interfere with binding of residues otherwise interacting on M•HLA-G. Presumably, such interactions would involve the $\alpha 3$ domain, considering its proximity to the carboxyl terminus of S•HLA-G. Whether the ability to bind TAP-dependent peptide without the TAP interaction is unique to S•HLA-G remains to be seen. However, it is suggestive that a subset of class I can bind cytosolic peptide without this physical interaction.

The sequences listed in Table 1 and the analysis of the pooled peptides suggest several characteristics of peptide binding to HLA-G. Like classical class I molecules, HLA-G preferentially binds peptide nonamers. Only 1 of 13 peptides analyzed herein contains more than nine residues. Similar exceptions have been observed for HLA-A2 (Collins et al., 1994) and HLA-B27 (Urban et al., 1994). The preference of HLA-G for nonamers is consistent with the conservation of the A and F pockets as suggested by model building. Two positions appear to be determinants of the HLA-G peptide specificity, as seen for other class I molecules (Rammensee et al., 1993). At the peptide carboxyl terminus, Leu is strongly preferred by HLA-G. The pooled sequences contained 90% Leu and 10% Ile at this position. Different from classical class I where position 2 serves as an anchor (Rammensee et al., 1993), HLA-G displays specificity for Pro at position 3, although the pooled sequences contain significant amounts of Ile and Ala at this position. Our HLA-G model suggests that specific residue changes in the B/C pockets are responsible for the anchor residue difference relative to HLA-A2 and other class I molecules. Preference for Tyr at position 3 has also been observed for HLA-B14 (DiBrino et al., 1994). Whereas most peptides shown in Table 1 are clearly derived from cytosolic proteins and thus are expected to bind class I in a TAP-dependent manner, the signal sequence from the ERP72 protein provides an illuminating exception. Like HLA-A2, HLA-G can apparently bind peptides derived from TAP-independent sources. The peptide binding results are representative of the peptides that bind HLA-G *in vivo*. Preliminary results from a subset of placental-derived HLA-G-bound peptides have identified two predominant peptides reported here (H. M., unpublished data).

In situ hybridization studies have shown that HLA-G mRNA is strongly expressed in the first trimester on villous cytotrophoblast and expression declines towards term (Yelavarthi et al., 1991). Protein expression has been detected on extravillous cytotrophoblast, but not on the underlying villous cytotrophoblast (Redman et al., 1984; Ellis et al., 1986; Hsi et al., 1984). HLA-G is highly homologous to the classical class I antigens and is able to bind CD8 (Sanders et al., 1991), suggesting that it can also present antigen to T cells. The transmission of infectious disease to the fetus through the placenta is of significant concern for certain viral infections, and in many placental cells, HLA-G is the only class I molecule expressed. For example, cytomegalovirus (CMV) is transmitted through the placenta in 1%–2% of births, often resulting in damaging congenital CMV infection in the newborn (Fowler et al., 1992). The critical protective immune response against adult CMV infections has been shown to be mediated by CD8⁺ CTL, restricted by MHC class I (Reddehase et al., 1987; Riddell et al., 1991). Thus, HLA-G may be implicated in presenting CMV antigens in placental viral infections. If so, it may be expected that HLA-G would bind a diverse array of peptides, similar to that of classical class I. The murine Qa-2 molecule is estimated to bind 200–1000 distinct peptides depending upon the criteria used for estimation (Joyce et al., 1994; Joyce and Nathenson, 1994). Our

data suggest that HLA-G binds peptides with diversity similar to that found for Qa-2. However, when we compare the HPLC profiles of HLA-G-derived peptides with those of HLA-A2, for example, the overall diversity is estimated to be about 5-fold lower for the HLA-G-derived peptides (H. M., unpublished data). It is possible that HLA-G has a higher affinity for certain peptides, as proposed for Qa-2 by Röttschke et al. (1993), and that while other peptides are in fact binding HLA-G, their amount as compared with the most abundant components is significantly lower. However, even if this interpretation is true, it is still possible that HLA-G is functioning as an antigen-presenting molecule. Placental tissue is exposed to a more limited diversity of viral infections and therefore an antigen-presenting function could be served by the somewhat lower diversity of peptides presented by HLA-G.

Since extravillous cytotrophoblasts are exposed to maternal blood, a second role for HLA-G is suggested by the ability of HLA class I to protect from natural killer (NK)-mediated cell lysis. Several studies have demonstrated the ability of HLA-G to protect transfected NK targets from lysis (Chumbley et al., 1994; Deniz et al., 1994). The presence of the S•HLA-G complex in placental tissue is suggestive of a significant role of this molecule in suppressing a maternal immune reaction. Soluble HLA-G may act in concert with M•HLA-G to protect cells from NK lysis, and also may help to protect those placental cells which express no class I. A second role for the S•HLA-G complex can be envisioned if an alloresponse to polymorphic paternal peptides presented by M•HLA-G or to polymorphic HLA-G heavy chain occurs (van der Ven and Ober, 1994), S•HLA-G may intervene to interact with the T cell receptor to anergize these unwanted T cell responses. Thus, S•HLA-G may play a critical role in maternal-placental immune tolerance.

Experimental Procedures

Cell Lines

The B-LCL lines 721.221 and .134 were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2 mM glutamine, and 1 mM Na pyruvate. HLA-G expressing retroviral constructs were described previously (Fujii et al., 1994). The TAP-negative .134 line was transfected with retroviral HLA-G1 and Gs cDNA as described (Miller et al., 1993). After G418 selection, stable cDNA transfectants of .221 and .134 lines were maintained as above.

Antibodies and Antisera

Antibody MAb HCA2 (Stam et al., 1990) reacts with the HLA-G heavy chain and was used in Western blot analysis. TAP antisera has been described (Spies et al., 1992). Details of the production and characterization of HLA-G-specific antibodies are described elsewhere (unpublished data). In brief, antibody 87G was produced by immunization of HLA-B27 transgenic mice with H-2 identical murine cells transfected with and expressing genes encoding HLA-G and human β_2m . Antibody 6Gs was generated from immunizations of BALB/c mice with a KLH-bound synthetic 20-mer peptide derived from the carboxy-terminal sequence of S•HLA-G (Fujii et al., 1994). Specificity for HLA-G was tested by four criteria: first, microcytotoxicity tests using over 60 cell lines from the tenth international workshop representing nearly all the known HLA class I specificities. Second, FACS analysis using 26 cell lines from the workshop covering most of the specificities. Third, FACS analysis using six LCLs of diverse HLA class I types transfected with the HLA-G gene in a comparative analysis with their untransfected counterparts. Fourth, immunoprecipitation of HLA-G from the transfected LCLs.

Monoclonal 87G showed no cross-reactivity with other class I when tested by these criteria. The 6Gs antibody showed complete specificity for S•HLA-G when tested for cross-reactivity with other class I proteins, including M•HLA-G. This analysis included Western blots and immunoprecipitation of protein from normal LCL from several individuals expressing distinct class I HLA types as compared with similar experiments using their Gs transfected counterparts.

Immunofluorescence Staining and FACS Analysis

Cells (5×10^5) per well of a 96-well microtiter plate were washed once with ice-cold phosphate-buffered saline (PBS) containing 1% FBS, 0.1% NaN₃, then incubated with 50 μ l of 87G or mouse immunoglobulin G2a (IgG2a) at a concentration of 30 μ g/ml for 30 min on ice. Cells were washed three times with ice-cold PBS-FBS and then incubated with 50 μ l fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin-specific antibody (TAGO) at a concentration of 23.4 μ g/ml for 30 min on ice. After three washes with PBS-FBS, cells were fixed with 100 μ l of 1% paraformaldehyde in PBS. Samples were analyzed on a FACScan flow cytometer (Becton Dickinson).

ELISA

Affinity-purified W6/32 was diluted with Na carbonate buffer (pH 9.6) at a concentration of 20 μ g/ml. Polystyrene plates (96-well) plates were incubated with 100 μ l of the antibody solution overnight at 4°C. The excess of the MAb was removed, the uncoupled sites were blocked with 5% skim milk in PBS, and the plates were incubated for 3 hr at room temperature. Cell cultures were prepared at a starting cell density of 2×10^5 cells/ml and culture supernatants were collected after 2 and 4 days in culture. Supernatants (100 μ l) were added to each well and the plates were incubated overnight at 4°C. Supernatants were removed and plates were washed four times with PBS + 0.05% (v/v) Tween 20 and the biotinylated-87G MAb was added. After 2 hr incubation at room temperature, the plates were washed and 100 μ l of avidin-alkaline phosphatase (Sigma) at 1:4000 dilution in PBS + 2% bovine serum albumin was added to each well. After 1 hr incubation at room temperature, the wells were washed and 100 μ l of 3 mg/ml p-NPP substrate (Southern Biotechnology) in 10% diethanolamine buffer (pH 9.5) was added to each reaction well. The relative concentration of Gs was estimated from absorbency of the yellow product at 405 nm using an EAR 400AT (SLT Lab Instruments) plate reader.

Labeling and Immunoprecipitation

The .221 transfectants and control cells were metabolically labeled with [³⁵S]methionine at a density of 5×10^7 /ml in methionine-free RPMI 1640 medium. After preincubation for 30 min at 37°C, 0.5 mCi of [³⁵S]methionine (trans label, ICN Biochemicals) was added to 1 ml of cell suspension, and the cells were incubated for 20 min at 37°C. Cells were subsequently washed twice with ice-cold 10 mM Tris (pH 7.8), 140 mM NaCl (TBS), and lysed. For immunoprecipitation, cells were lysed in 1% digitonin (Wako) in TBS, 0.1 mM PMSF, 10 μ g/ml pepstatin, 14 μ g/ml aprotinin. After incubation on ice for 30 min, the lysate was centrifuged at $10,000 \times g$ for 15 min at 4°C and the supernatant was collected. To 1 ml of this lysate was added 10 μ l of rabbit serum (Sigma). After continuous gentle mixing at 4°C for 4 hr, 80 μ l of 50% suspension of formalin-fixed *Staphylococcus aureus* (Pansorbin, Calbiochem) in TBS-digitonin was added. After incubation overnight at 4°C, *Staphylococcus aureus* was removed by a brief centrifugation. The resulting supernatant was made of 0.1% SDS and 5 mg/ml bovine serum albumin (final concentration), and 3 μ l of TAP antiserum was added. After continuous gentle mixing at 4°C for 2 hr, 35 μ l of a 50% suspension of prewashed protein A-Sepharose (Sigma) in lysis buffer was added. After this mixture was incubated at 4°C for 4 hr, the immune complex was washed five times with TBS, 0.1% digitonin and analyzed by 12% SDS-PAGE. For reprecipitation experiments, proteins were eluted from immunocomplexes three times by adding 0.5 ml TBS, 0.2% SDS, and incubated at 37°C for 30 min each time. The eluates were combined and NP-40 (Pierce) was added to a final concentration of 1%. The supernatants were reprecipitated with W6/32 and protein A-Sepharose and then washed as described above.

Western Blot Analysis

Cell culture supernatants (200 μ l) were collected from 2 and 4 day cultures and concentrated by speed vacuum and cells were solubilized

in NP-40. Samples were run on 10% SDS-PAGE and transferred to nitrocellulose membrane (S and S). Soluble HLA-G was detected with MAb 6Gs, followed by HRP-goat anti mouse IgG + IgM (Jackson), and finally with an enhanced chemiluminescence system (ECL, Amersham). TAP-associated HLA-G was detected by monomorphic MAb HCA2 (Stam et al., 1990; Grandea et al., 1995) and the same secondary reagents. For analysis of TAP immunocomplexes (Figure 2B), two TAP immunoprecipitation times were used. A short incubation was performed with precleaning for 1 hr followed by incubation with antiserum for 1 hr. A long incubation was performed by precleaning overnight and followed by incubation with antisera for 4 hr. After TAP immunoprecipitation, samples were run on SDS-PAGE and to membranes, and HLA-G protein detected as above.

Isolation and Purification of HLA-G-Associated Peptides

M•HLA-G was purified from the detergent lysate of .221-G1 cells, and S•HLA-G from the medium conditioned by .221-Gs cells. In brief, 14 g cell pellets (approximately 6×10^9 cells) of cultured cells were washed with PBS and mixed with an equal volume of solubilization buffer containing 2% NP-40, 0.8 M NaCl, 20 mM Na₂EDTA, PMSF (2 mM), iodoacetamide (100 μ M), pepstatin A (10 μ g/ml), leupeptin (10 μ g/ml), and aprotinin (5 μ g/ml) in PBS. The cells were gently agitated for 30 min at 4°C, nuclei removed by low-speed centrifugation at 1,500 \times g for 5 min at 4°C, and the total protein concentration adjusted to 2 mg/ml. The cell lysate was clarified by centrifugation at 100,000 \times g, for 90 min.

S•HLA-G was purified from the conditioned medium of 5×10^8 cells. Medium (7 l) was collected and clarified by low speed centrifugation and concentrated in a Pellicon filter cassette (Millipore) with a 10,000 molecular weight cut-off. The concentrated medium was stored in the presence of protease inhibitors. The M•HLA-G and S•HLA-G complexes were then purified by immunoaffinity chromatography using MAb W6/32 coupled to CNBr-activated Sepharose 4B. MHC complexes were eluted with 1% trifluoroacetic acid (TFA), peptides recovered by ultrafiltration using an Amicon cell with a 10,000 molecular weight cut-off and concentrated by vacuum centrifugation. Peptides were purified on a model 140B narrow-bore HPLC system (Applied Biosystems, Incorporated) using a 1 \times 100 mm C18 Vydac column at a flow rate of 100 μ l/min at 40°C. Indicated fractions were rechromatographed using a 1 \times 100 mm Spheri-5, RP-18 (5 μ m) column (ABI) at a flow rate of 40 μ l/min at 40°C. Linear acetonitrile gradients from solvent A (0.1% TFA) to solvent B (0.085% TFA in acetonitrile) were used for elution.

Amino Acid Sequence Analysis

Automated sequence analysis was performed in a pulsed-liquid protein sequencer (model 476A, ABI), as described (Maresh et al., 1994). Proteins were recovered from SDS-polyacrylamide gels by electroblotting onto a Problott membrane (ABI) and quantitation was based on amino acid sequence analysis. Peptides were sequenced on polybrene-coated glass fiber discs. A data base search of peptide sequences was performed using the Wisconsin Genetics Computer Group sequence analysis package (Devereux et al., 1984).

Mass Spectrometric Analysis of Peptides

Mass spectra were recorded on a Biolon20 plasma desorption mass spectrometer (ABI). A 5 μ l aliquot of sample (3–10 pmol of peptide in TFA/acetonitrile) was applied to a nitrocellulose-coated target. Spectra were acquired for 10^8 fission events at 15 kV acceleration voltage.

Molecular Modeling

A molecular model of the HLA-G μ domains was constructed on the basis of an HLA-A2-peptide complex crystal structure (Madden et al., 1993). Side chain replacements were carried out, using interactive computer graphics, in most similar rotamer conformations. Steric constraints introduced in the assembly of the model were relieved by some minor energy refinement. Peptide nonamers were modeled into the HLA-G binding site by transfer of crystallographic peptide conformations following superposition of HLA-G on the HLA-A2 crystal structure. Predicted changes in specificity pockets in HLA-G compared with HLA-A2 were analyzed using computer graphics.

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