CMLS, Cell. Mol. Life Sci. 59 (2002) 1460–1466
1420-682X/02/091460-07

CMLS Cellular and Molecular Life Sciences

Review

HLA-G protein processing and transport to the cell surface

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Received 4 June 2002; accepted 2 July 2002

Abstract. Data are presented on the intracellular trafficking of HLA-G protein, taking the unique features of this non-classical molecule into consideration: the existence of seven isoforms resulting from alternative splicing (HLA-G1 to G7), and reduced tail length compared with HLA class I antigens. Biochemical studies and analysis of viral strategies for escaping the host immune system led to the demonstration that (i) both the membrane-bound (HLA-G1) and the soluble (HLA-G5) forms of the molecule require peptide association for cell surface expression, using TAP-dependent or TAP-independent pathways; (ii) peptide loading onto the HLA-G protein plays a critical role in controlling the quality of the molecule reaching the cell surface; (iii) surface expression of truncated HLA-G molecules is possible, and (iv) HLA-G expression may be restricted to soluble HLA-G5. These data reveal that HLA-G presents specific cell trafficking pathways and strongly support the contention that the primary function of HLA-G is as of an inhibitor ligand for immune-competent cells.

Keys words. HLA-G isoform; intracellular traffic; function.

Introduction

Data concerning cell processing and transport of HLA-G protein continue to accumulate, highlighting the importance of the unique features of this non-classical MHC class I molecule. HLA-G protein is expressed as seven isoforms generated by alternative splicing of the primary transcript: four membrane-bound forms (HLA-G1 through G4) and three soluble forms (HLA-G5 through G7). The structure of the HLA-G1 protein is similar to those of classical class I antigens, consisting of three extracellular domains: α1, α2, and α3 (the latter associated with β_2-microglobulin), plus one transmembrane and one cytoplasmic domain. However, a unique feature of HLA-G is the presence of a stop codon in exon 6, which reduces the length of its protein tail [1]. In addition to this latter characteristic, one extracellular domain is deleted in HLA-G2 (α2), two in HLA-G3 (α2 and α3), and one in HLA-G4 (α3) [2, 3] (fig. 1). Soluble HLA-G isoform C terminals are encoded by intron 4 for HLA-G5 (full-length counterpart) and HLA-G6 (minus α2), and by intron 2 for HLA-G7 (consisting only of α1) [4–6] (fig. 1).

The HLA-G gene exhibits limited polymorphism: 14 alleles encoding six proteins with minor variations located outside the HLA-G groove [7, 8], and one 'null' allele with a deletion in exon 3 that generates a stop codon at the beginning of exon 4 [9]. Basal levels of the HLA-G gene are transcribed in almost all tissues studied; however, the HLA-G protein displays local expression only in cytotrophoblasts [10], amnion epithelial cells [11], chorionic villi endothelial cells [12], and adult thymic epithelial cells [13]. HLA-G protein expression may be up-regulated following exposure to interleukin (IL)-10 [14, 15], interferon (IFN)-γ [16], IFN-γ + granulocyte/macrophage-colony-stimulating faktor (GM-CSF), IFN-γ + IL-2 [17], and

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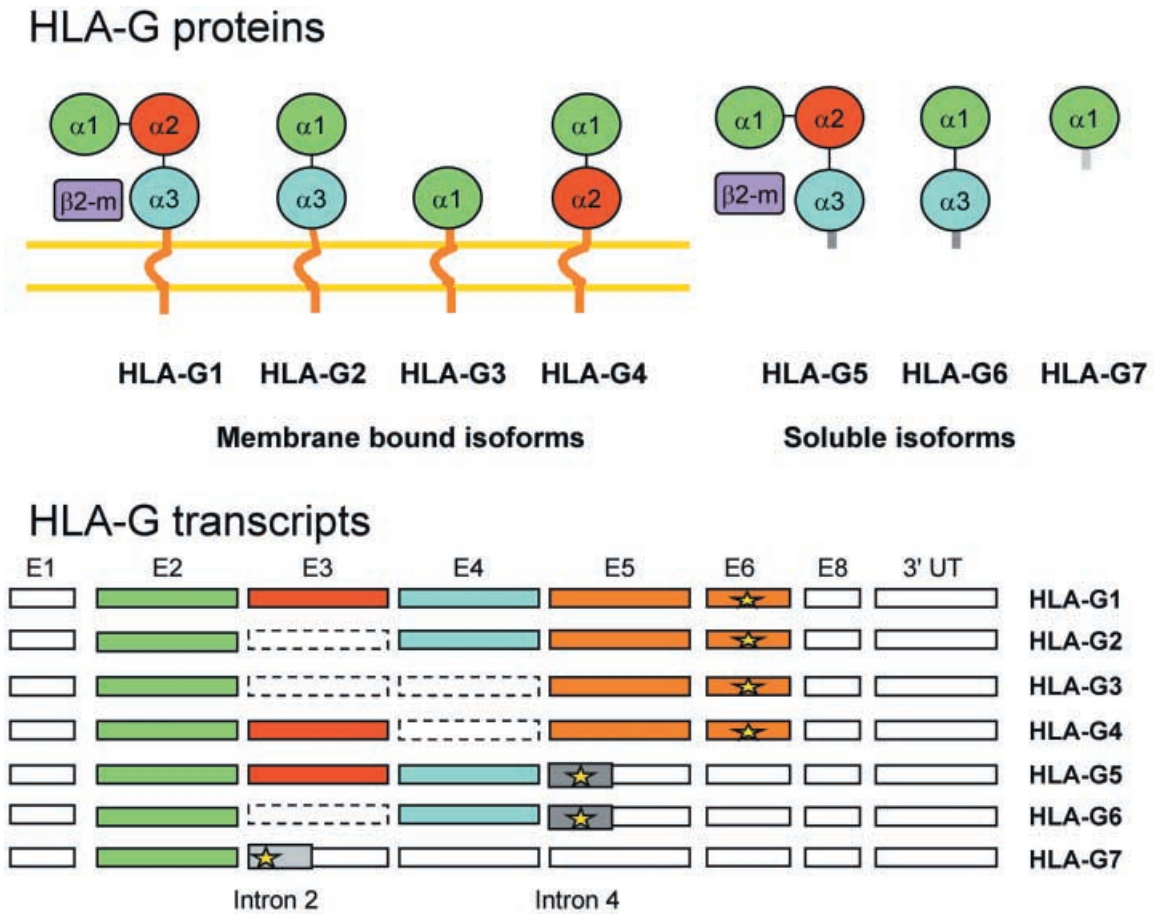

Figure 1. Alternatively spliced HLA-G mRNA and HLA-G protein isoforms. Exon 1 (E1) encodes leader peptide, exon 2 to exon 4 (E2–E4) encode α1 to α3 extracellular domains, exon 5 (E5) encodes the transmembrane region, and exon 6 (E6) encodes the reduced cytoplasmic domain of the HLA-G protein. Translation of HLA-G1 to HLA-G4 transcripts generates membrane-bound forms of HLA-G proteins. Introns 4 and 2 are retained in HLA-G5 and G6, and G7, respectively, thus generating soluble forms of HLA-G proteins. In these introns, open reading frames give rise to 21-amino-acid-specific tails for HLA-G5 and HLA-G6 proteins and a 2-amino-acid-specific tail for the HLA-G7 isoform (tails colored in gray). The star indicates the position of a stop codon.

IFN-β [18]. HLA-G protein is also expressed in certain tumors [19–21], in HCMV- and HIV-infected cells [22, 23], in transplanted heart tissue [24], and in skin with inflammatory pathologies, such as psoriasis and eczema [25–27].

Regulation of HLA-G gene expression is also atypical. Indeed, almost all classical HLA class I gene promoter regulatory sequences are disrupted in the HLA-G gene promoter [28]. Moreover, HLA-G participates in immune tolerance by inhibiting the function of cytotoxic lymphocytes (CTL) and natural killer (NK) cells [29, 30] and is able to inhibit the CD4+ alloproliferative response [31, 32]. Other functions have been proposed for HLA-G, notably in antigen presentation [33], inhibition of rolling adhesion of activated human NK cells [34], and as a shield against inflammation aggression [27].

Lessons learned from classical HLA class I antigens indicate that folding, assembly, and peptide loading of HLA molecules are critical for the regulation of their cell surface expression and involve intracellular protein complexes and chaperone molecules [35]. Cytosolic and nuclear proteins are first degraded by the proteasome, then transported into the lumen of the endoplasmic reticulum (ER) by a heterodimeric peptide transporter which consists of TAP1 and TAP2. At the same time, the class I chain and β_2-microglobulin are assembled in the ER with the help of calnexin and calreticulin. The peptides are then loaded into the groove of the HLA class I heavy chain, which in turn associates with β_2-microglobulin. The glycosylation pattern of the class I heavy chain is modified once the complex has exited the lumen of the ER and the heterotrimer is transported through the Golgi to the cell surface.

Biochemical approaches and the use of viruses that cause down-regulation of classical HLA class I protein expression have revealed shared pathways in the assembly and transport of HLA-G and HLA class I molecules [36]. Nevertheless, the HLA-G protein displays unique struc-

tural properties and presents a large panel of isoforms, suggesting specific HLA-G trafficking pathways. In the present article, we review recent findings relevant to these points and discuss them in the context of HLA-G function. Most of the data presented concern HLA-G1 and HLA-G5, for which suitable reagents are readily available.

HLA-G1 and G5 grooves contain a nanopeptide

The first indication of peptide presentation by the HLA-G1 molecule was provided by DNA sequence analysis and comparison with the HLA-A2 molecule. Several similarities were found, particularly in the α1 and α2 domains, which form the peptide pocket, suggesting that some peptides presented by HLA-A2 might also be presented by HLA-G1 [1]. Biochemical analysis of the HLA-G1 membrane-bound and the HLA-G5 soluble forms of the molecule have demonstrated that both proteins expressed in the LCL721.221 lymphoblastoid cell line (HLA class I negative) consist of heavy chain/β_2-microglobulin/peptide in a 1:1:1 ratio. Peptide elution experiments confirm the peptides presented by HLA-G1 and HLA-G5 to be the same size as those carried by HLA-A2, consisting of nine amino acids with the consensus sequence XI/LPXXXXXL [37, 38]. In accordance with the observed sequence similarities between HLA-G1 and HLA-A2, anchoring positions are located on amino acids 2 (isoleucine or leucine), 3 (proline), and 9 (leucine), although positions 2 and 9 are enough for efficient anchoring [37].

The diversity of eluted peptides from HLA-G1 and HLA-G5 was found to be approximately fivefold lower than with classical HLA class I [37]. Such limited diversity might be due to the cell type used, and the repertoire of individual peptides bound may well be different in vivo, however, this remains to be determined. Nevertheless, in view of the limited peptide diversity observed, Le Bouteiller and Blaschitz [39] suggested that viral peptides, a limited number of which infect trophoblasts, may be presented to allow HLA-G anti-viral function. Of note is that the HLA-G molecule displays invariance in the region of the peptide-binding groove and may limit the number of peptide combinations.

Finally, although there is strong evidence that HLA-G1 and HLA-G5 bind naturally processed peptides, the question is still open as to whether there are peptides that bind to the other HLA-G isoforms. The α1 and α2 domains, which form the peptide groove, are present only in HLA-G4, which might therefore be a candidate peptide presenter. Whether other isoforms are capable of binding peptide remains to be determined. One hypothesis to explore is the possible formation of HLA-G2, HLA-G4, and HLA-G3 homodimers, which might permit peptide loading, similar to HLA class II antigens [2].

TAP deficiencies down-regulate HLA-G1 and HLA-G5 expression

Is peptide loading of HLA-G a TAP-dependent mechanism? The role of TAP in HLA-G cell surface expression was firstly reported by Lee et al. [37], using modified lymphoblastoid cell lines. The TAP1-negative B-LCL 721.134 cell line was transfected with either HLA-G1 or HLA-G5 cDNA and compared with the TAP-positive B-LCL 721.221 cell line. In the transfected TAP-negative cell line, HLA-G1 expression was reduced to about 20% compared to the TAP-positive cell line, whereas no HLA-G5 protein could be detected in supernatant. HLA-G therefore uses both TAP-dependent (HLA-G1 and HLA-G5) and TAP-independent (HLA-G1) pathways to bind peptide [37].

Immunoprecipitation studies confirmed the existence of an interaction between the TAP complex and HLA-G1 [37, 40]. However, the lack of a detectable association of TAP with HLA-G5 led to the hypothesis of possible binding of cytosolic peptide without interaction [37]. This suggests either that the residues that interact with TAP are located in the cytoplasmic tail of HLA-G1, or that the unique 21 amino acids in the C-terminal of HLA-G5 interfere with TAP association [37]. The peptide-loading process thus remains to be investigated and seems to be associated with either the soluble or membrane form status of the molecule.

The results of Lee et al. [37] are consistent with several reports concerning the elucidation of pathways that lead to the cell surface expression of HLA-G1 in the context of Herpes simplex virus (HSV) and human cytomegalovirus (HCMV) infections, which have been associated with pregnancy loss. HSV produces the 9-kDa viral protein ICP47, which has been shown to block the TAP transporter, and therefore the translocation of peptide into ER, leading to the retention of the HLA class I heavy chain [41, 42]. Data obtained with the JEG-3 choriocarcinoma cell line have shown that HLA-G1 in HSV-infected or in ICP47-transfected cells fails to acquire endoglycosidase-H (endo-H) resistance, and thus is retained in the ER [43]. HLA-G1 cell surface analysis after ICP47 gene transfection of HLA-G-transfected LCL721.221 cells yielded results that agreed with those of Lee et al. [37], in TAP-negative cells, since tenfold down-regulation was observed with residual HLA-G1 cell surface expression [44]. In agreement with HSV-related experiments, HLA-G1 was shown to be sensitive to endo-H treatment in JEG-3 cells transfected with the HCMV US6 gene, which encodes a glycoprotein that also prevents peptide loading of the HLA class I molecule by inhibiting the TAP complex [45]. Using US6-expressing Hela cells, Park et al. [46] demonstrated that these peptide-deficient HLA-G1 molecules are rapidely degraded in the ER. We also recently investigated cells with both TAP and proteasome deficien-

cies, showing that HLA-G1 cell surface expression was entirely abolished [Rouas-Freiss et al., unpublished results]. This strongly supports the contention that whatever the loading mechanism involved may be, the HLA-G heavy chain requires peptide association for membrane expression.

HLA-G traffic presents unique features

First, HLA-G is likely to have evolved as a more resistant molecule than classical class I antigens. Indeed, using SDS-PAGE and immunoblotting analysis, McMaster et al. [47] observed that HLA-G molecules produced by cytotrophoblasts and found in amniotic fluid bear unusual carbohydrate structures, leading to a broad molecular-weight range of HLA-G-immunoreactive bands. Like classical HLA class I molecules, HLA-G contains a single N-linked glycosylation site. However, digestion of HLA-G proteins from placenta by endo-β D-galactosidase suggests that these molecules specifically carry *N*-acetyllactosamine units, which might stabilize the molecule [47]. Furthermore, in both JEG-3 cells and 2 A2 porcine bone-marrow-derived stromal cells, HLA-G1 has been shown to be resistant to dislocation and degradation mediated by HCMV US2 and US11 gene products, sharing this property with HLA-Cw3,-Cw4 [48]. In vitro co-transcription/translation of class I heavy chains with US2 and US11 gene products followed by immunoprecipitation experiments have demonstrated that these proteins do not associate with either HLA-G or HLA-C molecules [48].

A recent study regarding protein traffic has demonstrated that the shortened cytoplasmic domain of the HLA-G1 molecule plays a critical role in quality control of that molecule in post-ER compartments [46]. First, the absence of endocytosis signals was demonstrated to severely reduce the spontaneous endocytosis of HLA-G1 compared to classical class I molecules and resulted in the prolonged retention of molecules at the cell surface [49]. Second, due to the truncated tail of HLA-G1, a dilysine residue motif is positioned two to three amino acids from the C-terminal end of the cytoplasmic domain, thereby allowing interaction with the coat protein complex (COP) [46]. This complex is known to mediate retrieval from post-ER compartments [50]. In JEG-3 and LCL721.221 transfected cells that stably express HLA-G1, the dilysine motif function acts as a retrieval signal for HLA-G from the post-ER compartment to the ER and is responsible for the slow transport kinetics of HLA-G [46]. Third, loading of HLA-G1 with high-affinity peptides (KIPAQFYIL) instead of low-affinity ones (KGGAQFYIL) prevents retrieval of the HLA-G molecule, resulting in increased cell surface expression of HLA-G1. Park et al. [46] therefore proposed that the loading of these high-affinity peptides into the HLA-G groove could induce conformational changes that might alter recognition of the dilysine motif. This in turn might inactivate the retrieval motif and result in up-regulation of HLA-G1 cell surface expression.

In summary, HLA-G differs from the other HLA class I molecules by its limited polymorphism, the restricted number of peptides that bind to it by both TAP-dependent and TAP-independent pathways, and by quality control through high-affinity peptides. These data all suggest a crucial role for peptide binding to HLA-G1 and G5 in stabilizing these molecules and favor transport to the cell surface.

HLA-G2, G3 and G4 cell traffic

One striking feature of HLA-G is that in addition to HLA-G1, it may be expressed as three other membrane-bound isoforms: HLA-G2, G3, and G4, all of which are present in cytotrophoblasts [51]. The question of their expression at the cell surface has been investigated by several groups. Mallet et al. [52] used targeted enhanced green fluorescence protein (EGFP)-HLA-G fusion cDNA to track HLA-G isoform expression in murine (J26 fibroblasts transfected with the human β_2-microglobulin), and human (JAR choriocarcinoma) transiently transfected cells, observing that HLA-G2, G3, and G4 were retained in the ER. Using cDNAs ligated to an epitope tag, then transfected into the human B-lymphocyte C1R cell line (HLA-A, B negative, C positive), Bainbridge et al. [53] drew the same conclusion. In contrast, using untagged vector constructions, we recently observed that 2 h after synthesis, HLA-G2, G3 and G4 isoforms reached the cell surface of an HLA-A, B, C, E-positive melanoma cell line as endo-H-sensitive (immature) proteins [54]. Although this latter feature is unusual, it has already been reported for the HLA class-I-like molecule CD1d [55]. Most important is that the truncated HLA-G isoforms were shown to inhibit both NK and antigen-specific CTL cytolysis, suggesting that the functional epitope is the α1 domain, which is the one present in the HLA-G3 isoform [54].

The apparent discrepancies between previous studies and our results may be explained by differences in the experimental procedures. In particular, the use of tagged transfected constructions may affect protein structure and lead to the elimination of misfolded proteins by cytoplasmic proteases. Another hypothesis is that an unknown chaperone protein that would be absent in JAR, J26, and C1R is required for cell surface expression of short HLA-G isoforms. In our model, HLA-G2, G3, and G4 were co-expressed with other HLA class I molecules and therefore might be candidate chaperone molecules. Of note is that the mouse CMV glycoprotein gp34 associates with folded class I MHC molecules and exhibits two endo-H-sensitive oligosaccharides at the cell surface [56].

HLA-G5 expression may be favored

Carrying out mixed lymphocyte cultures, we found that the soluble HLA-G5 protein, but not the membrane-bound HLA-G1 isoform, was secreted by allo-specific CD4+ T cells from the responder population, which in turn suppressed the allogeneic proliferative T cell response [32]. The production of HLA-G5 protein only was also observed in HCMV-infected U-373 MG astrocytoma cells, which constitutively express HLA-G mRNAs. Accordingly, U-373 MG astrocytoma cells stably transfected with both IE-pp72 and IE-pp86 HCMV proteins are capable of specifically expressing HLA-G5 [22]. In situations in which HLA-G1 protein expression is absent, HLA-G5 expression might be associated with the HLA-G allele, which is a 'high secretor' of soluble forms (G*01041), rather than with 'low secretor' alleles, such as G*01013, for example [57]. The cell genotype may influence alternative splicing, mRNA stability, and post-transcriptional regulation of protein translation.

Do HLA-G5 properties render its transport to the cell surface easier? First, the HLA-G5 protein is shorter than HLA-G1 and does not possess a tail endowed with dilysine residues involved in quality control for reaching the cell surface. Second, HLA-G5 exibits a unique 21-amino acid carboxyl terminus, which confers solubility to the molecule [4]. One is therefore tempted to suggest that these structural properties could render certain viral protein/HLA-G1 interactions ineffective. In agreement with this hypothesis is the absence of interaction of HLA-G5 with TAP [37], as mentioned above. Finally, of note is that the soluble HLA-G6 isoform shares common C-terminal properties with HLA-G5 and may thus also enjoy favored expression. In particular, the HLA-G6 protein isoform has been shown to circulate in maternal blood during pregnancy [58].

Conclusion

Convergent data show that HLA-G1 exhibits the typical structure of an antigen-presenting element. The demonstration that the HLA-G-restricted specific CD8+ T cell repertoire is selected in HLA-G transgenic mice [59] may argue in favor of HLA-G protein being a restricting element. Furthermore, a recent study has shown that mother-child pairs in which both carry the identical mutation in HLA-G exon 2 may be at higher risk for mother-to-child transmission of HIV-1 [60]. This observation might involve the presence of HLA-G-restricted CD8+ CTLs in combinaison with viral peptides; however, this exon 2 mutation is silent and may not modify the HLA-G presentation pocket. On the other hand, we now have an array of evidence suggesting that the primary function of HLA-G might not be peptide presentation to the T cell receptor. Indeed, HLA-G seems to reveal a low peptide diversity and exhibits slow turnover, which would be inefficient in presenting exogenous peptides. As recently proposed, peptide loading could be a key mechanism in the transport of HLA-G to the cell surface, acting in a quality-control process. The loading of HLA-G1 is not stricktly TAP dependent (as it is for HLA-G5) and may vary according to cell type. This suggests the existence of an alternative pathway for reaching the cell surface, as described for classical HLA class I molecules.

CMV has a biologically active IL-10 homolog that can up-regulate HLA-G protein expression at the monocyte cell surface [15] and might be involved in HLA-G induction in activated macrophages infected with HCMV [22]. HLA-G expression is also up-regulated in the monocytes and T lymphocytes of HIV+ patients [23]. These results suggest that the specific properties and transport pathway of HLA-G, which differ from those of class I antigens, might aid the virus to escape the immune system. On the other hand, HCMV infection down-regulates HLA-G expression in cytotrophoblasts, even after infection with CMV depleted in genes that down-regulate classical class I molecule expression [61]. The differences in HLA-G modulation might thus be due to the CMV strain used, or to cell-specific regulation of CMV genes. We also hypothesize the role of HLA-G structure, which might differ with respect to peptide-loading according to the cell type, and might influence interactions with CMV gene products.

The truncated HLA-G2 and HLA-G3 isoforms are capable of reaching the cell surface under certain circumstances, depending on the cell type. As previously suggested, these isoforms may be helpful against maternal rejection when the fetus is homozygous for the 'null' allele (G*0105N), and do not allow HLA-G1, G5, or G4 translation in the placenta [62]. Moreover, although the HLA-G6 and HLA-G7 transport pathways have not yet been investigated, these isoforms might also be helpful in overcoming the presence of a homozygous 'null' allele. Therefore, isoforms with a conserved α1 domain might be barriers against mutations, serving to conserve an important function in immune tolerance.

Considered together, these data strongly support the contention that the primary function of the processing and transport of quasi-monomorphic HLA-G is to act as an inhibitory ligand to at least three receptors: ILT2 [63, 64], ILT4 [65], and KIR2DL4/p49 [66, 67]. Because at least one of these receptors is present at the cell surface of T, B, and NK cells, monocytes and dendritic cells, the HLA-G protein can bind all these cells in exercising its immune function.

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