Nonclassical HLA-G molecules are classical peptide presenters

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Background: The physiological functions of the classical HLA (human leukocyte antigen) molecules, HLA-A, HLA-B and HLA-C, are to present peptides to T cells and to inhibit the activity of natural killer cells. In contrast, the functions of nonclassical HLA-molecules, such as HLA-E, HLA-F and HLA-G, remain to be established. The expression of HLA-G is largely limited to the placental trophoblast, where it might mediate protection of the fetus from rejection by the mother. Achieving the aim of understanding the function of HLA-G should be facilitated by information on the biochemical properties of HLA-G molecules, especially on their potential ability to act as peptide receptors.

Results: To study peptide presentation by HLA-G, we used stably transfected LCL721.221 cells as a source of HLA-G molecules and analysed the spectrum of extracted peptides by individual and pool sequencing. Our results indicate that HLA-G molecules, like classical HLA molecules, are associated with a wide array of peptides derived from cellular proteins. Peptides presented by HLA-G usually consisted of 9 amino acids, and adhered to a specific sequence motif, with anchor residues at position 2 (isoleucine or leucine), position 3 (proline) and the carboxy-terminal position 9 (leucine). Thus, the HLA-G peptide ligand motif follows the principles of classical HLA motifs, although it displays its own unique features. Peptide-binding assays indicated that two of the three anchor residues were sufficient for binding, and that the three natural HLA-G ligands that we identified bound, not only to HLA-G, but also to HLA-A2. This was not surprising, because the binding pockets of HLA-A2 and HLA-G overlap in their ability to recognize anchor residues at positions 2 and 9. Likewise, some, but not all, HLA-A2 peptide ligands could also bind to HLA-G.

Conclusions: Nonclassical HLA-G molecules present peptides essentially in the same way as classical HLA molecules do. We determined the peptide motif that is specifically recognized by HLA-G; its basic features are described by the sequence XI/LPXXXXXL. This information should help to elucidate the physiological role of HLA-G molecules at the fetal–maternal interface. Most likely, this role is to protect fetal cells from lysis by natural killer cells, and possibly to present foreign peptides to a class of T cells that has not yet been identified.

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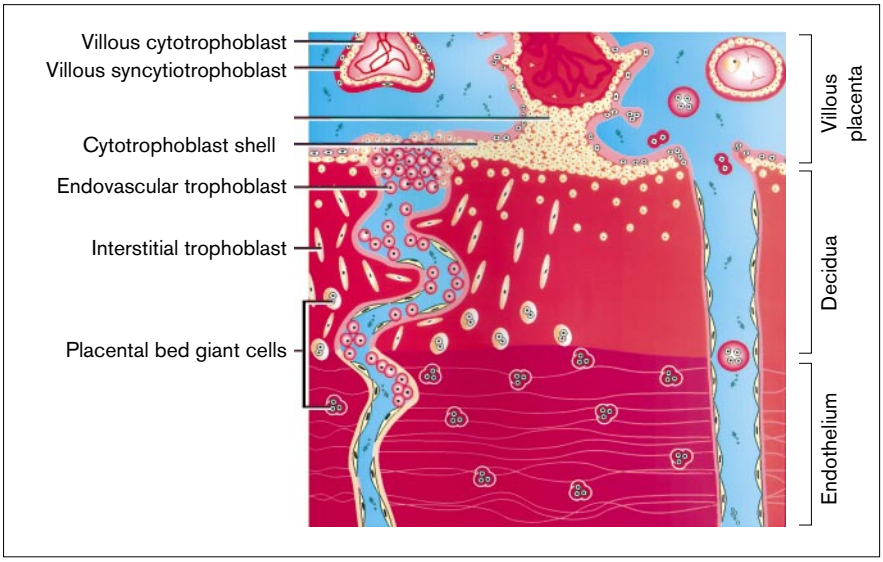
Received: **17 November 1995**
Revised: **19 January 1996**
Accepted: **29 January 1996**

Current Biology 1996, Vol 6 No 3:305–314

Background

The classical genes of the human class I major histocompatibility complex (MHC) — the human leukocyte antigen (HLA)-A, HLA-B and HLA-C genes — had been known about for a long time when molecular biology techniques led to the discovery of the nonclassical HLA genes (reviewed in [1]). Although the function of the classical HLA molecules as polymorphic peptide receptors is well documented (reviewed in [2,3]), the function of the nonclassical HLA molecules remains to be established. In the mouse, some of the nonclassical MHC molecules display specialized antigen-presentation functions; for example, some present antigenic peptides containing an N-formyl group and others present lipid antigens (reviewed in [4]). By analogy, specialized functions have been suggested for the nonclassical HLA proteins [5], although there is no homology between these molecules and the nonclassical mouse MHC proteins [1]. Of the nineteen nonclassical HLA sequences known to date, three — HLA-E, HLA-F and HLA-G — are expressed (reviewed in [6]). The almost monomorphic HLA-G was found to be expressed in the trophoblast of human placenta [7,8], predominantly at the fetal–maternal interface along the invasive pathway. There, HLA-G can mainly be found on the extravillous cytotrophoblast and the amnion [9–12] (Fig. 1). Apart from this extraordinary tissue distribution, the existence of five differentially spliced HLA-G mRNAs, lacking none, one, or two exons of the genomic HLA-G sequence, is remarkable [13,14]. In addition, the largest mRNA, which encodes all of the α domains (α_1, α_2 and α_3), gives rise to five isoforms of the HLA-G α chain [15]. All but one of the HLA-G mRNA products seem to stay at the cell surface, whereas the

Figure 1



A placental section showing the different trophoblast populations. Villous cytotrophoblast and villous syncytiotrophoblast are both HLA-G negative. All the other trophoblast populations are HLA-G positive. Decidual NK cells are found in the decidua basalis in close contact with interstitial trophoblast cells. (Adapted from [41].)

37 kDa HLA-G α chain seems to form a soluble MHC class I molecule [16]. The expression of HLA-G at the fetal–maternal interface has led to the suggestion that it plays a role in maternal tolerance against the fetal semiallograft. Three mechanisms for this tolerance have been suggested: first, the presence of HLA-G on the cell surface might prevent lysis by decidual $CD56^+$ natural killer (NK) cells [17,18]; second, γδ T cells, which make up 6–9 % [19], or even 27 % [20], of the $CD3^+$ T cell population in the decidua during early pregnancy, may be induced by HLA-G to suppress rejection of the semiallograft by $CD8^+$ cytotoxic T cells; and finally, soluble HLA-G might have some suppressive effect on T cells.

Apart from these putative specific functions for HLA-G, parallels to classical HLA molecules have been found. Firstly, an interaction between HLA-G and CD8 has been described [21]. Secondly, in the tamarin *Saguinus oedipus*, an ortholog of HLA-G serves as a restricting element [22,23]. Thirdly, limited polymorphism of HLA-G was detected in Afro–Americans [24]. Finally, indirect evidence for peptide loading by HLA-G was provided by the finding that the expression of TAP, the transporter associated with antigen presentation, parallels HLA-G expression in the human trophoblast [25,26]. We therefore investigated the capacity of HLA-G molecules to present peptides. Here we report the isolation of HLA-G peptide ligands from transfected cell lines and their characterization by sequencing, mass spectrometry and peptide-binding studies.

Results

Isolation of HLA-G associated peptides

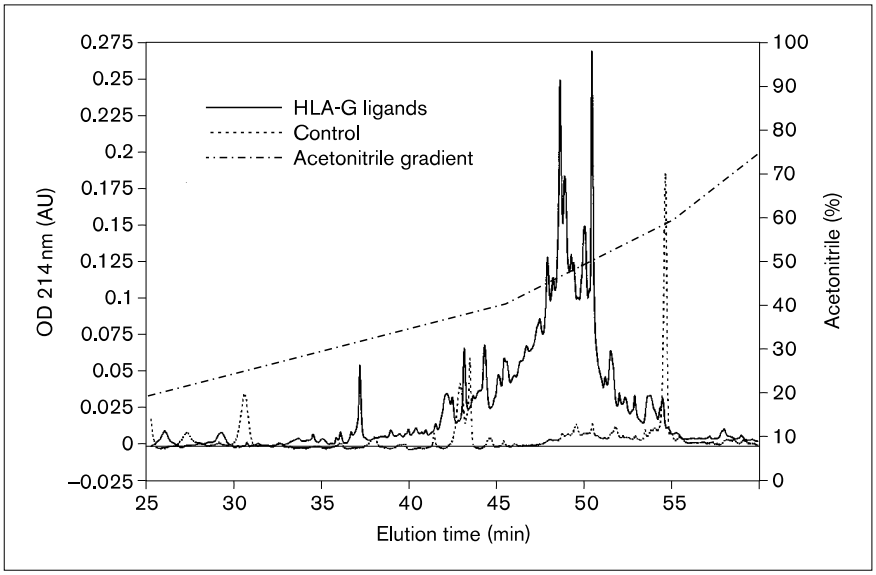
As a source of HLA-G, we used the HLA-null lymphoblastoid cell line LCL721.221 transfected with a plasmid encoding the HLA-G gene [17,27,28]. Transfectants were grown in liquid roller bottle cultures to 10^{10} cells and more. HLA-G molecules were isolated from cell pellets by detergent extraction followed by immunoprecipitation with the solid-phase-bound antibody W6/32 [29], which is specific for a monomorphic determinant on HLA-molecules. Peptides associated with HLA-G were extracted from the HLA–antibody complexes by treatment with trifluoroacetic acid (TFA), as described for other MHC molecules [30], and separated by reversed-phase high-performance liquid chromatography (HPLC) (Fig. 2). In order to control for material sticking nonspecifically to the solid phase, the cell lysate was also mock-precipitated with beads coupled to glycine instead of antibodies. Material extracted from the mock-precipitate was also separated by HPLC (Fig. 2, dashed line). The HPLC profile clearly indicates that a considerable amount of heterogeneous material, most probably a complex mixture of peptides, had been specifically recovered from HLA-G molecules. As a further control, non-transfected LCL721.221 cells were detergent-extracted and immunoprecipitated with W6/32 antibodies. TFA extraction and HPLC separation of this material did not yield any HPLC profile over background (data not shown). In order to compare the peptides extracted from classical HLA molecules, LCL721.221 cells were transfected with a plasmid encoding HLA-A2.1 and subjected to the same procedure. The HPLC profile of the peptides eluted from HLA-A2.1 (data not shown) looked very similar to those eluted from HLA-G. Thus, HLA-G and HLA-A2.1 both seem to be associated with a mixture of peptides.

Pool sequencing of HLA-G associated peptides

Pool sequencing of class I ligands is a fast and convenient way to obtain the features, or motif, shared by all peptides

Figure 2

HPLC profile of the HLA-G ligand separation. HPLC separations were carried out as described [30]. The control trace represents material non-specifically bound by glycine-blocked sepharose.



associated with a particular MHC class I molecule [30]. After setting aside several well separated peak fractions for individual sequencing, all remaining fractions of the HLA-G extracted material, corresponding to elution times of 40–55 minutes, were combined and subjected to pool sequencing. This was performed in a total of four independent experiments. Table 1 shows the raw data of two of these.

The most striking feature of the pool sequencing patterns (Table 1) is that the HLA-G ligands preferentially contain hydrophobic residues at positions 2, 7 and 9, and a proline residue at position 3. Position 2 is occupied by isoleucine or leucine at a ratio of 2:1, position 7 is preferentially occupied by isoleucine or valine (or, to a lesser extent, leucine or phenylalanine). Position 9 represents the carboxyl terminus of the ligands, as indicated by the absence of signals in later positions and by the rapid, nonlinear decrease of all amino acids. This position seems to be exclusively occupied by leucine, but this anchor residue was only visible in two out of four pool sequencing experiments. The reason for this inconsistency is probably technical, because Edman degradation is difficult to perform at carboxy-terminal sites of peptides. As in many HLA class I ligand motifs, mostly hydrophilic residues are favoured in the central region of the ligands, around positions 4 and 5. The pool sequencing experiments also revealed other preferred amino acids: lysine or arginine at position 1, tyrosine at position 6, and glutamine or methionine (or sometimes lysine) at position 8. In addition, many weaker signals for various amino acids at positions 4 through 8 were detected. Thus, these data clearly indicate that HLA-G molecules are associated with a complex mixture of peptides with a specific sequence pattern.

For comparison, the peptide mixture extracted from LCL721.221-derived HLA-A2.1 molecules was also subjected to pool sequencing. The result was consistent with that found earlier for HLA-A2.1 ligands obtained from JY cells [30] (data not shown). The comparison of pool sequences for HLA-G and HLA-A2.1 ligands indicates that peptides associated with HLA-G have a distinct sequence motif, which overlaps with the peptide motif recognized by HLA-A2.1.

Identification of individual HLA-G ligands

The prominent HPLC peak fractions that were excluded from the pool were sequenced individually. We identified three individual peptides: RIIPRHLQL, which was derived from histone H2A, and RLPKDFRIL/KLPAQFYIL, both of which were from unknown sources (Table 2). The mass of the histone-derived peptide was confirmed by mass spectrometry. All three peptides were 9 amino-acids long, had a leucine residue at position 9 and a positively charged amino acid at the amino terminus. Thus, the aliphatic residues at position 2 and 9 of all three ligands confirmed the anchor residues determined by pool sequencing. The proline anchor residue at position 3 was also present in two of the three ligands; the remaining peptide had its proline residue at position 4.

A clear-cut HLA-G ligand motif emerged when the pool sequencing data and the sequences of the individual ligands were combined (Table 2). HLA-G ligands consisted of 9 amino acids, with the following anchor residues: isoleucine or leucine at position 2, proline at position 3 and leucine at the carboxyl terminus. In addition, there was a preference for a positively charged residue at position 1, as confirmed for all three ligands, for an aromatic

residue at position 6, for leucine at position 7 and for glutamine at position 8, as indicated by pool sequencing. The latter preferences are also reflected by the sequences of individual ligands: the peptides KLPAQFYIL and RLPKDFRIL have an aromatic residue at position 6, and the peptide RIIPRHLQL has a leucine residue at position 7 and a glutamine at position 8.

Peptide-binding studies.

Incubation with binding peptides increases the cell-surface density of at least some MHC molecules [31]. We therefore tested whether this might also be the case with HLA-G on LCL721.221 cells. Figure 3 shows that the density of HLA-G on the cell-surface increased 2–3-fold upon incubation with the HLA-G ligand RIIPRHLQL, as indicated by FACS (fluorescence-activated cell sorting) analysis.

Although it was not expected, we found that the density of HLA-A2 increased after incubating HLA-A2.1 expressing LCL721.221 cells with synthetic HLA-A2.1 ligands (Fig. 3e,g). The HLA-G ligand RIIPRHLQL also bound to HLA-A2.1 (Fig. 3c), and conversely, the HLA-A2.1 ligand ILKEPVHGV bound to HLA-G (Fig. 3d). This overlap of binding specificities was not unexpected, because the main features of the HLA-A2.1 peptide motif — a length of 9 amino acids and aliphatic anchor residues at positions 2 and 9 — were reproduced in the HLA-G motif. The overlap was not complete, however, as illustrated by the HLA-A2.1 ligand YMNGTMSQV, which bound to HLA-A2.1 but not to HLA-G (Fig. 3f,g). That the peptide-binding assay used here was peptide-specific was further demonstrated by the lack of binding of an HLA-B27 ligand, GRLTKHTKF, to both HLA-G and

Table 1

Pool sequencing of the ligand mixture eluted from HLA-G molecules.

Experiment 1

	Amino-acid residues (in pmol)																		
Cycle no.	Ala A	Arg R	Asn N	Asp D	Glu E	Gln Q	Gly G	His H	Ile I	Leu L	Lys K	Met M	Phe F	Pro P	Ser S	Thr T	Trp W	Tyr Y	Val V
1	56.0	22.5	128.8	52.5	–	7.6	–	–	13.7	2.6	69.3	7.9	11.0	9.1	47.9	–	5.0	12.1	30.8
2	29.7	10.4	46.6	25.3	–	4.4	–	–	**54.5**	**37.4**	17.8	6.0	8.3	5.7	28.7	–	3.9	15.5	18.1
3	21.2	4.7	22.1	19.3	–	2.8	–	–	41.4	5.0	8.2	0.9	2.1	**70.6**	11.4	–	5.3	5.3	15.9
4	28.8	3.3	14.8	19.6	–	2.5	–	–	5.3	**10.4**	**29.6**	2.3	2.4	29.0	7.2	–	7.1	3.4	9.1
5	12.0	4.7	13.9	19.6	–	3.7	–	–	4.8	**15.8**	23.5	2.4	4.1	11.6	8.0	–	7.0	5.4	11.5
6	14.0	5.5	10.8	12.8	–	2.1	–	–	5.7	8.4	23.6	2.3	**7.2**	6.9	6.6	–	5.8	**12.4**	10.8
7	6.4	3.4	7.6	8.9	–	1.3	–	–	**24.7**	12.3	7.6	1.4	**7.1**	3.9	6.2	–	4.0	5.0	**23.8**
8	5.8	3.6	5.0	7.2	–	**4.0**	–	–	6.0	7.6	8.9	**3.1**	2.9	2.7	5.1	–	3.6	4.3	14.4
9	2.9	2.6	4.7	5.4	–	1.5	–	–	3.0	**15.4**	3.7	1.6	1.6	2.2	5.6	–	3.7	1.6	5.1
10	1.8	2.0	4.2	3.5	–	0.7	–	–	0.4	4.8	1.8	0.5	0.8	1.3	4.9	–	3.1	1.0	2.7

Experiment 2

	Amino-acid residues (in pmol)																		
Cycle no.	Ala A	Arg R	Asn N	Asp D	Glu E	Gln Q	Gly G	His H	Ile I	Leu L	Lys K	Met M	Phe F	Pro P	Ser S	Thr T	Trp W	Tyr Y	Val V
1	24.7	91.2	6.4	4.1	13.7	3.9	56.5	7.9	13.5	8.8	45.2	26.4	7.8	8.4	16.1	–	0.4	9.0	36.8
2	23.0	44.9	4.3	4.1	15.6	**12.5**	70.3	1.3	**135.8**	59.4	15.9	15.6	13.3	13.3	13.0	–	0.2	8.9	40.8
3	34.8	24.5	5.6	**9.8**	17.3	6.8	65.3	2.6	106.5	13.4	8.9	5.0	4.5	**105.8**	7.4	–	0.3	5.1	35.6
4	44.3	31.3	**10.8**	**21.0**	28.4	9.5	51.6	2.0	21.9	15.9	**50.4**	6.6	5.4	74.4	7.6	–	0.2	8.6	20.9
5	28.8	**57.8**	9.8	**37.1**	**41.8**	15.4	56.3	**5.2**	9.7	23.6	**52.9**	5.3	7.1	34.4	10.8	–	0.6	8.4	26.5
6	36.1	**65.8**	10.3	33.0	34.1	12.6	49.3	**9.0**	7.1	19.3	**56.9**	4.2	7.6	21.5	9.2	–	0.2	**23.7**	17.5
7	23.8	**71.8**	8.6	29.3	25.2	9.6	40.2	**9.6**	**35.9**	38.6	34.6	6.2	11.8	17.3	6.3	–	0.4	13.5	**46.9**
8	15.9	**79.3**	8.5	24.0	30.2	**19.5**	35.9	7.5	21.3	30.5	36.4	**9.7**	8.0	12.5	4.9	–	0.3	9.6	35.2
9	12.2	**83.1**	7.7	21.4	29.8	17.8	33.5	6.1	14.8	29.2	23.1	9.2	9.1	12.9	4.6	–	0.2	7.2	22.0
10	10.8	45.6	6.2	12.0	20.1	11.9	30.3	3.5	6.0	19.3	10.6	9.1	3.5	9.1	3.5	–	0.1	5.6	11.7
11	9.2	57.9	6.6	12.0	17.4	12.2	27.1	2.3	5.1	12.6	8.7	4.8	4.7	8.3	3.5	–	0.4	4.9	8.1
12	6.5	54.8	5.2	12.4	10.2	8.4	25.2	1.8	3.2	8.6	7.7	2.8	3.6	7.8	3.0	–	0.3	5.3	5.9

HLA molecules were precipitated from transfected LCL721.221 cells and the peptides extracted. Raw data of peptide pool sequencing are shown [30]. Values showing > 50 % increase compared to the previous (or pre-previous) cycle are defined as signals and are underlined; values with > 100 % increase are referred to as strong signals and are shown in bold [30]. In addition, positions following a signal are defined as a signal of the same quality if the pmol amount of the respective PTH-amino acid (the derivative detected in Edman degradation) is constant or still increasing (for example, F6 in experiment 1, or K6 and H7 in experiment 2). PTH-T coeluted with a major contaminant and its values are therefore omitted from the table. In the first experiment, PTH-E, PTE-G and PTE-H were hidden under contaminant peaks, so they could not be evaluated.

HLA-A2.1. The other two HLA-G ligands, RLPKDFRIL and KLPAQFYIL, also bound to both HLA-G and HLA-A2.1 (data not shown), confirming the overlap of both motifs.

Further peptide-binding studies confirmed the predicted anchor positions of HLA-G peptide ligands. The amino acids at positions 2, 3 and 9 of the peptide KIPAQFYIL (a variant of the ligand KLPAQFYIL, designed to express the best motif match at every anchor position) were substituted with glycine, and the original anchor residues were successively reintroduced. KIPAQFYIL itself strongly stabilized MHC class I density on LCL721.221.G cells upon overnight incubation (Fig. 4a). However, KGGAQFYIG had no stabilizing effect at all. We found that stabilization could be achieved to some extent with KGGAQFYIL, and that the stabilizing effect of KGPAQFYIL was similar to that of KIPAQFYIL (Fig. 4b). Therefore, the three anchor positions, 2, 3 and 9, as determined by ligand analysis, were confirmed by peptide-binding analysis; moreover, two out of the three anchor residues were sufficient for reasonable binding.

Table 2

Comparison of HLA-G and HLA-A2 peptide motifs.

HLA-G	Position								
	1	**2**	**3**	4	5	6	7	8	**9**
Anchor residues		**I** **L**	**P**						**L**
Preferred residues	R K					Y	I V L F	Q M	
Individual ligands	*R	**I**	I	P	R	H	L	Q	**L**
	†R	**L**	**P**	K	D	F	R	I	**L**
	‡K	**L**	**P**	A	Q	F	Y	I	**L**

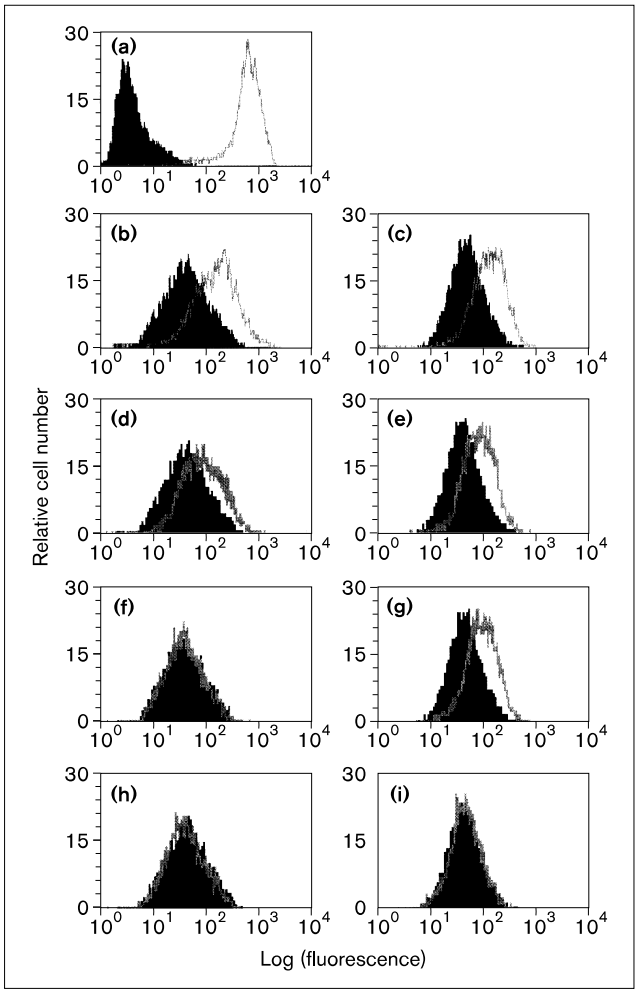
HLA-A2	Position								
	1	**2**	3	4	5	6	7	8	**9**
Anchor residues		**L** **M**							**V** L
Preferred residues				E K		V		K	

The technique of pool sequencing and the criteria for evaluating the raw sequencing data are described in Table 1 and in [30]. Increases of pmol values, compared to previous cycles, that were reproduced in four independent experiments and/or the individual ligands were judged significant. Bold letters represent amino acids that are considered to be anchor residues. The candidate protein for the individual ligand was assigned by database searching. For comparison, the peptide motif of HLA-A2 is shown. Anchor residues are shown in bold and preferred residues are underlined. Source: *histone H2A, amino-acids 77–85; †‡unknown.

Discussion

HLA-G molecules are peptide receptors. The peptide specificity of HLA-G molecules is similar to that of classical MHC molecules [32]. HLA-G molecules present peptides that are predominantly 9 residues in length, as indicated by ligand sequencing. Histone H2A, identified as the donor protein for one ligand, is also a common ligand source for classical MHC molecules [3]. The sequence criteria for peptides to be recognized by HLA-G

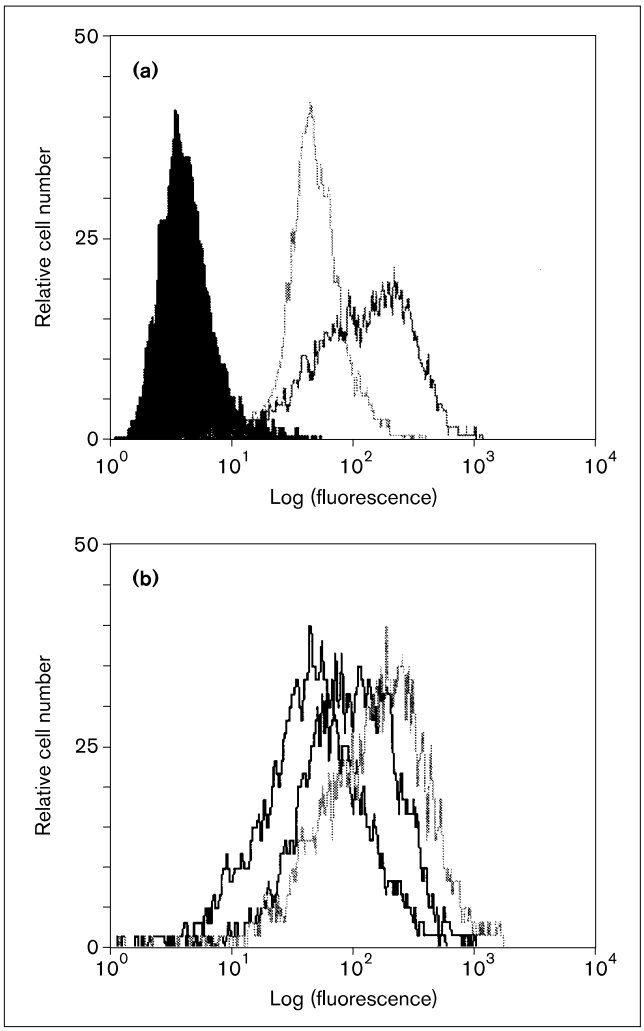
Figure 3



Peptide-binding assay: comparison of peptide binding between HLA-G and HLA-A2.1. Cells were incubated overnight with peptide. MHC class I surface expression was measured by FACS analysis, using W6/32 as the primary antibody and synthetic peptides. **(a)** LCL721.221 cells (black curve) and JY cells (light curve) were stained to serve as negative and positive controls, respectively. **(b,d,f,h)** LCL721.221 cells expressing HLA-G were incubated with the following peptides: RIIPRHLQL (b), ILKEPVHGV (d), YMNGTMSQV (f), or GRLTKHTKF (h). **(c,e,g,i)** LCL721.221 cells expressing HLA-A2.1 were incubated with the peptides used in (b,d,f,h), respectively. In (b–i), black curves denote incubations carried out in the absence of peptide; light curves represent incubations in the presence of peptide.

— the combination of three restricted anchor residues (isoleucine or leucine at position 2, proline at position 3 and leucine at position 9) and six more variable positions — are comparable in stringency to those of classical MHC molecules, implying that the peptide repertoire that is presented has a similar diversity. In addition to the main anchors, preferences for non-anchor residues are observed: position 1 is preferentially positively charged and position 7 is hydrophobic in most ligands.

Figure 4



Peptide-binding assay: analysis of the anchor residues of an HLA-G binding peptide by glycine substitution. Cells were incubated overnight with peptide. MHC class I surface expression was measured by FACS analysis using W6/32 as the primary antibody. **(a)** LCL721.221 cells expressing HLA-G, labelled with (light grey curve) and without (black curve) primary antibody served as controls. The dark grey curve shows the increase of MHC I surface expression upon incubation with the peptide KIPAQFYIL. **(b)** Effect of substituting predicted anchor residues on MHC class I surface stabilization. LCL721.221 cells expressing HLA-G were incubated with KGGAQFYIG (left black curve), KGGAQFYIL (right black curve) or KGPAQFYIL (grey curve).

Although the carboxy-terminal anchor position could only be detected in two of the pool sequencing experiments, all individual peptide ligands had a leucine residue at the carboxyl terminus. The failure to detect a carboxy-terminal amino acid in some of the peptide pools is probably technical, perhaps a result of inefficient fixation of peptides in the sequencer. Peptide-binding studies further confirmed that the amino acids at positions 2, 3 and 9 were anchor residues. Substituting the residues at positions 2, 3 and 9 with glycine gradually diminished the stabilizing effect of the HLA-G binding peptide KIPAQFYIL on MHC class 1 density. Substitution of a single anchor residue had virtually no effect in our binding assay, whereas substitution of two anchors reduced the stabilization effect by 50 %. Peptide KGGAQFYIG, in which glycine replaces all three anchor positions, loses the MHC I surface density stabilizing effect entirely. Glycine was chosen for these substitution experiments because alanine, which is commonly used for such purposes, might have met the requirements (at least partially) for a hydrophobic amino acid at anchor positions 2 and 9.

If the principles of the HLA-A2.1 structure [33,34] are applied to HLA-G molecules, the peptide-binding pockets of HLA-G can be predicted (Table 3) and the accommodation of the HLA-G ligand side chains in the binding groove may be explained as follows. The amino-terminal residue of the ligands is preferentially positively charged, probably because of the negative character of the likely contact site (formed by residues 5, 7, 59, 63, 66, 99, 159, 163, 167 and 171), which is negatively charged because of a glutamate residue at position 63. The anchor at position 2 of HLA-G ligands occupied by the aliphatic amino acids isoleucine or leucine is likely to be accommodated by the B pocket (formed by residues 7, 9, 24, 34, 45, 63, 66, 67, 70 and 99), as in HLA-A2.1 [34]. The preference of HLA-G for isoleucine side chains and of HLA-A2.1 for leucine is probably influenced by an amino-acid substitution at residue 9 of the HLA molecule. In HLA-G, the bulky phenylalanine at position 9 is substituted by the smaller serine residue, leading to a more spacious pocket which accommodates isoleucine with a different configuration of the methyl groups in the side chain. The proline residue at anchor position 3 probably sets conformational constraints for the peptide, which then fits more easily into the peptide-binding groove of HLA-G. Hydrophobic residues at position 7 are probably not accommodated in a specific pocket either, but make contact with the HLA α2-helix [34]. Carboxy-terminal anchor side chains are accepted by the F pocket (formed by residues 77, 80, 81, 84, 116, 123, 143, 146 and 147) in HLA-A2 molecules [34]. Pocket F in HLA-A2.1 and in HLA-G differ only slightly at the pocket rim. Therefore, leucine should be accepted by pocket F of HLA-G, as in the case of the HLA-A2.1 F pocket [34]. HLA-G preferentially recognizes ligands containing a leucine residue at position 9, whereas HLA-A2.1 prefers a

valine residue at this position; this seems to correlate with the downward orientation of the side chains at position 7 of the ligand, and the substitution of arginine for tryptophan at position 97 of HLA-G, which results in the amino-terminal orientation of tyrosine at position 116 [34]. In addition, some HLA-A2.1 ligands with carboxy-terminal valine residues are able to bind to HLA-G in our peptide-binding assay. In summary, the assumed HLA-G pocket structure well reflects the ligand motif requirements.

Our peptide-binding assays confirm the sequence-specificity of the HLA-G ligand motif, in that a HLA-B27 ligand and one of the HLA-A2.1 ligands do not bind. Furthermore, the peptide-binding experiments indicate that the binding requirements of HLA-G and HLA-A2.1 overlap. All individual HLA-G ligands have hydrophobic amino acids in positions 2 and 9. It is therefore not surprising that they also bind to HLA-A2.1. Likewise, some of the HLA-A2.1 ligands bind to HLA-G, exemplified by the peptide ILKEPVHGV in our assay. On the other hand, other HLA-A2.1 ligands do not bind to HLA-G, as represented by the peptide YMNGTMSQV. Thus, the partial overlap in peptide-binding requirements for HLA-A2.1 and HLA-G emphasizes the fact that the general criteria for peptide accommodation are shared between these classical and nonclassical HLA molecules.

HLA-G is an ortholog of the class I molecule of the New World monkey, *Saguinus oedipus*, which diverged from the human line 40 million years ago [22]. The HLA-G-related class I molecules serve as classical restriction elements in *Saguinus oedipus* [23]. The *Saguinus oedipus* class I molecule is a peptide receptor with classical peptide-binding specificities (B. Grahovac, K. Falk, G. Rötzschke, G. Jung, S.S. and H-G.R., unpublished observations). We have shown that the HLA-G motif is also classical; however, the anchor positions and chemistry of the ligands are not conserved between this and the *Saguinus oedipus* class I molecule. This finding is not unexpected, because amino-acid substitutions inside the peptide-binding pockets alter their environment substantially.

HLA-G is a nonclassical MHC molecule. Other nonclassical MHC molecules with peptide-presenting functions

Table 3

Comparison of the amino acids predicted to be contained within the peptide-binding pockets of HLA-G and HLA-A2.

(a) Predicted pockets contacting HLA-G ligands

Pocket A			Pocket B			Pocket F		
Position	HLA-G	HLA-A2	Position	HLA-G	HLA-A2	Position	HLA-G	HLA-A2
5	Met	Met	7	Tyr	Tyr	77	**Asn**	**Asp**
7	Tyr	Tyr	9	**Ser**	**Phe**	80	Thr	Thr
59	Tyr	Tyr	24	Ala	Ala	81	Lys	Lys
63	Glu	Glu	34	Val	Val	84	Tyr	Tyr
66	Asn	Lys	45	Met	Met	116	Tyr	Tyr
99	Ile	Tyr	63	Glu	Glu	123	Tyr	Tyr
159	Tyr	Tyr	66	**Asn**	**Lys**	143	**Ser**	**Thr**
163	Thr	Thr	67	**Thr**	**Val**	146	Lys	Lys
167	Trp	Trp	70	His	His	147	**Cys**	**Trp**
171	Tyr	Tyr	99	**Ile**	**Tyr**			

Predicted contact-forming residues of the peptide ligands

Pocket A			Pocket B			Pocket F		
Position	HLA-G	HLA-A2	Position	HLA-G	HLA-A2	Position	HLA-G	HLA-A2
1	Arg, Lys	–	2	Ile, Leu	Leu, Met	9	Leu	Val, Leu

(b) Predicted pockets which presumably do not interact with peptide ligands of HLA-G molecules

Pocket C			Pocket D			Pocket E		
Position	HLA-G	HLA-A2	Position	HLA-G	HLA-A2	Position	HLA-G	HLA-A2
9	**Ser**	**Phe**	99	**Ile**	**Tyr**	97	**Trp**	**Arg**
70	His	His	114	**Glu**	**His**	114	**Glu**	**His**
73	Thr	Thr	155	Gln	Gln	133	Trp	Trp
74	**Asp**	**His**	156	**Arg**	**Leu**	147	**Cys**	**Trp**
97	**Trp**	**Arg**	159	Tyr	Tyr	152	Val	Val
			160	Leu	Leu	156	**Arg**	**Leu**

The relevant residues were identified on the basis of the HLA-A2.1 crystal structure [33]. Differences are highlighed in bold.

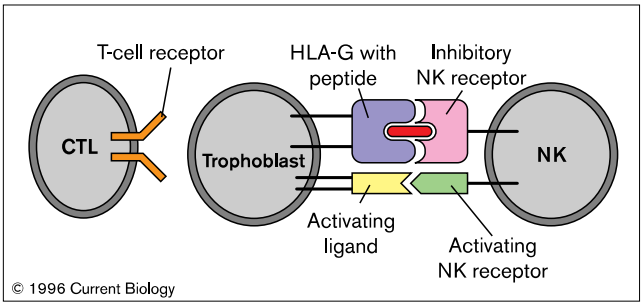
have been described only in the mouse so far (reviewed in [4]). The nonclassical mouse and human MHC molecules are not homologous [1]. The mouse molecules function as specialized peptide receptors — H2-M3 is especially equipped to present N-formylated peptides [35,36], Qa-2 is specialized for histidine-containing peptides [37,38] and Qa-1 preferentially presents signal sequences [39].

In comparison, nonclassical HLA-G molecules resemble classical peptide receptors and the classical ortholog of *Saguinus oedipus*, but not the nonclassical peptide receptors of the mouse. In humans, however, no HLA-G-restricted T lymphocytes have been reported yet [1]. Thus, we are left with the question as to what physiological role HLA-G molecules may play. The 'null hypothesis' suggests that HLA-G has no function at all (reviewed in [40,41]), but is an evolutionary relict. Another suggestion is that HLA-G has a non-immunological function. Because HLA-G molecules are selectively expressed on extravillous trophoblast, which invades uterine tissues during the process of implantation, we have proposed that the unusual $CD56^{bright}$ $CD16^{-}$ $CD3^{-}$ NK cells, which populate the uterine mucosa at this time, control trophoblast invasion by interacting with HLA-G [41,42]. We now find, however, that HLA-G molecules present peptides, and are very similar to classical HLA-A and HLA-B molecules. It therefore seems likely that HLA-G has an immunological function, as discussed below.

HLA-G molecules are selectively expressed at the fetal–maternal interface. Because the fetus can be considered to be a semiallograft — it expresses paternal antigens foreign to the mother's immune system — there must be a mechanism that prevents fetal rejection by the maternal immune system. One obvious mechanism by which fetal rejection can be prevented is by not expressing HLA-A and HLA-B (and perhaps HLA-C) on the outer fetal lining, the trophoblast. Thus, T cells alloreactive against paternal HLA-A and HLA-B, as well as minor-histocompatibility-antigen-specific T cells, which recognize paternal peptides presented by HLA-A or HLA-B molecules that are shared between father and mother, can neither recognize nor attack the intact fetus. Because HLA-G itself exhibits limited polymorphism, it should not be a target for alloreactivity by itself. However, the lack of HLA-A, HLA-B, or HLA-C expression on a cell draws the attention of another immune cell type, the NK cells.

NK cells are specialized to kill cells that no longer express one or more of the HLA-A, HLA-B or HLA-C alleles [43,44]. They perform this function by using two sets of receptors, one of which is activating, the other inhibiting. The activating NK receptors, which probably recognize carbohydrate structures expressed on a wide array of target cells, and the inhibitory receptors, which recognize structures on HLA-B and HLA-C (and probably HLA-A) molecules, are in a balanced state if either sees their ligands. If the ligands if the inhibitory receptors are missing, however, the target cell is killed [45]. This has led to the formulation of the 'missing-self' hypothesis [46].

Figure 5



A hypothesis for HLA-G function. Maternal cytotoxic T cells (CTLs) directed against paternal HLA-A or HLA-B antigens cannot kill the trophoblast because it lacks HLA-A and HLA-B. NK cells, which usually recognize and kill cells that do not express HLA-A or HLA-B molecules, are inhibited by HLA-G expression on trophoblast cells.

NK cells are found in great numbers in the decidua, a tissue in intimate contact with the extravillous trophoblast. NK cells freshly isolated from the decidua do not kill trophoblast targets; they do so, however, after *in vitro* culture with interleukin 2 [41,45]. Thus, trophoblast cells have the potential to be lysed by NK cells, implying that they express structures recognizable by activating NK receptors. Because HLA-G is the only HLA molecule shown to be expressed on trophoblast cells, it is likely that this molecule serves as an NK inhibitor. Whether the peptides presented by HLA-G are important for NK recognition, or merely act to stabilize the surface expression of HLA-G, remains to be determined.

Thus, according to our hypothesis, the fetus protects itself from being destroyed by alloreactive maternal T cells by shielding itself with a layer of cells lacking HLA-A and HLA-B expression. To avoid destruction of this layer by NK cells, the trophoblast expresses the almost monomorphic HLA-G in order to stimulate every single one of the inhibitory NK receptors, so that the balance is shifted towards inhibition (Fig. 5).

In addition to inhibiting NK cells, peptide presentation by HLA-G molecules could allow T cells to survey trophoblast cells for viral infection or malignant transformation, as HLA-A, HLA-B or HLA-C restricted T cells do with other cell types. This possibility had been discussed by Schmidt and Orr [47] even before the peptide receptor nature of HLA-G was discovered. A principal problem here, however, is the obvious lack of HLA-G expression in the thymus [41], which makes positive selection of HLA-G restricted T cells an unlikely event. It remains to be

seen, therefore, whether HLA-G restricted T cells, perhaps virus-specific, will be found in the future.

During preparation of this manuscript, Lee *et al.* [48] reported an HLA-G peptide motif similar to ours, based on ligand sequencing but without confirmation of the anchor residues by binding assays.

Conclusions

HLA-G molecules are peptide receptors presenting peptides derived from cellular proteins at the cell surface. Like the classical HLA class I molecules, HLA-A, HLA-B and HLA-C, HLA-G molecules present peptides with a specific sequence motif, the basic features of which are XI/LPXXXXXL. This information should be of great value for determining the physiological function of HLA-G molecules selectively expressed on the outermost layer of the fetus, which is exposed immediately to maternal blood and immune cells. Most likely, HLA-G molecules function as inhibitors of NK cells, and thus avoid destruction of the fetus by the maternal immune system. In addition, peptide presentation by HLA-G expressed on the trophoblast might allow T cell-mediated surveillance of viral infection or malignant transformation, although HLA-G restricted T cells have not yet been identified.

Materials and methods

Cell lines and antibodies

HLA-G transfectants [17] of the LCL721.221 HLA-null lymphoblastoid cell line [27] were used as a source of HLA-G. HLA-A2.1 transfected LCL721.221 cells [17], and untransfected parental cells served as controls. Antibodies were affinity purified on protein A–Sepharose beads by standard procedures from culture supernatants of the hybridomas W6/32 (anti-HLA-class-I) [29] and BB7.2 (anti-HLA-A2/A69) [49,50]. Transfectants were regularly analysed for transgene expression by FACS analysis (data not shown).

Isolation of HLA-G molecules and ligands

HLA-G molecules and peptide ligands were isolated essentially as described for HLA-A2 [30]. Cells were expanded at 37 °C in 2 l roller bottles, containing Dulbeccos's modified Eagle's medium supplemented with 25 mM Hepes, 2 mM glutamine, 5 % fetal calf serum, 5 % newborn calf serum and 75 μg ml^{-1} hygromycin, to 1.5×10^6 cells per ml. In some experiments, RPMI1640 medium containing 10 % FCS and supplements was used. For each experiment, $1–4 \times 10^{10}$ cells were lysed in PBS, containing 1 % NP40 and protease inhibitors, for 1 h, while stirring on ice. The lysate was precleared at $350 \times g$ for 10 min and then ultracentrifuged at $100\,000 \times g$ for 1 h. The cleared lysate was sterilized by filtration and passed over immunoaffinity columns. After washing away unbound material, ligands were eluted from the precipitated HLA molecules by treatment with 0.1 % TFA at pH 2. TFA-extracted material was subjected to ultrafiltration through Centricon 10 (Amicon) membranes and the permeate was SpeedVac concentrated. The extracted material of molecular weights below 10 kDa was separated by reversed-phase HPLC (Pharmacia SMART system) on a narrow-bore HPLC-column (2.1 mm, C2/C18; Pharmacia).

Peptide analysis

Pooled HPLC fractions [30] and individual fractions were sequenced by Edman degradation on a protein sequencer (model 476A, Applied Biosystems). Masses of individual fractions were determined by ESI- or MALDI-MS on Finnigan TSQ 7000 and Finnigan Vision 2000 mass spectrometers, respectively. Candidate protein sources were identified by matching individual sequences in protein or nucleotide databases.

Peptide-binding assay with synthetic peptides

Synthetic peptides were synthesized by the Fmoc solid phase method on a peptide synthesizer (model 432 A; Applied Biosystems). The peptides were analyzed by reversed-phase HPLC and mass spectrometry. We used three HLA-G binding peptides (RIIPRHLQL, RLPKDFRIL and KLPAQFYIL), two HLA-A2 binding peptides (ILKEPVHGV and YMNGTMSQV [3]), and one HLA-B27 binding peptide (GRLTKHTKF [3]). In addition, the variant peptide KIPAQFYIL, and its glycine-substituted analogues, KGGAQFYIG, KGGAQFYIL and KGPAQFYIL, were tested. The binding assays were performed with LCL721.221 cells transfected with plasmids encoding HLA-G or HLA-A2.1. Untransfected LCL721.22 cells served as a negative control, and JY cells were used as a positive control. Cells (1×10^6) were pelleted for 10 min at 1 000 rpm, resuspended in 1 ml RPMI (containing 10 % FCS and supplements), and incubated overnight for approximately 15 h with 200 μg peptide at 37 °C in 24-well plates. FACS analysis was performed using W6/32 as the primary antibody and a fluorescein isothiocyanate-labelled goat anti-mouse antibody as the secondary antibody.

Acknowledgements

M.D. and C.M. contributed equally to this work. We thank S. Faath, G. Pougialis and K. Gunst for technical assistance, T. Ruppert for mass spectrometry and J. Dietl for helpful discussions. This work was supported by the Deutsche Forschungsgemeinschaft (Leibnizprogramm and Großgerätesachbeihilfe to H-G.R.), grants from the Bundesministerium für Bildung und Forschung, and grants from the Medical Research Council, UK (Y.W.L. and N.H.) and from the Special Program of Research, Development and Research Training in Human Reproduction, World Health Organisation (Y.W.L.). N.H. is a Fellow of the Lister Institute for Medical Research.

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