Trophoblast Class I Major Histocompatibility Complex (MHC) Products Are Resistant to Rapid Degradation Imposed by the Human Cytomegalovirus (HCMV) Gene Products US2 and US11

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Summary

US11 and US2 encode gene products expressed early in the replicative cycle of human cytomegalovirus (HCMV), which cause dislocation of human and murine major histocompatibility complex (MHC) class I molecules from the lumen of the endoplasmic reticulum to the cytosol, where the class I heavy chains are rapidly degraded. Human histocompatibility leukocyte antigens (HLA)-C and HLA-G are uniquely resistant to the effects of both US11 and US2 in a human trophoblast cell line as well as in porcine endothelial cells stably transfected with human class I genes. Dislocation and degradation of MHC class I heavy chains do not appear to involve cell type–specific factors, as US11 and US2 are fully active in this xenogeneic model. Importantly, trophoblasts HLA-G and HLA-C possess unique characteristics that allow their escape from HCMV-associated MHC class I degradation. Trophoblast class I molecules could serve not only to block recognition by natural killer cells, but also to guide virus-specific HLA-C– and possibly HLA-G–restricted cytotoxic T-lymphocytes to their targets.

Key words: trophoblast • cytomegalovirus • human • MHC class I • HLA-G

During human pregnancy, allogenic trophoblast cells are exposed directly to maternal immune effector cells. For many years, the absence of MHC-restricted rejection of the implanting trophoblast was attributed to the lack of class I MHC expression on these cells. It is now clear that trophoblast cells express both classical (HLA-C; references 1–4) and nonclassical (HLA-G; references 5–9) class I products. To date, the function of these molecules and their involvement in creating an immunologically privileged site at the maternal–fetal interface remains elusive. Sequence similarity of HLA-G to HLA-A and -B molecules suggests that HLA-G (7) may function in a manner analogous to its classical counterparts: HLA-G may have a role in antigen presentation (10–12) and/or in evading NK cell–mediated lysis (13, 14), although the existence of HLA-G–restricted T cells in vivo remains to be established. Trophoblast HLA-C locus products might have a complementary or overlapping purpose. However, the unique and limited localization of HLA-G (15–18), its restricted polymorphism (19–23), and the known reduced surface stability of HLA-C locus products (24–27) suggest that human trophoblast MHC class I molecules may possess attributes that allow a unique function at the maternal–fetal interface.

Human cytomegalovirus (HCMV)[1] is a common human pathogen that typically causes morbidity only in the immunocompromised host or when infection occurs during pregnancy. HCMV infects trophoblast (28), it is a known teratogen, and HCMV infection during pregnancy has been linked to spontaneous pregnancy loss (29, 30). HCMV has proven interesting from an immunologic viewpoint, as the virus has developed a number of strategies that enable it to evade immune detection (31, 32). Of interest for this report, HCMV-infected cells synthesize two gene products early in the HCMV infectious cycle, US2 and US11, which cause the dislocation of newly synthesized class I heavy chain from the endoplasmic reticulum (ER) to the cytosol. Cytosolic class I heavy chains are deglycosylated by

[1]*Abbreviations used in this paper:* CM, complete medium; ER, endoplasmic reticulum; HCMV, human cytomegalovirus; MOI, multiplicity of infection.

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N-glycanase, and rapidly degraded by the proteasome. Therefore, US2 and US11 may be partly responsible for the downregulation of MHC class I surface expression in HCMV-infected cells (33, 34). These effects of US2 and US11 have been reported for human HLA-A and -B locus products, as well as for murine class I molecules expressed in human cells. Allelic preferences have been described for US2- and US11-mediated degradation of the murine class I products (35). The effects of US11 and US2 on the trophoblast class I molecules HLA-G and HLA-C are of particular interest, both to further delineate in humans the substrate specificity of these viral gene products, and to evaluate potentially unique characteristics of trophoblast MHC class I products.

We report that, unlike any previously described murine or human class I products, both HLA-G and HLA-C appear fully resistant to the rapid degradation associated with the US11 and US2 HCMV gene products. This suggests that trophoblast MHC class I molecules possess characteristics that allow escape from HCMV-associated MHC class I degradation, and could serve not only to block recognition by NK cells, but also to guide virus-specific, HLA-C– or HLA-G–restricted CTL to their targets.

Materials and Methods

Cell Lines. The JEG 3 choriocarcinoma cell line was obtained from American Type Culture Collection (Rockville, MD). The porcine, bone marrow–derived stromal cell line 2A2, immortalized by transformation with SV40 large T antigen, was generated (36) and characterized. Both cell lines were maintained in complete medium (CM):DME (Sigma Chemical Company, St. Louis, MO) supplemented with 10% heat-inactivated fetal bovine serum/calf serum (1:1 vol/vol; Sigma Chemical Co.), 2 mM L-glutamine, 1/1,000 dilution U/ml penicillin (GIBCO BRL, Gaithersburg, MD), and 100 μg/ml Streptomycin (GIBCO BRL). Cells were grown at 37°C in humidified air with 5% CO_2 to ~90% confluency before infection.

Transfection Experiments. Stable transfection of 2A2 cells with the full-length class I cDNAs encoding HLA-A2 or HLA-Cw3 resulting in surface expression was carried out as described (37). HLA-G cDNA under the control of the SRα promoter in the expression vector pBJ5 containing a neomycin resistance gene (14) was a kind gift from Dr. L. Pazmany (Harvard University, Boston, MA). The 2A2 cells were selected in CM containing 0.25 mg/ml G418 (GIBCO BRL). Surface expression of HLA-G on transfected 2A2 cells was established by indirect immunofluorescence and flow cytometry using both the mAb W6/32 and the allele-specific antibody 87G (kind gift from Dr. D.E. Geraghty, Fred Hutchinson Cancer Research Center, Seattle, WA; data not shown). HLA-expressing 2A2 clones (2A2-HLA-A2/8.3, 2A2-HLA-Cw3/7, and 2A2-HLA-G/1.2) were obtained by FACS® sorting of G418-resistant, W6/32-positive 2A2 cells (FACS® Star Plus; Becton Dickinson Immunocytometry Systems, San Jose, CA) and subcloning by limiting dilution in the presence of irradiated (40 cGy) 2A2 feeder cells.

Antibodies. W6/32 is a murine mAb recognizing the properly folded heavy chain of class I MHC molecules when associated with β_2m (38–40). The polyclonal rabbit anti–mouse class I heavy chain antiserum (RafHC) recognizes nonassembled and unfolded murine heavy chains (35). The polyclonal rabbit anti–human class I heavy chain antiserum (RαHC) was raised against purified HLA-A2 and -B27 human class I heavy chains. This antibody exhibits no detectable cross-reactivity with porcine class I heavy chains. The polyclonal antiserum detecting US11 (αUS11) was raised against bacterially produced full-length US11; the antiserum detecting US2 (αUS2) was raised against the luminal NH_2-terminal portion of US2, again produced in bacteria.

Viruses and Viral Infection. Recombinant vaccinia viruses expressing murine K^b (lacking the cytoplasmic tail), murine D^d, US11, and US2 were a generous gift from J. Bennink and J. Yewdell (National Institutes of Health, Bethesda, MD). Cells were infected with recombinant vaccinia viruses at a multiplicity of infection (MOI) of 5 for 1 h in 500 μl of PBS supplemented with 10% BSA (Boehringer Mannheim, Indianapolis, IN) at 37°C. CM was then added to cultures for an additional 3 h at 37°C to allow viral replication and expression of recombinant proteins. Carboxybenzyl-leucyl-leucyl-leucinal (aldehyde; ZL_3H) was synthesized as described (34). Cells were starved in the presence of 25 μM ZL_3H in cysteine- and methionine-free DMEM (Sigma Chemical Co.) for 45 min at 37°C before metabolic labeling and analysis. For double infections, JEG 3 cells were infected with VVUS11 or VVUS2 at a MOI of 3 for 1 h at 37°C, followed by the addition of VVK^b or VVD^d at a MOI of 3 for an additional 1 h. Thereafter, CM was added and cells starved as described above.

In Vitro Transcription and Translation. In vitro transcription was performed essentially as described (41) on 5 μg of the pSP72 plasmid (Promega Corp., Madison, WI) containing either cloned HLA-A2, or JEG 3 cell–derived HLA-G or HLA-Cw*0401 cDNA. Templates were linearized at the HindIII (New England Biolabs, Beverly, MA) restriction site downstream from the HLA-C and HLA-G inserts. In vitro translations were performed as described (41) in a total reaction mixture of 25 μl, containing 17.5 μl Flexi™ Rabbit Reticulocyte Lysate (Promega Corp.), 0.8 μl KCl (2.5 M; Promega Corp.), 0.5 μl amino acid mixture minus methionine (1 mM; Promega Corp.), 2.5 μl [^{35}S]methionine (10 mCi/m, translation grade; Dupont, Boston, MA), 0.25 μl RNase inhibitor (40 U/μl; Promega Corp.), 1.5 μl dog pancreas microsomes and RNA. Translations were performed for 1.5–2 h at 30°C.

Immunoprecipitation and Gel Electrophoresis. Sedimented microsomes (4 min, 14,000 rpm) from in vitro translations were lysed in 1% digitonin in 25 mM Hepes, 150 mM KOAc, pH 7.7 (Boehringer Mannheim Corp., Indianapolis, IN) and lysates precleared with normal mouse serum as described (41). Immunoprecipitations of the precleared lysates were performed using the mAb W6/32, at 4°C for 1 h before the addition of 100 μl inactivated *Staphylococcus aureus* (*Staph A*) for an additional 1 h at 4°C. The immunoprecipitates were washed four times in ice-cold wash buffer (0.2% digitonin in 25 mM Hepes, 150 mM KOAc, pH 7.7), resuspended in sample buffer (62.5 mM Tris-HCl, pH 6.8, 5% 2-ME, 10% glycerol, 4% SDS, and bromophenol blue) and boiled at 95°C for 5 min before SDS-PAGE.

Pulse–chase analyses were performed on $\sim 10 \times 10^6$ metabolically labeled cells as described (42). Lysis and immunoprecipitation techniques were performed as described for in vitro translated products with two modifications: two additional normal rabbit serum/normal mouse serum preclearing cycles were included in the cellular samples, and cell lysates were normalized for protein synthesis before loading on PAGE. When reimmunoprecipitation was performed, the Staph A pellet from primary immunoprecipitations was denatured by boiling in ~50 μl PBS plus

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1.0% SDS for 10 min. The supernatant containing denatured antigens was then transferred to 1.0 ml 1.0% NP-40 in water (final SDS concentration ~0.1%). 10 μl 10% BSA and 5 μl RafHC were added and the reaction was incubated for 45 min at 4°C. Antibody/antigen complexes were then captured with 100 μl *Staph A* for 45 min at 4° C. Complexes were washed and denatured as described above.

Results and Discussion

The immunoevasive strategies used by clinically important viruses have been used as tools that allow us to better understand the similarities and differences between trophoblast class I molecules and those present in nonreproductive tissues (43). Surface expression of HLA-G and HLA-C products in the trophoblast-derived cell line, JEG 3, is susceptible to inhibition by the HSV-encoded ICP47 product (43), as previously described for classical class I molecules (44–46). The mechanism for this downregulation involves the abrogation of peptide loading of class I heavy chains in the lumen of the ER, thereby causing ER retention of class I complexes. Retention of HLA-G and HLA-C in the ER of HSV-infected JEG 3 cells confirms the dependence of trophoblast class I products on peptide loading for proper maturation (12) and surface expression, consistent with close structural and functional similarities to other classical human class I molecules, such as HLA-A and -B.

Human cytomegalovirus is another pathogen known to infect trophoblast (28) and to act as a teratogen. HCMV has been associated with spontaneous pregnancy loss (29). In contrast to our findings for HSV, however, the biochemical effects of HCMV gene products on HLA-G and HLA-C suggest that trophoblast MHC class I products possess novel characteristics of structure or trafficking, which allow them to escape those immunoevasive strategies of HCMV that involve the US11 and US2.

JEG 3-derived HLA-G and HLA-C Are Resistant to the Rapid Degradation Associated with the HCMV Gene Products US2 and US11. JEG 3 cells (which express HLA-G and HLA-Cw*0401) were infected either with a vaccinia virus recombinant driving the expression of mouse K^b (known to be sensitive to rapid degradation associated with US 11; reference 35), or with both a vaccinia virus driving K^b expression (VVK^b) and a separate vaccinia virus recombinant driving the expression of the HCMV-protein, US11 (VVUS11; Fig. 1). Infected cells were then metabolically labeled in a pulse–chase experiment and lysates of these cells immunoprecipitated sequentially with RafHC, αUS11, and W6/32 antibodies. Immunoprecipitates were analyzed by PAGE.

In the absence of VVUS11 infection, significant amounts of K^b were detected with the anti-heavy chain reagent (RafHC) at the end of both the pulse and the 30-min chase. In contrast, in cells infected with VVUS11, despite similar rates of synthesis of K^b heavy chains during the pulse, nearly all K^b was degraded at the 30-min chase point.

W6/32 recognizes both HLA-G and HLA-C (3, 43, 47–49), and W6/32 reactive, properly formed HLA-C and HLA-G from JEG cells accumulate during the 30-min chase, both in the absence and the presence of US11. No US11-associated degradation of either HLA-C or HLA-G was observed. Immunoprecipitation with the polyclonal antibody αUS11 revealed the presence of US11 only in VVUS11-infected cells. Similar experiments using VVUS2 revealed that HLA-G and HLA-C in JEG 3 cells were also resistant to the rapid degradation of class I heavy chain typically associated with the HCMV gene product US2 (data not shown).

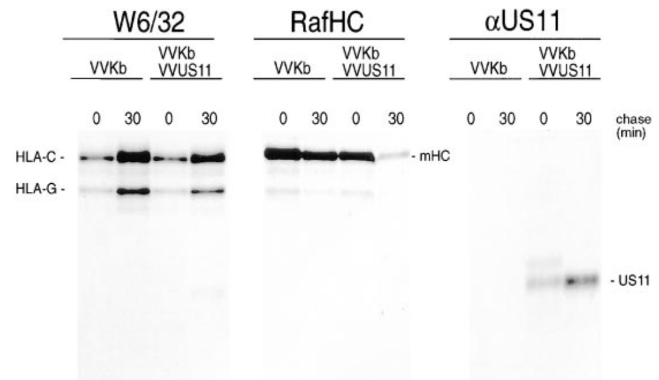

Figure 1. HLA-G and HLA-Cw*0401 from JEG 3 trophoblast-derived cells are not degraded in the presence of the HCMV gene product US11. Approximately 10^7 JEG 3 cells were either infected with a vaccinia virus expressing the murine class I heavy chain, K^b (lacking its cytoplasmic tail, VVK^b, MOI = 5), or doubly infected with VVK^b (MOI = 5) and a vaccinia virus expressing the HCMV gene product US11 (VVUS11, MOI = 5). Cells were metabolically pulse labeled for 15 min with [^{35}S]methionine and chased with unlabeled media for 0 and 30 min. Lysates of these cells were sequentially immunoprecipitated with an antibody against mouse heavy chain (*RafHC*), with W6/32, and with αUS11.

Trophoblast MHC Class I Products Associate with β_2m but Not with US2 and US11. To further evaluate the mechanism underlying the unique resistance of trophoblast class I molecules to degradation in the presence of both US11 and US2, we addressed the physical association of US2 and US11 with MHC class I products translated in vitro. HLA-A2 and trophoblast HLA-G and HLA-C locus products were cotranslated in vitro with the light chain β_2m under conditions promoting assembly, together with either US11 or US2. The products of these translations were then either denatured and analyzed directly by PAGE or lysed in digitonin, immunoprecipitated with the mAb W6/32, and the resultant immunoprecipitations analyzed by SDS-PAGE (Fig. 2 *a*). For "direct load" samples, class I heavy chain, US2, and β_2m, were detected at the appropriate positions by SDS-PAGE. W6/32 immunoprecipitates of the HLA-A2 translation mixture reveal HLA-A2, β_2m, and US2 coprecipitation, indicating the physical association of these three components. In contrast, for both HLA-G and HLA-C translation mixtures, W6/32 immunoprecipitated only class I heavy chains and associated β_2m. When the amounts of US2 cotranslated with equivalent amounts of HLA-G and β_2m were optimized (Fig. 2 *b*), W6/32 still immunoprecipitated only class I heavy chain and associated

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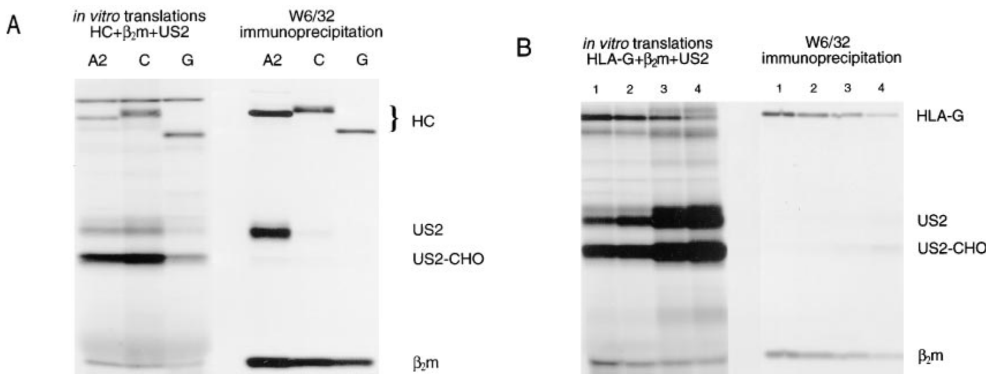

Figure 2. In vitro–translated HLA-A2 associates with cotranslated US2 and β_2m; in vitro–translated trophoblast MHC class I heavy chains do not. (*A*) Human MHC class I heavy chains (HLA-A2, HLA-G, and HLA-C) were cotranslated in vitro with human β_2m and US2. Radioactively labeled translation products were either separated directly by SDS-PAGE, or were immunoprecipitated with W6/32 before electrophoretic analysis. As indicated, components of the cotranslation are present at the anticipated position in the direct load samples. (*B*) US2 fails to be coprecipitated with HLA-G and β_2m, despite optimization of the relative quantities of translated heavy chain, β_2m, and US2. Increasing amounts of transcribed US2 were added to the cotranslation mixture of HLA-G, β_2m, and US2 to allow relative quantities of class I heavy chain to US2 similar to those demonstrated for HLA-A2 in *A*. Lanes *1–4* represent separate translation mixtures in which the amounts of transcribed HLA-G and β_2m template were constant but transcribed US2 template was present at a ratio of 2:1, 5:1, 10:1, and 20:1, respectively. W6/32 immunoprecipitation and electrophoresis were as described above.

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β_2m, despite the translation of US2 in relative amounts greater than or equal to those observed for HLA-A2/US2/β_2m cotranslations (Fig. 2 *a*). We observed similar results for US11 (data not shown). In short, US2 and US11 failed to be coimmunoprecipitated with HLA-C or HLA-G, indicating their lack of association with trophoblast MHC class I heavy chains in the in vitro system.

HLA-G and HLA-C Expressed Stably in the Porcine Endothelial Cell Line, 2A2, Are Resistant to Rapid Degradation of Class I Heavy Chain Associated with the HCMV Gene Products US11 and US2. To rule out cell type-specific protection of HLA-C and HLA-G from US11 and US2-associated degradation in JEG 3 cells, the susceptibility to degradation of human MHC class I locus products stably transfected into 2A2 porcine cells was analyzed. HLA-A2–expressing porcine cells (2A2-A2/8.3) were infected either with VVUS2 or VVUS11. Metabolic labeling was conducted in the presence of the proteasome inhibitor carboxybenzyl-leucyl-leucyl-leucinal (ZL_3H), which retards the degradation of dislocated class I heavy chains and results in the cytosolic accumulation of an unfolded, deglycosylated class I heavy chain intermediate (33, 34). RαHC recovered HLA-A2 mainly in its glycosylated form at both 0 and 30 min of chase in uninfected 2A2-A2/8.3 cells (Fig. 3 *a*). In contrast, in either VVUS2- or VVUS11-infected cells, accumulation of the HLA-A2 heavy chain intermediate occurs at the 30-min chase point. RαHC immunoprecipitates of metabolically labeled nontransfected, parental 2A2 cells (2A2) demonstrate that this antibody does not cross-react with endogenous porcine class I heavy chains. Thus, in the transfected porcine cell model, HLA-A2 is susceptible to rapid degradation associated with either US2 or US11. Immunoprecipitations with αUS2 and αUS11 antibodies demonstrate expression of these molecules in infected cells (Fig. 3 *b*). Results from 2A2-A2/8.3 cells confirm that the porcine endothelial cell transfectant is an appropriate model

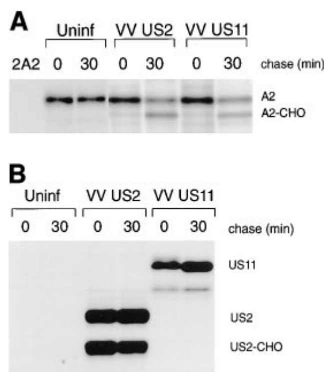

Figure 3. HLA-A2 is degraded in porcine endothelial cells in the presence of either US2 or US11. Approximately 10^7 2A2-A2/8.3, HLA-A2–expressing porcine endothelial cells were either infected with a vaccinia virus driving the expression of US2 (VVUS2, MOI = 5), with VVUS11 (MOI = 5), or left uninfected (*Uninf*). Cells were pretreated with the proteasome inhibitor, ZL_3H, for 45 min, pulse labeled for 15 min with [^{35}S]methionine, and then chased with unlabeled media for 0 and 30 min (all in the presence of ZL_3H). Lysed cells were sequentially immunoprecipitated with a polyclonal antibody against class I heavy chain, RαHC, and with αUS11 and αUS2. 10×10^6 parental 2A2 cells were included as a specificity control for the RαHC antibody and were pretreated, pulse labeled, and lysed as above. These lysates were immunoprecipitated with RαHC only. (*A*) Immunoprecipitations with the polyclonal anti–human MHC class I heavy chain antibody, RαHC. (*B*) Immunoprecipitations with polyclonal antibodies raised against US2 and US11 (αUS2 and αUS11, respectively).

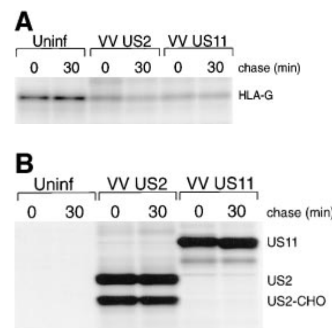

Figure 4. HLA-G stably expressed in porcine endothelial cells is resistant to class I heavy chain degradation associated with US2 and US11. Identical experiments to those described for Fig. 3 were performed using 2A2-HLA-G/1.2 cells, a porcine endothelial cell line stably transfected with HLA-G. For these experiments, primary RαHC immunoprecipitates were reimmunoprecipitated with RαHC before SDS-PAGE. (*A*) Immunoprecipitations with the polyclonal anti–human MHC class I heavy chain antibody, RαHC. (*B*) Immunoprecipitations with polyclonal antibodies raised against US2 and US11 (αUS2 and αUS11, respectively). *Uninf*, Uninfected.

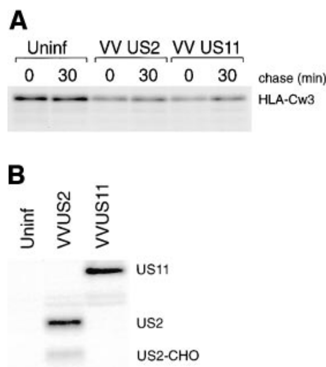

Figure 5. HLA-Cw3 stably expressed in porcine endothelial cells is resistant to class I heavy chain degradation associated with US2 and US11. Identical experiments to those described for Figs. 3 and 4 were performed using 2A2-HLA-Cw3/7 cells, a porcine endothelial cell line stably transfected with HLA-Cw3. (*A*) Immunoprecipitations with the polyclonal anti–human MHC class I heavy chain antibody, RαHC. (*B*) Immunoprecipitations with polyclonal antibodies raised against US2 and US11 (αUS2 and αUS11, respectively). *Uninf,* Uninfected.

for the study of US2- and of US11-associated immunoevasion, and documents the HCMV-associated dislocation reaction in a nonhuman cell system.

Similar experiments were then performed using porcine endothelial cells transfected with HLA-G (2A2-G/1.2) and HLA-Cw3 (2A2-Cw3/7). In contrast to the results obtained with HLA-A2 transfectants, neither VVUS11 nor VVUS2 infection affected the fate of the class I heavy chains in HLA-G or -Cw3 transfectants (Figs. 4 *a* and 5 *a*). In short, consistent with findings from JEG 3 cells, HLA-G in porcine endothelial cells is resistant to US11- and to US2-associated degradation. HLA-Cw3 is similarly resistant to US11- and US2-associated degradation in this xenogeneic model.

We have previously shown that the HLA-C locus product isolated from JEG 3 cells is HLA-Cw*0401 (4). In JEG 3 cells, this product is resistant to US2 and US11. HLA-Cw3 expressed in the porcine endothelial cells is similarly resistant. Therefore, we propose that escape from dislocation and degradation associated with the HCMV gene products US2 and US11 may be a more generalized characteristic of HLA-C locus products. Thus, HLA-G and HLA-C locus products appear unlike any previously described human or murine class I molecule, in that each can evade the degradation associated with both US11 and US2.

The results presented here are of interest from several viewpoints. First, we have shown that dislocation of MHC class I heavy chain associated with the presence of the HCMV gene products US2 and US11 can be detected in a nonhuman model. Dislocation and degradation appear to be neither cell type nor species specific. From the viewpoint of the virologist, the resistance of HLA-G and HLA-C to both US11 and US2 suggests that there might be yet another product within the genome of HCMV involved in interrupting surface expression of HLA-G and HLA-C locus products. Potential candidates might include US6, which has been shown to inhibit the TAP complex in a manner somewhat analogous to the HSV protein ICP47 (50–52), or US3, which has also been associated with retention of class I heavy chain in the ER (53, 54). It is possible that another, yet to be described, product may be dedicated to the specific elimination of trophoblast class I products. The demonstrated lack of coimmunoprecipitation of HLA-G and HLA-Cw*0401 with US11 and US2 prompt one to consider structural attributes that might disallow either direct interaction or interactions via a molecular intermediate. The analysis reported here provides a starting point for a more precise delineation of those elements of class I molecules recognized by US2 and US11.

Alternatively, and of interest to the reproductive biologist, trophoblast MHC class I molecules may be uniquely resistant to immunoevasive strategies used by HCMV. However, the ability of HCMV to infect trophoblast cells (28) and the association of HCMV infection during early pregnancy with both teratogenesis and spontaneous pregnancy loss (29, 30) argue against this. Nonetheless, the resistance of HLA-G and HLA-C locus products to both US11- and US2-associated degradation indicates that trophoblast class I products possess characteristics of structure and/or processing that distinguish them from other, classical MHC class I molecules. Could the actions of viral products like US2 and US11 constitute a selective force that favors evolution of HLA-G or -C like products to counteract such viral strategies?

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