# Effect of proteasome inhibitors on expression of HLA-G isoforms*

K. POLÁKOVÁ[1], E. BANDŽUCHOVÁ[2], M. BYSTRICKÁ[3], H. PANČUCHÁROVÁ[3], G. RUSS[3]

*[1]Cancer Research Institute, e-mail: exonpola@savba.sk, Slovak Academy of Sciences 833 91 Bratislava, Slovak Republic; [2]Slovak Medical University, Bratislava, and [3]Institute of Virology, Slovak Academy of Sciences, Bratislava, Slovak Republic*

**Received April 19, 2006**

HLA-G primary transcript is alternatively spliced into a number of mRNAs. In addition to full length HLA-G1 protein isoform these mRNAs might also encode truncated HLA-G protein isoforms lacking one or two extracellular domains. Whereas HLA-G1 protein isoform is regularly identified, truncated HLAG protein isoforms are not detected even if all alternative spliced mRNAs are present in cells. The absence of entire domain(s) renders the truncated HLA-G protein isoforms incapable of binding peptide and β2-microglobulin. These features of truncated HLA-G protein isoforms may result in their rapid degradation by proteasomes. Here we show that despite the presence of all alternatively spliced HLA-G transcripts in JEG-3 cells pretreated with proteasome inhibitors only a full length HLA-G1 protein isoform was regularly detected. Interestingly, immunoblot analysis showed slight increase of HLA-G1 protein in cells pretreated with proteasome inhibitors, although the expression of HLA-G1 transcript was basically not affected. Expression of HLA-G3 transcript increased in JEG-3 cells pre-incubated with LLL, however, neither HLA-G3 nor other HLA-G short protein isoform was regularly detected. In K562 transfectants proteasome inhibitor LLL greatly enhanced expression of the HLA-G1 and -G2 transcripts as well as corresponding protein isoforms. Flow cytometry analysis showed that in cells pre-treated with proteasome inhibitors cell surface expression of HLA-G1 protein decreased but the quantity of intracellularly localized HLA-G antigens increased. Altogether our results suggest that truncated HLA-G proteins isoforms are not detected in JEG-3 cells as a result of their instability and the low translation efficiency of truncated HLA-G transcripts.

*Key words: HLA-G, isoforms, proteasome inhibitors*

HLA-G is a non-classical HLA-class I antigen whose normal expression is restricted to extravillous cytotrophoblast, thymic epithelial cells and cornea. HLA-G apparently play an important role in the implantation process, in protection of semi-allogenic fetus from maternal immune attack, and in those pathological conditions which are associated with unique HLA-G protein expression. Importantly, tumor cells frequently associated with partial or total loss of classical HLA class I expression may still express non-classical HLA-G class I antigens. Such peculiar expression of HLA class I antigens is postulated to permit tumor cells to escape from both, CD8+T and NK cell cytotoxicity [1–10].

HLA-G is characterized by low polymorphism and alternative splicing of HLA-G primary transcript producing a number of mRNAs coding four potentially membrane bound and three secreted protein isoforms (see Fig. 1). The structure of the full length HLA-G1 protein isoform is similar to those of classical class I antigens, consisting of three extracellular domains: α1, α2, and α3 (the later associated with β2-microglobulin), plus one transmembrane and one cytoplasmic domain. Due to the presence of a stop codon in exon 6 the length of cytoplasmic tail as compared with classical HLA class I antigens is reduced. HLA-G1 protein isoform is found in dimers on the cell surface formed via disulfide bridges between two unique cysteine residues located at positions 42 and 147 [11, 12].

In addition to full length HLA-G1 protein isoform, HLA-G2 lacking the α2 domain, HLA-G3 devoid of the α2 and α3 domains, and HLA-G4 without the α3 domain are also considered to be membrane bound protein isoforms. It is likely that HLA-G2 protein isoform homodimerizes to form an HLA

*This work was supported by the grants 2/5020/25 and 2/6022/6 from the Scientific Grant Agency of Ministry of Education of Slovak Republic and Slovak Academy of Sciences.

Abbreviations: HLA – human leukocyte antigen; mAb – monoclonal antibody; β2m – β2-microglobulin; RT-PCR – reverse transcriptase-polymerase chain reaction; LC – lactacystin; LLL – N-CBZ-LEU-LEU-LEU-AL (MG132); EPOX – epoxomicin.

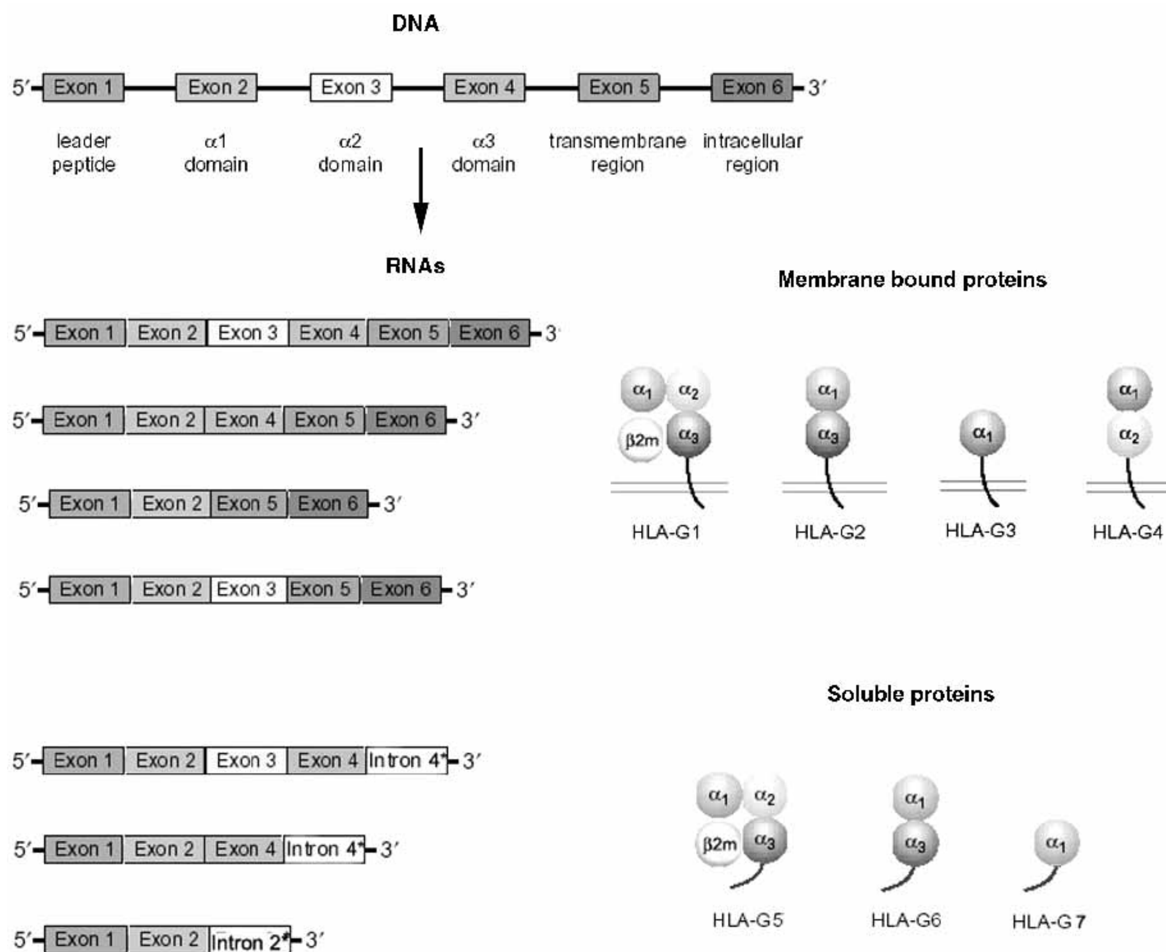


**Figure 1. The mRNA organization and proposed structure of the HLA-G isoforms. Exon 1 encodes leader peptide, Exon 2 to Exon 4 encode α1 to α3 extracellular domains, Exon 5 encodes the transmembrane region, and Exon 6 encodes the cytoplasmic domain of the HLA-G protein. Translation of HLA-G1 to HLA-G4 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. Asterisk indicates stop codon.**

class II-like structure [11]. Soluble forms of HLA-G can be encoded by HLA-G mRNA containing intron 4 (or intron 2). However, introns containing soluble HLA-G protein isoforms have not been convincingly detected, therefore it is still unclear whether soluble HLA-G5 is mainly generated by alternative splicing or rather by membrane bound HLA-G molecules simply released from the surface of cells [3].

HLA-G1 protein isoform has been extensively studied in terms of both expression and function, mainly because in most cases only this isoform is detected even if all alternative spliced mRNA are present in cells. There are only very rare exceptions. In IGR melanoma cell line, bands apparently corresponding to the HLA-G2 protein isoform and/or HLA-G4 and HLA-G3 protein isoforms were detected by western blotting. Interestingly, HLA-G1 protein isoform was not expressed in IGR cells, even though these cells express the corresponding mRNA [13]. Another example is HS738 eye cell line expressing HLA-G2 protein isoform [11]. Interesting observation linking the short HLA-G protein isoform detection with the effect of long term in vitro cultivation on HLA-G expression was described recently. In this case a melanoma cell line named Fon, derived from an HLA-G-positive surgically removed primary melanoma lesion, switched from cell-surface HLA-G1 to intra-cellular HLA-G2 expression upon long-term *in vitro* propagation [14].

Biochemical analysis of cells expressing truncated HLA-G protein isoforms produced from the beginning the conflicting results [3, 12]. First of all the question of their expression at the cell surface has been investigated by several groups. MALLET et al [15] used targeted enhanced green fluorescence protein (EGFP)-HLA-G fusion DNA to track HLA-G protein isoform expression in murine J26 fibroblasts

transfected with the human β2-microglobulin, and human JAR choriocarcinoma cells transiently transfected, observing that HLA-G2, -G3, and -G4 were retained in the ER. BAINBRIDGE et al using cDNAs ligated to an epitope tag, then transfected into the human B-lymphocyte C1R cell line (HLA-A, -B negative, -C positive) drew the same conclusion [16]. In contrast, using untagged vector constructions, RITEAU et al observed that HLA-G2, -G3 and -G4 protein isoforms reached the cell surface of the HLA-A, -B, -C and -E positive melanoma cell line M8 as endo-H-sensitive (immature) proteins [17]. Although this latter feature is unusual, it has already been reported for the HLA class I-like molecule CD1d [18]. The apparent discrepancies between these studies may be explained by differences in the experimental procedures. In particular, the use of tagged versus non tagged transfected constructions as well as cells with or without co-expression of other HLA class I antigens might affect expression of short isoforms. The current status about the truncated HLA-G2 and HLA-G3 isoforms is that under certain circumstances and depending on the cell type they are capable of reaching the cell surface [3, 19].

HLA-G1 protein isoform exhibits the typical structure of an antigen presenting molecule, nevertheless the primary function of HLA-G1 might not be peptide presentation to the T cell receptor. The primary function of HLA-G1 may be to act as an inhibitory ligand to at least three receptors: ILT2, ILT4 and KIR2DL4 [20]. There is still a dispute as to whether any of the truncated protein isoforms display a major immunological function. The truncated HLA-G isoforms were shown to inhibit both NK and antigen-specific CTL cytolysis. 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 [21]. Notably, some immuno-inhibitory function was observed in cells with no surface expression of truncated protein isoforms [10, 17, 22]. One possible function of the short forms of HLA-G is that they support the expression of HLA-E on trophoblast cells. The cellular expression of HLA-E is uniquely dependent on the concurrent expression of other class I molecules, such as HLA-C or -G, because the exon-1-encoded leader sequence of these molecules is cleaved to provide the peptide that stabilizes the mature HLA-E molecule. All known forms of HLA-G mRNAs encode this leader sequence, and therefore, all could contribute to supporting the expression of HLA-E. However, although this could explain the experimental finding that the short forms of HLA-G suppress NK cell-mediated lysis (by stabilizing HLA-E), their physiological role is probably extremely limited. Although in early trophoblasts the truncated HLA-G protein isoforms are co-expressed usually with the full-length form of HLA-G1, they are expressed at lower concentrations than HLA-G1, making them unlikely to play a major role in supporting the expression of HLA-E [23].

Many studies have shown expression of HLA-G1 antigen in cells and tissues but none has explored carefully why truncated isoforms are usually not detected even if cells express corresponding transcripts. Truncated isoforms are not detectable in cells perhaps because of the low steady state level of the corresponding mRNAs, the low translation efficiency or because they are unstable. Actually, truncated isoforms might be unstable, because they each lack at least one structural component required to allow the formation of a stable class I molecule, such as a complete peptide-binding groove, or the regions that bind to β2-microglobulin or the appropriate chaperone molecules in the endoplasmic reticulum [16]. Folding abnormalities of truncated isoforms may result in their rapid degradation by proteasomes. In agreement with this is the observation by BAINBRIDGE et al that although all truncated membrane-bound isoforms of HLA-G were detected within transfected cells, the amount of protein yielded by transfectants was considerably lower for the truncated spliced variants than for HLA-G1, suggesting that they may be translated less efficiently or that their protein products or mRNA may be less stable [16]. The stability of the truncated, soluble protein isoforms of HLA-G has not been investigated specifically. Here we analyzed whether pre-treatment of JEG-3 cells with proteasome inhibitors can result in an accumulation of shorter isoforms to the level allowing their detection.

## Material and methods

*Cell cultures*. The following HLA-G positive cell lines were used: choriocarcinoma cell line JEG-3 (ATCC, Rockville, MD, USA), and transfectants K562-G1 and K562-G2 (kindly provided by Dr. E. Weiss, Munich, Germany). The JAR, or K562 cell lines were used as negative controls. Cells were propagated in DMEM medium with 10% fetal calf serum.

*Treatment of cells by proteasome inhibitors*. Cells were incubated overnight in the presence of 20 µM LLL (Sigma) or 10 µM lactacystin-synthetic (BostonBiochem-USA), or 5 µM epoxomicin (BostonBiochem-USA).

*Monoclonal antibodies*. Monoclonal antibody 4H84 recognizing free heavy chain of all HLA-G protein isoforms was used. This antibody was a generous gift from Dr. McMaster (San Francisco, CA, USA). Monoclonal antibody 87G specific for intact HLA-G antigens was kindly provided by Dr. Geraghty (Seattle, WA, USA).

*Flow cytometry*. Live or paraformaldehyde-fixed and Triton-X-100 permeabilized cells were incubated with the first antibody for 1 hour at 4 °C, then washed and stained with FITC-conjugated rabbit anti-mouse IgG (Dako, Hamburg, Germany) for 30 minutes at 4 °C. Cytometric analysis was performed on a Coulter Epics Altra Flow Cytometer (Becman Coulter, Florida, USA). The live cells were gated using forward and side scatter and staining with propidium iodide.

*Protein extraction and western blotting*. Cells were lysed in TENN buffer (50-mM Tris-HCl, pH 7.4, 150-mM NaCl,

1-mM EDTA, and 1% Nonidet P-40) supplemented with protease inhibitors. Cell extracts were boiled in SDS-PAGE reducing sample buffer and separated on 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were electroblotted onto a nitrocellulose membrane. The blots were blocked with 5% nonfat powder milk in PBS (containing 0.01% Tween 20) and then incubated with specific mAbs. After washing, the blots were incubated with peroxidase-conjugated rabbit anti-mouse IgG antibodies (Dako) and immunoreactive bands were visualized with the ECL detection system (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) [8, 9, 24].

*RNA isolation and semiquantitative RT-PCR analysis*. Total RNA was extracted from 5–10x$10^6$ cells using the Trizol reagent (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. Briefly, the cell pellet was lysed in 1000 µl of Trizol and then centrifuged at 12,000 *g* for 10 minutes. The supernatant was mixed with 200 µl of chloroform, centrifuged again, and total RNA was precipitated from the aqueous phase with 500 µl isopropyl alcohol. The RNA pellet was washed with 75% ethanol, air-dried, and resuspended in 20 µl diethyl pyrocarbonate (DEPC) treated water. Specific amplification of HLA-G transcripts was performed on cDNA. cDNA was prepared from 2 µg of total RNA by reverse-transcription (RT) in a 20-µl reaction volume using Reverse transcription reagents from Applied Biosystems: 1x PCR buffer II, 1 mM of each dNTP, 5 mM $MgCl_2$, 10-U RNase inhibitor, 50-U MultiSribe reverse transcriptase, and 2.5 µM oligo $d(T)_{16}$. The reaction was carried out at 42 °C for 60 minutes, and stopped by heating at 99 °C for 5 minutes. PCR was performed in total volume of 30 µl containing 6 µl of RT product, 4 mM $MgCl_2$, 0.75 U of AmpliTaq Gold polymerase (Applied Biosystems), 200 µM of each dNTP, and 7 pM of each primer. The following set of primers was used: G.257F (exon 2; 5'-GGA AGA GGA GAC ACG GAA CA) and G.1004R (exon 5 and exon 6 junction; 5'-CCT TTT CAA TCT GAG CTC TTC TTT) [25] As an internal control the ß-actin gene amplification was carried out for each sample using following primers: BGU; 5'-ATG TTT GAG ACC TTC AAC AC and BGL; 5'-CAC GTC ACA CTT CAT GAT GG. The PCR conditions were 15 minutes at 95 °C for DNA polymerase activation, followed by PCR cycles of 1 minute at 94 °C, 1 minute and 30 seconds at 62 °C, and 2 minutes at 72 °C, with a final extension at 72 °C for 7 minutes. The thermal cycler used was Perkin Elmer 9600. PCR product accumulation was measured during the exponential phase and the following numbers of PCR cycle were determined experimentally: 26 or 28 cycles for JEG-3 and 16 for K562-G1 and 20 cycles for K562-G2, respectively. PCR products were visualized by UV light after electrophoresis in 2% agarose gels stained with ethidium bromide [8, 9, 24].

## Results

*Effect of proteasome inhibitors on expression of HLA-G transcripts.* First we analyzed the effect of proteasome inhibitors on the expression of alternatively spliced HLA-G transcripts in JEG-3 cells. The RT-PCR products were obtained by amplification of cDNA prepared from total RNA by reverse transcription. As seen in Figure 2A, the specific amplification of HLA-G transcripts of JEG-3 cells revealed three bands corresponding to HLA-G1 (770 bp), HLA-G2 /HLA-G4 (490 bp) and HLA-G3 (220 bp) cDNA. RT-PCR analysis carried out with a large number of amplification cycles (37) did not show any effect of proteasome inhibitors. Nevertheless, if the number of amplification cycles was decreased to 26 or 28 cycles effect of proteasome inhibitors was visible. Particularly expression of HLA-G3 transcript increased after pre-incubation with proteasome inhibitor LLL, as demonstrated by the corresponding band intensity (Fig. 2A).

As expected in K562-G1 and K562-G2 transfectants only single band corresponding to 770 bp and 490 bp, respectively, were detected (Fig. 3A). Pre-incubation of transfectants with proteasome inhibitor LLL greatly enhanced expression of corresponding transcripts, particularly of HLA-G2. This increase was clearly visible when the number of cycles decreased from 28 to 20 in the case of HLA-G1 and to 16 for HLA-G2. In control JAR and K562 cells no HLA-G specific transcripts were detected.

Altogether these results show that JEG-3 cells express all alternatively spliced HLA-G transcripts and that proteasome inhibitors do not negatively affect the level of their expression. To address the possibility that truncated HLA-G transcripts are translated into unstable proteins rapidly degraded by proteasomes we turned to immunoblot analysis of JEG-3 cells pretreated with proteasome inhibitors. In addition we analyzed the effect of proteasome inhibitors on expression of HLA-G proteins in K562-G1 and K562-G2 transfectants.

*Effect of proteasome inhibitors on expression of HLA-G protein isoforms.* We carried out immunoblot analysis of JEG-3 cells using the 4H84 mAb that recognizes an epitope located in the $\alpha 1$ domain shared by all of the HLA-G protein isoforms. Comparison was made with the positive control transfectants K562-G1 and K562-G2 and with negative JAR and K562 control cells that do not transcribe any HLA-G mRNA. Immunoblot analysis results are shown in Figs. 2B and 3B. As expected, in untreated JEG-3 cells and K562-G1 cells 4H84 mAb detected one protein of 39 kDa corresponding to the membrane bound HLA-G1 protein isoform. In K562-G2 cells 4H84 mAb detected a protein of the predicted size (26 kDa) of HLA-G2 protein isoform. This confirmed that mAb 4H84 recognizes not only HLA-G1 but also HLA-G2 isoforms. Although in JEG-3 cells pre-treated with proteasome inhibitors the intensity of the band corresponding to HLA-G1 slightly increased, no band corresponding to any of truncated HLA-G isoforms was constantly visible. Although in JEG-3 cells a proteasome inhibitor LLL faintly enhanced the level of HLA-G3 transcript and decreased the level of HLA-G2/G4 transcripts, a weak band corresponding

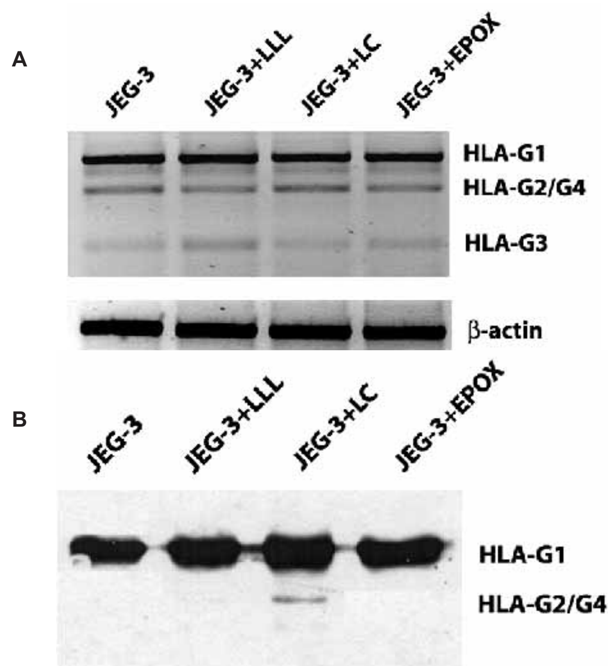


**Figure 2. RT-PCR analysis of HLA-G mRNA expression (A) and immunoblot analysis of HLA-G protein isoforms (B) in JEG-3 cells treated with proteasome inhibitors. RT-PCR products were obtained by amplification of cDNA prepared from untreated JEG-3 cells and from cells incubated overnight with LLL or lactacystin or epoxomicin. β-actin cDNA amplification was used as internal control. Lysates were prepared from untreated control JEG-3 cells and from cells incubated overnight with LLL (LLL) or lactacystin (LC) or epoxomicin (EPOXO) and assayed for HLA-G protein isoforms by immunoblot analysis.**

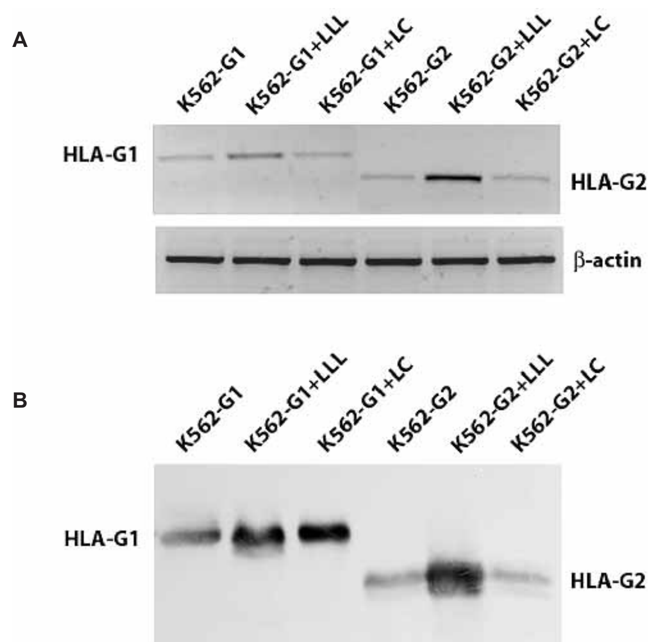


**Figure 3. RT-PCR analysis of HLA-G mRNA expression (A) and immunoblot analysis of HLA-G protein isoforms (B) in K562-G1 and K562-G2 transfectants treated with proteasome inhibitors. RT-PCR products were obtained by amplification of cDNA prepared from untreated K562-G1 and K562-G2 cells and from cells incubated overnight with LLL or lactacystin. β-actin cDNA amplification was used as internal control. Lysates were prepared from untreated control K562-G1 and K562-G2 cells and from cells incubated overnight with LLL (LLL) or lactacystin (LC) and assayed for HLA-G protein isoforms by immunoblot analysis.**

to HLA-G2/G4 proteins on the odd occasion appeared in cells pre-treated with lactacystin (Fig. 2B). Pre-treatment of K562-G1 and K562-G2 transfectants with proteasome inhibitor LLL greatly enhanced expression of HLA-G proteins, particularly of HLA-G2 (Fig. 3B).

Altogether these data show that the truncated HLA-G protein(s) was not detected in JEG-3 cells pre-treated with proteasome inhibitors, even though these cells expressed the corresponding mRNAs. The results of immunoblot analysis are consistent with low translation efficiency of truncated HLA-G transcripts and by instability of truncated HLA-G protein isoforms which even in the cells pre-treated with proteasome inhibitors do not reach the level sufficient for immunoblot detection.

*Effect of proteasome inhibitors on the cell surface and intracellular expression of HLA-G antigens.* We examined the quantity of intracellularly localized HLA-G antigens by flow cytometry of fixed and permeabilized K562 transfectants using mAb 4H84. As shown in Figure 4 pretreatment of K562 transfectants with proteasome inhibitors enhanced the level of HLA-G1 (Fig. 4A) as well as HLA-G2 protein isoform (Fig. 4B). This increase is in agreement with immunoblot analysis and can be explained by the inhibition of HLA-G antigens degradation by proteasomes. To get information of proteasome inhibitor effect on cell surface HLA-G antigen expression, we carried out flow cytometry analysis of live cells using mAb 87G.

As only HLA-G1 protein isoform can reach the cell surface only JEG-3 cells and K562-G1 transfectant were included into this analysis. As shown in Figure 5 proteasome inhibitors decrease expression of HLA-G1 protein isoform on the cell surface of JEG-3 as well as on K562-G1. Such decrease is in full agreement with current view about the formation of HLA-G/β2m/peptide complex and its transport to the cell surface.

## Discussion

In the present study for the first time was examined stability of the truncated HLA-G protein isoforms. The truncated HLA-G protein isoforms might be unstable, because they each lack at least one structural component required to allow the formation of a stable class I molecule, such as a complete peptide-binding groove, or the regions that bind to

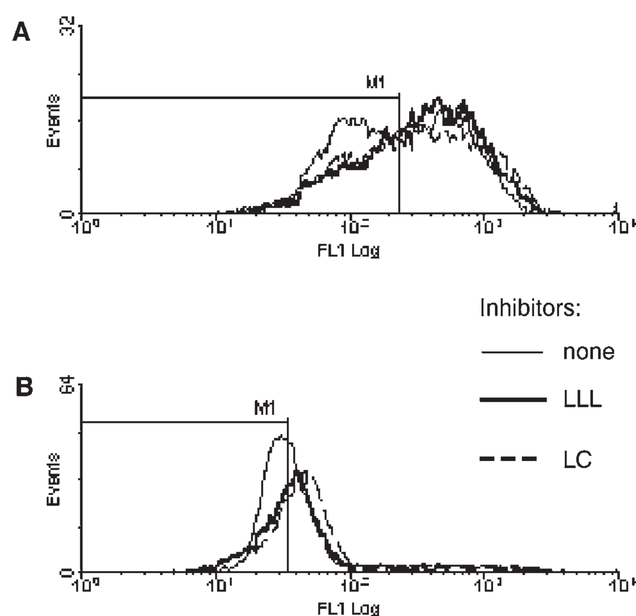


Figure 4. Flow cytometry analysis of HLA-G antigens in cells treated with proteasome inhibitors. K562-G1 (A) and K562-G2 (B) transfectants were first incubated with or without proteasome inhibitor (LLL or LC). Then the cells were fixed with 3% paraformaldehyde and permeabilized with Triton-X-100, and finally analyzed with 4H84 monoclonal antibody recognizing HLA-G free heavy chain. The cells were gated using forward and side scatter. Vertical lines indicate mean fluorescence intensity for cells incubated in the absence of proteasome inhibitor.

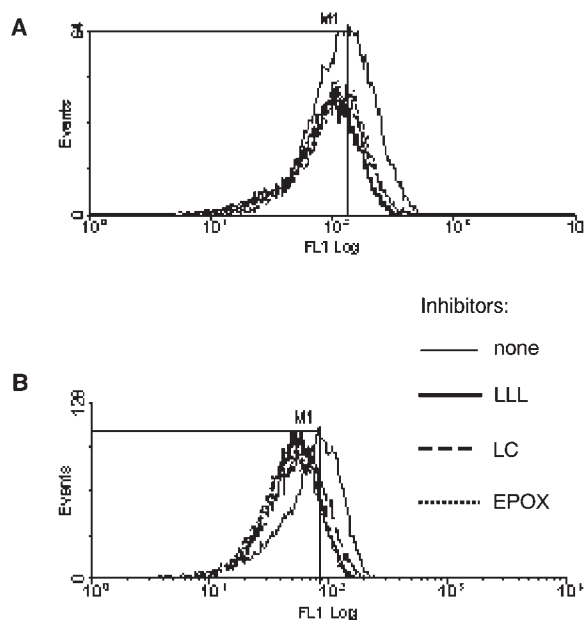


Figure 5. Flow cytometry analysis of surface HLA-G antigens on cells treated with proteasome inhibitors. JEG-3 cells (A) and K562-G1 transfectant (B) were first incubated with or without proteasome inhibitor (LLL, LC or EPOX) and finally analyzed with mAb 87G recognizing native HLA-G/β2m/peptide complex. The cells were gated using forward and side scatter and staining with propidium iodide. Vertical lines indicate mean fluorescence intensity for cells incubated in the absence of proteasome inhibitor.

β2-microglobulin [3, 16]. Provided that short HLA-G isoforms are actually unstable we would predict that they would be degraded by proteasomes. Therefore we analyzed whether pre-treatment of JEG-3 cells with proteasome inhibitors can result in an accumulation of truncated HLA-G protein isoforms to the level allowing their detection. To address this question we used JEG-3 cells which express all alternatively spliced HLA-G transcripts. To our best knowledge only a single exception was reported when a band corresponding to HLA-G2/G4 protein isoform was visible [23]. We found that JEG-3 cells pretreated with proteasome inhibitors express all alternatively spliced transcripts, and that proteasome inhibitors do not negatively affect the level of their expression. In addition, flow cytometry confirmed intracellular accumulation of HLA-G protein in cells pre-treated with proteasome inhibitors. Nevertheless, the truncated HLA-G protein(s) was not constantly detected in JEG-3 cells pre-treated with proteasome inhibitors, even though these cells express the corresponding mRNAs.

Although our data are by no means quantitative, nevertheless they clearly show that pre-treatment of K562 transfectants with proteasome inhibitor LLL enhanced expression of both, the HLA-G1 and -G2 transcripts as well as corresponding protein isoforms. This increase was detected only with proteasome inhibitor LLL and was more pronounced for HLA-G2. Increase in the protein levels can be explained by an inhibition of their degradation, and increase in RNA transcripts might be related to expression in K562 transfectants. Interestingly LLL increased in parallel recombinant adeno-associated virus transgene mRNA and protein levels [26].

Alternative splicing of MHC class I pre-mRNA molecules is not unique to HLA-G. For example, alternative splicing of HLA-A and -B mRNA has been reported to occur spontaneously during HLA down-regulation in tumors, following infection by intracellular bacteria and as a result of stimulation by interferon-γ [27, 28, 29]. However, no specific functions have been ascribed to these molecules nor the truncated proteins encoded by these transcripts have been identified, thereby questioning the biological significance of these observations.

Altogether our results suggest the low translation efficiency of mRNAs corresponding to short HLA-G isoforms (this is supported by the fact that transcripts are detected by RT-PCR) as well as instability of short HLA-G proteins which is documented by the effect of proteasome inhibitors particularly in transfectants.

## References


[1] ALGARRA I, CABRERA T, GARRIDO F. The HLA crossroad in tumor immunology. Hum Immunol 2000; 61: 65–73.

[2] TAIT BD. HLA class I expression on human cancer cells. Implication for effective immunotherapy. Hum Immunol 2000; 61: 158–165.

[3] BAINBRIDGE D, ELLIS S, LE BOUTEILLER P, SARGENT I. HLA-G remains a mystery. Trends in Immunol 2001; 22: 548–552.

[4] DAVIES B, HIBY S, GARDNER L, LOKE YW, KING A. HLA-G expression by tumors. Am J Reprod Immunol 2001; 45: 103–07.

[5] MAIER S, GERAGHTY DE, WEISS EH. Expression and regulation of HLA-G in human glioma cell lines. Transpl Proc 1999; 31: 1849–1853.

[6] CHANG CC, MURPHY SP, FERRONE S. Differential in vivo and in vitro HLA-G expression in melanoma cells: potential mechanisms. Hum Immunol 2003; 64: 1057–1063.

[7] PANGAULT C, AMIOT L, CAULET-MAUGENDRE S, BRASSEUR F et al. HLA-G protein expression is not induced during malignant transformation. Tissue Antigens 1999; 53: 335–336.

[8] POLÁKOVÁ K, KŔČOVÁ M, KUBA D, RUSS G. Analysis of HLA-G expression in malignant hematopoietic cells from leukemia patients. Leukemia Res 2003; 27: 643–648.

[9] POLÁKOVÁ K, BANDŽUCHOVÁ E, HOFMEISTER V, WEISS EH et al. Binding analysis of HLA-G specific antibodies to hematopoietic cells isolated from leukemia patients. Neoplasma 2000; 50: 331–338.

[10] ROUAS-FREISS N, MOREAU P, FERRONE S, CAROSELLA ED. HLA-G proteins in cancer: Do they provide tumor cells with an escape mechanism? Cancer Res 2005; 65: 10139–10144.

[11] ISHITANI A, GERAGHTY DE. Alternative splicing of HLA-G transcripts yields proteins with primary structures resembling both class I and class II antigens (mRNA/nonclassical class I antigen/placenta). Proc Natl Acad Sci USA 1992; 89: 3947–3951.

[12] MCMASTER M, ZHOU Y, SHORTER S, KAPASI K et al. HLA-G isoforms produced by placental cytotrophoblasts and found in amniotic fluid are due to unusual glycosylation. J Immunol 1998; 160: 5922–5928.

[13] PAUL P, ROUAS-FREISS N, KHALIL-DAHER I, MOREAU P et al. HLA-G expression in melanoma: A way for tumor cells to escape from immunosurveillance. Proc Natl Acad Sci USA 1998; 95: 4510–4515.

[14] ROUAS-FREISS N, BRUEL S, MENIER C, MARCOU C et al. Switch of HLA-G alternative splicing in a melanoma cell line causes loss of HLA-G1 expression and sensitivity to NK lysis. Int J Cancer 2005; 117: 114–122.

[15] MALLET V, PROLL J, SOLIER C, AGUERRE-GIRR M et al. The full length HLA-G1 and no other alternative form of HLA-G is expressed at the cell surface of transfected cells. Hum Immunol 2000; 61: 212–224.

[16] BAINBRIDGE DR, ELLIS A, SARGENT IL. The short forms of HLA-G are unlikely to play a role in pregnancy because they are not expressed at the cell surface. J Reprod Immunol 2000; 47: 1–16.

[17] RITEAU B, ROUAS-FREISS N, MENIER C, PAUL P et al. HLA-G2, -G3, and -G4 isoforms expressed as nonmature cell surface glycoproteins inhibit NK and antigen-specific CTL cytolysis. J Immunol 2001; 166: 5018–5026.

[18] KIM HS, GARCIA J, EXLEY M, JOHNSON KW et al. Biochemical characterization of CD1d expression in the absence of β2 Microglobulin. J Biol Chem 1999; 274, 9289–9295.

[19] MOREAU P, ROUSSEAU P, ROUAS-FREISS N, LE DISCORDE M et al. HLA-G protein processing and transport to the cell surface. Cell Mol Life Sci 2003; 59: 1460–1466.

[20] ROUAS-FREISS N, MARCHAL RE, KIRSZENBAUM M et al. The alfa 1 domain of HLA-G1 and HLA-G2 inhibits cytotoxicity induced by natural killer cells: Is HLA-G the public ligand for natural killer cell inhibitory receptor? Proc Natl Acad. Sci USA 1997; 94: 5249–5254.

[21] MORALES PJ, PACE JL, PLATT JS, PHILLIPS TA et al. Placental cell expression of HLA-G2 isoforms is limited to the invasive trophoblast phenotype. J Immunol 2003; 171: 6215–6224.

[22] MENIER C, RITEAU B, DAUSSET J, CAROSELLA ED, ROUAS-FREISS N. HLA-G Truncated isoforms can substitute for HLA-G1 in fetal survival. Hum Immunol 2000; 61: 1118–1125.

[23] ULBRECHT M, MAIER S, HOFMEISTER V, FALK CS et al. Truncated HLA-G isoforms are retained in the endoplasmic reticulum and insufficiently provide HLA-E ligands. Hum Immunol 2004; 65: 200–208.

[24] POLÁKOVÁ K, BENNINK JR, YEWDELL JW, BYSTRICKÁ M et al. Mild acid treatment induces cross-reactivity of 4H84 monoclonal antibody specific to nonclassical HLA-G antigen with classical HLA class I molecules. Hum Immunol 2003; 64: 256–264.

[25] PAUL P, ROUAS-FREISS N, MOREAU P, CABESTRE FA et al. HLA-G, -E, -F preworkshop: tools and protocols for analysis of non-classical class I genes transcription and protein expression. Hum Immunol 2000; 61: 1177–1195.

[26] JENNINGS K, MIYAMAE, T, TRAISTER R, MARINOV A et al. Proteasome inhibition enhances AAV-mediated transgene expression in human synoviocytes in vitro and in vivo. Mol Therapy 2005; 11: 600–607.

[27] DUBOIS V, TIERCY JM, LABONNE MP, DORMOY A, GEBUHRER L. A new HLA-B44 allele (B*44020102S) with a splicing mutation leading to a complete deletion of exon 5. Tissue Antigens 2004; 63: 173–180.

[28] NORGAARD L, FUGGER L, MADSEN HO, SVEJGAARD A. Identification of 4 different alternatively spliced HLA-A transcripts. Tissue Antigens 1999; 54: 370–378.

[29] WANG Z, MARINCOLA FM, RIVOLTINI L, PARMIANI G, FERRONE S. Selective histocompatibility leukocyte antigen (HLA)-A2 loss caused by aberrant pre-mRNA splicing in 624MEL28 melanoma cells. J Exp Med 1999; 190: 205–215.