The HCMV membrane glycoprotein US10 selectively targets HLA-G for degradation

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Human cytomegalovirus (HCMV) encodes an endoplasmic reticulum (ER)–resident transmembrane glycoprotein, US10, expressed early in the replicative cycle of HCMV as part of the same cluster that encodes the known immunoevasins US2, US3, US6, and US11. We show that US10 down-regulates cell surface expression of HLA–G, but not that of classical class I MHC molecules. The unique and short cytoplasmic tail of HLA–G (RKKSSD) is essential in its role as a US10 substrate, and a tri–leucine motif in the cytoplasmic tail of US10 is responsible for down-regulation of HLA–G. Both the kinetics of HLA–G degradation and the mechanisms responsible appear to be distinct from those used by the US2 and US11 pathways, suggesting the existence of a third route of protein dislocation from the ER. We show that US10-mediated degradation of HLA–G interferes with HLA–G–mediated NK cell inhibition. Given the role of HLA–G in protecting the fetus from attack by the maternal immune system and in directing the differentiation of human dendritic cells to promote the evolution of regulatory T cells, HCMV likely targets the HLA–G–dependent axis of immune recognition no less efficiently than it interferes with classical class I MHC–restricted antigen presentation.

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Abbreviations used: β_2m, β_2–microglobulin; CTL, cytotoxic T-lymphocyte; endo H, endoglycosidase H; HCMV, human cytomegalovirus; shRNA, short hairpin RNA; US, unique short region protein.

Cytotoxic T-lymphocytes (CTL) are essential for limiting and clearing viral infections (Doherty et al., 1992). CTLs are restricted by class I MHC molecules, which are a frequent target of viral strategies for their down-regulation or even elimination. The unique short region of human cytomegalovirus (HCMV) genome contains the US2–US11 genes, a region predicted to encode at least eight small glycoproteins of only limited homology (Weston and Barrell, 1986; Kouzarides et al., 1988). Several of them interfere with class I MHC–restricted antigen presentation, through inhibition of the MHC-encoded TAP peptide transporter (HCMV US6; Ahn et al., 1997; Jun et al., 2000), retention of newly synthesized class I MHC products at their site of synthesis (HCMV US3; Jones et al., 1996; Jun et al., 2000), or dislocation of class I MHC products from the endoplasmic reticulum (HCMV US2 and HCMV US11; Jones et al., 1996; Wiertz et al., 1996a,b; Machold et al., 1997; Schust et al., 1998). The coordinate regulation of the genes contained in the unique short region protein (US) region and the

common theme of interference with class I MHC–restricted antigen presentation suggest the possibility that other members of the family, with as yet poorly defined functions, may affect class I MHC antigen presentation as well.

For US8 and US10, a physical interaction with classical class I MHC products occurs (Furman et al., 2002; Tirabassi and Ploegh, 2002), but neither show significant ER retention or down-regulation of class I MHC products to the extent seen for US3, US2, and US11. Although both US8 and US10 bind to classical class I MHC products, only the expression of US10 imposes a delay on their egress from the ER, without affecting overall turnover of assembled class I MHC complexes or free class I MHC heavy chains. Based on our experience with the US2 and US11 products, the observation window of these experiments was limited to short periods only, and was thus biased against the possibility of documenting changes that occur

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with slower kinetics, yet are quantitatively significant. These experiments also failed to take into account the possibility that some of the HCMV US gene products might target nonclassical class I MHC products, by analogy of the effects reported for US2 and HFE, a class I–like molecule involved in the trafficking of the transferrin receptor (Ben-Arieh et al., 2001; Vahdati-Ben Arieh et al., 2003).

HLA-G is a particularly interesting nonclassical class I MHC molecule. It shows restricted tissue distribution and has limited polymorphism (Shawar et al., 1994; Carosella et al., 2000). HLA-G has strong immunomodulatory properties with specific relevance at immune-privileged sites such as the trophoblast or thymus, and it inhibits proliferation of T cells (Riteau et al., 1999; Lila et al., 2001), natural killer cells (Pazmany et al., 1996; Rouas-Freiss et al., 1997; Khalil-Daher et al., 1999), and antigen-specific T cell cytotoxicity (Le Gal et al., 1999; Wiendl et al., 2002). HLA-G has aroused interest not only because of its role in feto-maternal interactions, but also because of its expression on subsets of human dendritic cells, in particular those implicated in the activation of regulatory T cells (Liang et al., 2008; Pazmany et al., 1996).

We report that, unlike any previously described nonclassical class I product, HLA-G is sensitive to proteasomal degradation in a HCMV US10-dependent manner. The underlying mode of degradation of HLA-G under the agency of US10 appears to be unique, despite similar subcellular localization and structural relatedness of US10 to US2 and US11. We suggest that HCMV-infected cells avail themselves of all possibilities to frustrate class I MHC–restricted antigen presentation, including the inhibition of pathways that concern nonclassical class I MHC products in the context of an HCMV infection.

RESULTS

HCMV US10 down-regulates surface presentation of JEG3-derived HLA-G by degradation

Although HCMV US10 binds to classical class I MHC molecules and delays their trafficking (Furman et al., 2002), it does not affect their steady–state cell surface levels (Ahn et al., 1997). To test whether US10 could interfere with the synthesis and stability of nonclassical class I MHC products, we expressed US10 in the HLA-G–positive choriocarcinoma cell line JEG3 and examined surface levels of HLA-G by cytofluorimetry using W6/32 or MEM-G/9, both of which recognize assembled heterodimers of heavy chain and β_2-microglobulin (β_2m; Fig. 1 A). We observed a significant reduction of surface expression of HLA-G in US10-expressing cells, whereas introduction of US9, used as a control, was without effect on HLA-G levels (Fig. 1 A, right). The effect of US10 on HLA-G expression is not unique to the naturally HLA-G–expressing choriocarcinoma cell line JEG3. Introduction of HLA-G into HeLa cells showed that US10 also down-regulates HLA-G in this setting (Fig. 1 B, top). US10 did not affect surface display of the classical class I MHC products endogenous to HeLa cells (Fig. 1 B, bottom). This result suggests that properties intrinsic to US10 are sufficient to account for down-regulation of HLA-G, and that no choriocarcinoma-specific factors contribute.
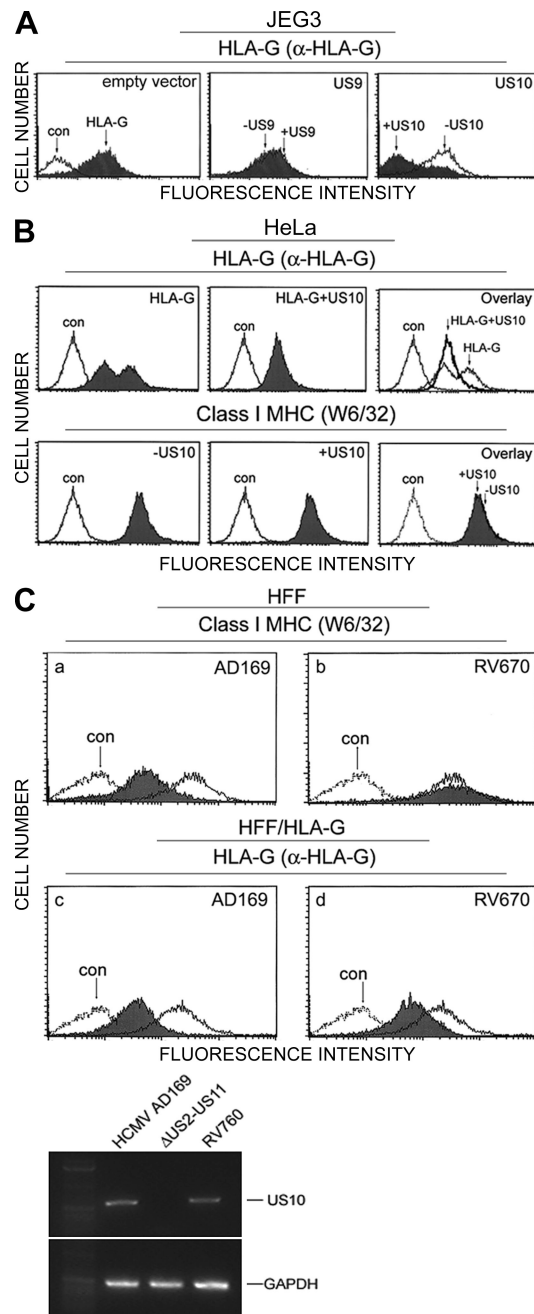
Figure 1. US10 down–regulates HLA-G molecules, but not classical class I MHC products, by degradation. (A) Cell surface expression of HLA-G in HCMV US9 and US10-expressing JEG3 cells. JEG3 was transiently transfected with empty vector, US9, or US10. After 48 h, cell surface expression of HLA-G was monitored by cytofluorimetry using mAb MEM-G/9. (B) US10 down-regulates HLA-G products. Both US10 and HLA-G or US10 alone were transiently transfected into HeLa cells. Surface expression of HLA-G or classical class I MHC was measured by cytofluorimetry using MEM-G/9 or W6/32. (C) Human foreskin fibroblasts (HFF) stably expressing HLA-G were infected with wild-type HCMV AD169 (a and c, shaded area), a HCMV mutant ΔUS2-US11 (a–d, solid black line), or RV670 (b and d, shaded area). Surface levels of either classical class I MHC or HLA-G by cytofluorimetry using W6/32 or MEM-G/9. US10 gene product is verified by RT-PCR in a HCMV AD169 or mutant virus-infected cells. The data shown are representative of two independent experiments with similar results.

To ascertain the physiological relevance of US10 activities in down-regulation of HLA-G, we examined whether US10 affects cell surface levels of HLA-G in the context of HCMV infection. Human foreskin fibroblasts stably expressing HLA-G were infected either with wild-type HCMV AD169, a HCMV deletion mutant lacking the US2-US11 region (ΔUS2-US11), or with a HCMV mutant virus, RV670, lacking all genes in the US2-US11 region with the exception of US10 (Jones and Muzithras, 1992; Jones et al., 1995). We then examined surface levels of either classical class I MHC or HLA-G by cytofluorimetry using W6/32 or MEM-G/9. In AD169-infected cells, surface levels of classical class I MHC and HLA-G were significantly reduced, whereas in ΔUS2-US11 AD169-infected cells (Fig. 1 C, a and c), they were comparable to levels observed in uninfected cells (Fig. 1 C). The HCMV mutant virus, RV670, down-regulated cell surface expression of HLA-G (Fig. 1 C, d), but did not affect levels of classical class I MHC products (Fig. 1 C, b). RV670-infected cells express the US10 gene product as do wild-type HCMV AD169-infected cells (Fig. 1 C, bottom). These findings show that US10 specifically down-regulates HLA-G, but not classical class I MHC products.

US10–dependent degradation of HLA–G involves a deglycosylated intermediate in the presence of proteasome inhibitor

To explore how US10 affects expression of HLA-G, we installed an HA epitope tag on US3, US9, and US10 to assess the levels of these HCMV products in transfectants. The use of the HA tag allowed us to select transfectants with comparable levels of expression for each of the viral products, as they are detected by one and the same antibody (Fig. 2 A). We quantitated the amount of HLA-G in US3-, US9-, and US10-expressing JEG3 cells by immunoblotting using MEM-G/1 antibody, which recognizes the denatured α_1 domain of HLA-G. HLA-G was barely detectable in the JEG3-US10 cells, but neither US3 nor US9 affected the robust HLA-G levels present in these transfectants (Fig. 2 A). We confirmed the results of immunoblotting by biochemical analysis on [^{35}S]-methionine and cysteine-labeled cells. In the absence of US10, significant amounts of HLA-G were detected at the end of both the pulse and the 6-h chase. In contrast, in JEG3 cells transfected with US10, despite similar rates of synthesis of HLA-G during the 30-min pulse, ~90% of labeled HLA-G was degraded after the 6-h chase (Fig. 2 B, lanes 7 and 8). Only a small portion of endoglycosidase H (endo H)–sensitive HLA-G complexes remains at 6 h of chase (Fig. 2 B, lanes 7 and 8). We conclude that surface expression and stability of HLA-G are sensitive to the presence of US10, whereas the classical class I MHC molecules are not affected by US10.

To investigate whether the proteasome is involved in US10-mediated degradation of HLA-G, we monitored HLA-G levels in US10-expressing JEG3 cells in the presence of the proteasome inhibitor ZL$_3$VS (Jones et al., 1996; Wiertz et al., 1996a). In JEG3 cells, HLA-G is a stable type I membrane glycoprotein with scant reduction in the amounts of biosynthetically labeled HLA-G, even after 12 h of chase. Little if any HLA-G remains after the 12-h chase period in

US10-expressing cells (Fig. 2 C, lane 6). Inclusion of ZL$_3$VS prevented the loss of labeled HLA-G in US10-expressing cells. Moreover, we observed a deglycosylated intermediate reminiscent of what is seen in US2 or US11-expressing cells for classical class I MHC products (Fig. 2 C, lanes 8 and 9). We obtained similar results for HeLa cell transfectants that express HLA-G and US10 (Fig. 2 D, lanes 8 and 9). We conclude that inclusion of proteasome inhibitors allows the visualization of the deglycosylated HLA-G intermediate in cells that express US10.
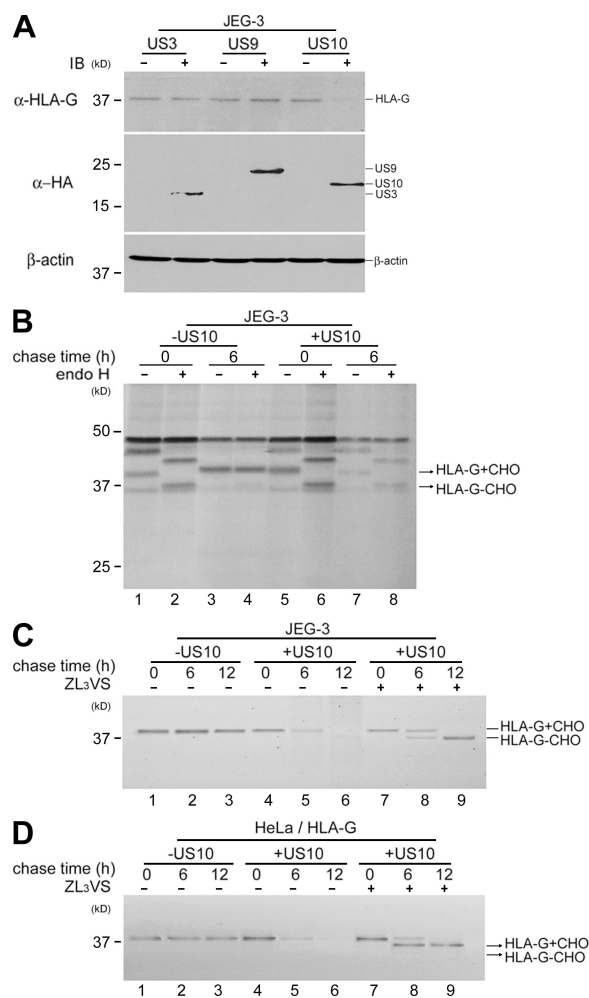

Figure 2. HLA-G dislocation and degradation in the presence of US10. (A) US10 degrades HLA-G. Lysates from JEG3 cells expressing HA-tagging US3, US9, or US10 were analyzed by immunoblotting with the indicated antibodies. (B) US10-expressing JEG3 cells were metabolically labeled for 20 min and chased for 6 h. Assembled HLA-G molecules were immunoprecipitated with MEM-G/9 and treated with endo H where indicated. Immunoprecipitation from NP-40 lysates of JEG3 cells (C) and HeLa cells (D) pulse-labeled for 20 min and chased for the indicated time points in the absence (lanes 1–6) or presence (lanes 7–9) of 50 µM ZL$_3$VS were performed with MEM-G/1 antibodies. A representative result of two independent experiments is shown.

Characterization of the cytoplasmic tail residues of US10 critical for degradation of HLA–G

To identify the regions of US10 responsible for mediating the degradation of HLA-G, we constructed two truncation mutants, US10ΔCT and ΔTM+CT, in which the cytoplasmic tail or both the transmembrane domain and the cytoplasmic tail of US10, respectively, were deleted (Fig. 3 A, top). These mutant proteins remain endo H sensitive during chase periods of up to 4 h (Fig. 3 B). In contrast to cells that express wild–type US10, cells that express the US10 truncation mutants
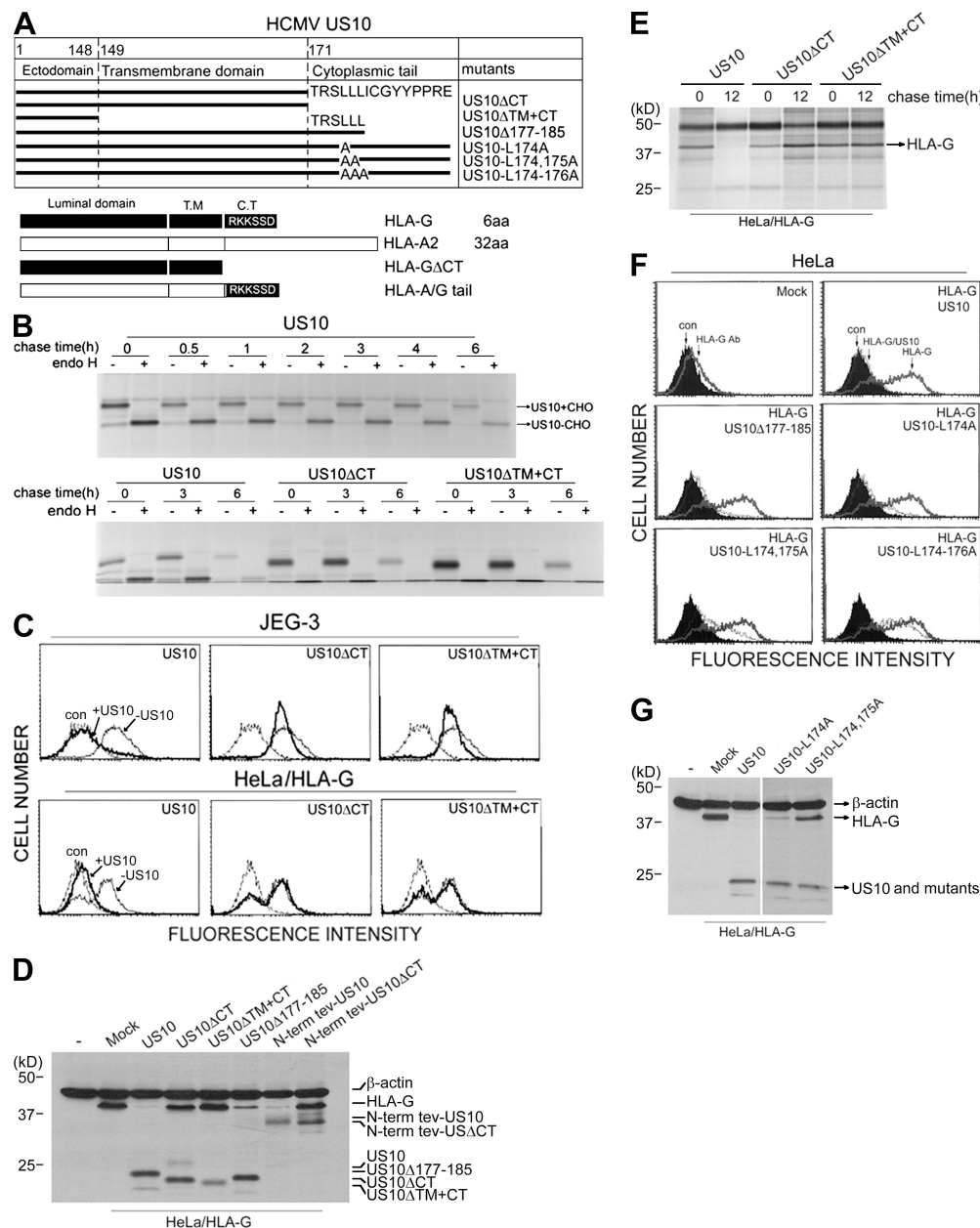
Figure 3. Characterization of the cytoplasmic determinants of US10 responsible for the degradation of HLA–G. (A) Schematic representation of the chimeras of US10 and HLA-G. TM, transmembrane; CT, cytoplasmic tail; filled, HLA-G; open, HLA-A2. (B) Stability and maturation of US10 and its deletion mutants. HeLa cells were transfected with US10, US10ΔCT or US10ΔTM+CT. Cells were labeled for 15 min and chased for the indicated times. Immunoprecipitated proteins were digested (+) or not digested (−) with endo H. (C) HLA-G surface expression in JEG3 and HeLa cells expressing US10 chimeras. HeLa and JEG3 cells were transiently transfected with the individual cDNAs encoding the US10 deletion mutants. After 48 h, cell surface expression of HLA-G was analyzed by cytofluorimetry using MEM-G/9. Dashed line, no US10; solid line, US10; bolded line, transfectants. (D and E) Stability of HLA-G in cells expressing wild-type US10, mutants, or HA-TEV epitope–tagged constructs of US10. (F and G) The tri-leucine motif of the US10 cytoplasmic tail is critical for degradation of HLA-G. Expression of HLA-G and the indicated point mutants of US10 were analyzed by cytofluorimetry and immunoblotting using MEM-G/9 and MEM-G/1 antibodies. Data are from three independent experiments.
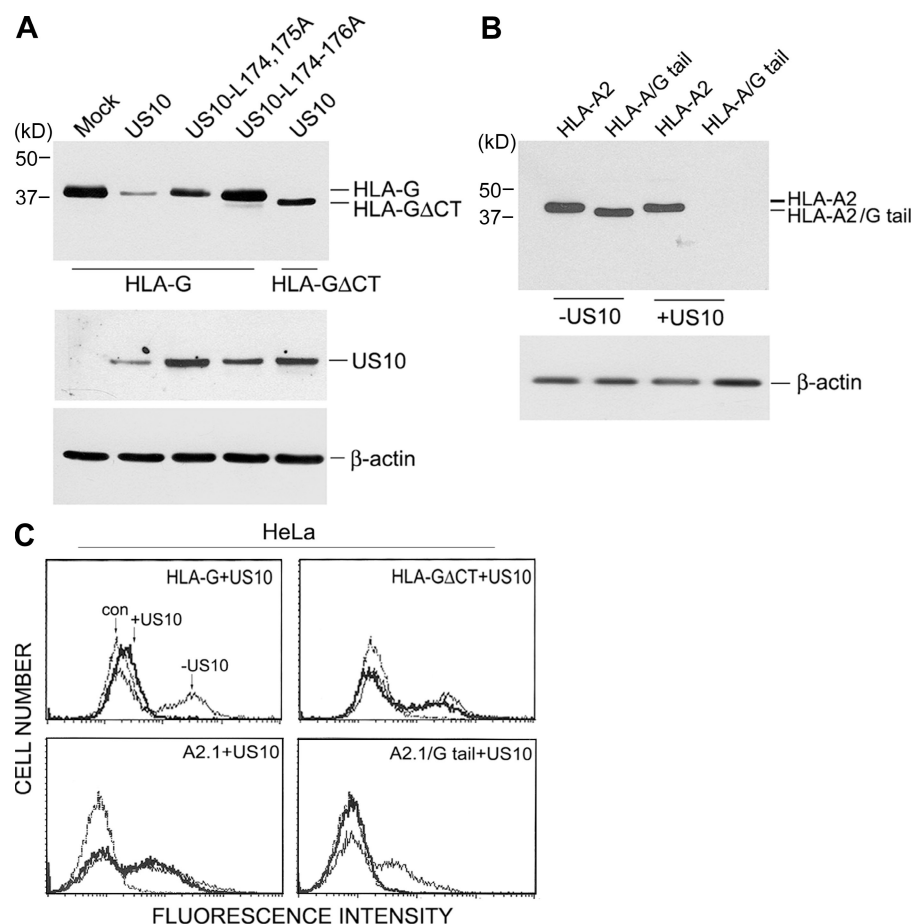
Figure 4. US10 uniquely targets the short cytoplasmic tail of HLA-G.
(A and C) HeLa cells expressed HLA-G, HLA-GΔCT, or HLA-A2.1 were transiently transfected with the indicated constructs and observed for protein expression or cell surface expression of HLA-G by immunoblotting or cytofluorimetry with the indicated antibodies (A: α-HLA-G, α-HA, or α-β-actin; C: α-HLA-G or BB7.2). (B) HeLa cells expressing US10 were transiently transfected with wild-type HLA-A2 or HLA-A/G tail and examined for protein expression of HLA-A2 by immunoblotting with HLA-A2 antibody. Dashed line, control Ab; solid line: transfectants alone; bolded line: transfectants+US10. Data are representative of similar experiments performed at least two times.

The cytoplasmic tail of HLA-G is required for US10 to exert its function

The sensitivity of HLA-G to degradation facilitated by US10 is intrinsic to HLA-G. The cytoplasmic tail of HLA-G is much shorter (6 amino acids; RKKSSD) than that of the classical class I MHC products (Geraghty et al., 1987; Fig. 3 A, bottom). To address the role of HLA-G's short cytoplasmic tail, we generated not only HLA-GΔCT, which lacks the cytoplasmic tail, but also HLA-A2/G tail, a version of the classical class I MHC product HLA-A2 equipped with the cytoplasmic tail of HLA-G (HLA-A2/G tail; Fig. 3 A, bottom). Expression levels of HLA-A2.1 or HLA-GΔCT were not affected by US10 (Fig. 4 A), whereas HLA-A2/G tail behaved like HLA-G (Fig. 4, B and C). We examined surface expression of these mutants in US10-expressing HeLa cells. Deletion of the cytoplasmic tail of HLA-G (HLA-GΔCT) prevented its down-regulation by US10, whereas surface expression of HLA-A2/G tail was affected by US10 like HLA-G (Fig. 4 C). HLA-E is a human nonclassical class I MHC molecule and is similar to HLA-G with the exception of an extension of the cytoplasmic tail when compared with HLA-G (32 aa instead of 6 aa). We examined whether the level of HLA-E protein expression is affected by US10 (Fig. S1). HLA-G or HLA-E–expressing HeLa cells were transfected with US10 and examined by immunoblotting with anti-HLA-G or anti-HLA-E antibodies. Protein levels of HLA-G were significantly affected by US10, whereas the HLA-E level was only slightly reduced. We conclude that within the context of a human class I MHC molecule the short cytoplasmic tail sequence of HLA-G is necessary and sufficient to allow US10-mediated degradation and that US10 specifically targets HLA-G.

showed normal levels of HLA-G at the cell surface (Fig. 3 C). Even though the levels of expression of wild-type and mutant proteins were comparable, the levels of HLA-G heavy chain, assessed by immunoblotting, were similar for cells that express the US10 truncation mutants and HLA-G transfectants produced in the absence of US10 (Fig. 3, D and E). The cytoplasmic tail of US10 thus functions as a signal that targets HLA-G for degradation.

To narrow the determinants within the cytoplasmic tail essential to the down-regulatory capacity of US10, we generated a construct that lacks residues 177–185 (US10Δ177-185; Fig. 3 A, top). This mutant showed a wild-type pattern of degradation of HLA-G (Fig. 3, D and F). Thus, the motif between amino acids 171 and 176 (TRSLLL) of US10 appeared critical for the degradation of HLA-G. We then established the importance of the leucine residues 174–176 for degradation of HLA-G. We substituted the first leucine residue (in bold; TRS**A**LL), the first and second leucine residues (TRS**AA**L), and all three leucine residues (TRS**AAA**) with alanine (Fig. 3 A, top). The version of US10 that lacks two leucine residues restored HLA-G protein levels, and its presence is permissive for cell surface expression of HLA-G. Replacement of all three leucines rendered US10 completely inactive (Fig. 3, F and G). We conclude that the tri-leucine motif in the cytoplasmic tail of US10 is crucial to target HLA-G for degradation.

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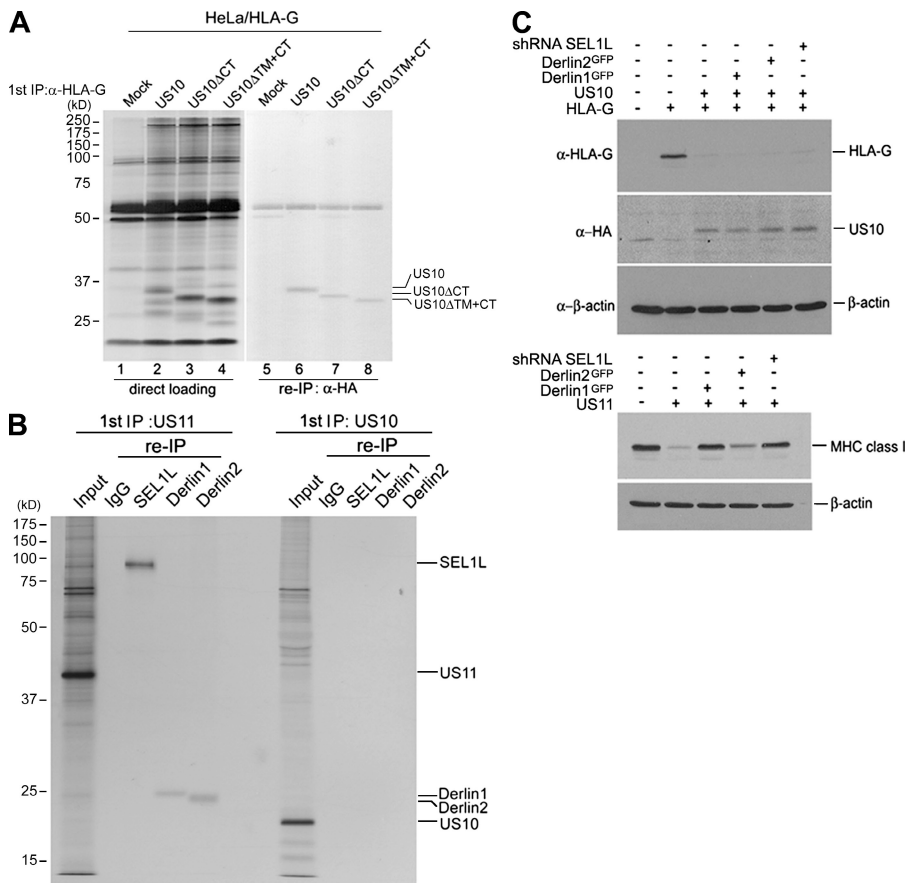
Figure 5. US10 selectively targets HLA–G molecules for degradation by a mechanism distinct from that used by HCMV US11. (A) The luminal domain of US10 is required for the interaction of HLA–G. Cells transfected with indicated cDNA were labeled with [^{35}S] methionine and cysteine for 1 h. The anti-HLA-G immunoprecipitate was analyzed directly (lanes 1–4) or reimmunoprecipitated after dissociation of the initial immunoprecipitate using anti-HA antibodies (lanes 5–8). (B) US10 does not obviously associate with the dislocation components recruited by US11: Sel1L and Derlin1, 2. Immunoprecipitations from digitonin lysates were performed with anti-HA antibodies. The anti-HA immunoprecipitates were either analyzed directly or reimmunoprecipitated using the indicated antibodies. (C) Expression of a Derlin1/2 dominant-negative construct or shRNA Sel1L does not inhibit US10-mediated HLA–G degradation. Data are representative of two experiments.

The mechanism of degradation of HLA-G by US10 is distinct from that used by US2 and US11 for classical class I MHC products

We next investigated the physical association of US10 and HLA-G in digitonin lysates of metabolically labeled cells. In anti-HLA-G immunoprecipitates, both US10 and its truncation mutants were present (Fig. 5 A, lanes 2–4), as verified by a second round of immunoprecipitation with antibodies that recognize the tagged versions of US10 (Fig. 5 A, lanes 6–8). The luminal segment of US10 is therefore sufficient for its interaction with HLA-G, a situation analogous to the interaction of US2, US3, and US11, all of which interact with their client proteins via their luminal domains.

We have mapped in some detail the protein complexes required for US11-dependent dislocation of classical class I molecules. US11 associates in stable fashion with Sel1L, Derlin1, and Derlin2 (Lilley and Ploegh, 2004; Mueller et al., 2006). A similar experiment conducted for US10 fails to recover Sel1L, Derlin1, or Derlin2 (Fig. 5 B). We confirmed these results by using short hairpin RNA (shRNA) for Sel1L and by transfection with a dominant-negative construct of Derlin1 or Derlin2 (Lilley and Ploegh, 2004; Mueller et al., 2006). In US11-expressing HeLa cells cotransfected with either Derlin1^{GFP} or Sel1L shRNA, we observed stabilization of the class I HC (Fig. 5 C, bottom). In contrast, HLA-G was degraded in US10-dependent fashion under the same conditions (Fig. 5 C, top).

Although the failure to recover these components of the dislocation complex is a negative result, we note that the recovery of US10 in these experiments is very similar to that recorded for US11, based on comparable methionine content of US11 and US10. We have thus far been unable to positively identify interactors of US10 that might account for its ability to dislocate HLA-G. The failure to recover the well-documented interactors of US11, as well as the strikingly different kinetics with which dislocation of HLA-G occurs relative to classical class I molecules, also compared with cells that express US2, suggests that US10 employs a mechanism distinct from that used by US2 and US11. In much the same way that the early aspects of US2- and US11-dependent dislocation operate through recruitment of different machinery (Lilley and Ploegh, 2004; Loureiro et al., 2006; Mueller et al., 2006), the US10 molecule may well use a different pathway to execute destruction of HLA-G molecules.

US10 prevents HLA-G–mediated NK cell inhibition

HLA-G has unique immunomodulatory properties through its ability to inhibit NK cell–mediated cytotoxicity (Pazmany et al., 1996; Navarro et al., 1999; Sasaki et al., 1999). We therefore examined the ability of US10 to interfere with HLA-G–mediated inhibition of NK cytotoxicity. In previous studies, killing of the class I–negative human B cell line 721.221 by peripheral NK cells is inhibited by the expression of HLA-G (Pazmany et al., 1996; Ponte et al., 1999; Söderström et al., 1997). Peripheral NK (pNK) cells were coincubated with 721.221, 721.221/HLA-G, or 721.221/HLA-G that

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coexpresses US10 at a 10:1 E/T ratio for 5 h at 37°C. We observed that US10 also degrades HLA-G in 721.221 cells (Fig. 6 A) and that HLA-G–expressing 721.221 cells are resistant to cytotoxic activity of pNK cells compared with the parental 721.221 cell line (Fig. 6 B). US10 blocked the inhibition of NK lytic activity by HLA-G, as did addition of mAb specific for HLA-G (Fig. 6 B). We conclude that US10 interferes with the ability of HLA-G to regulate NK cell activity.

DISCUSSION

A deglycosylated intermediate of HLA-G is present in US10-expressing cells exposed to proteasome inhibitors, but is evident only with considerable delay after synthesis. The properties of the tails of HLA-G and US10 are essential for dislocation to occur, yet neither the HLA-G tail nor the US10 tail resemble those of classical class I MHC molecules or those of US2 and US11, respectively: the tri-leucine cluster in the US10 cytoplasmic tail is without an obvious counterpart in US2 or US11. In addition, the physical association of US10 with HLA-G through the luminal domain of US10 may have contributed to a small extent to the retention of HLA-G complexes in the ER (Fig. 2 B).
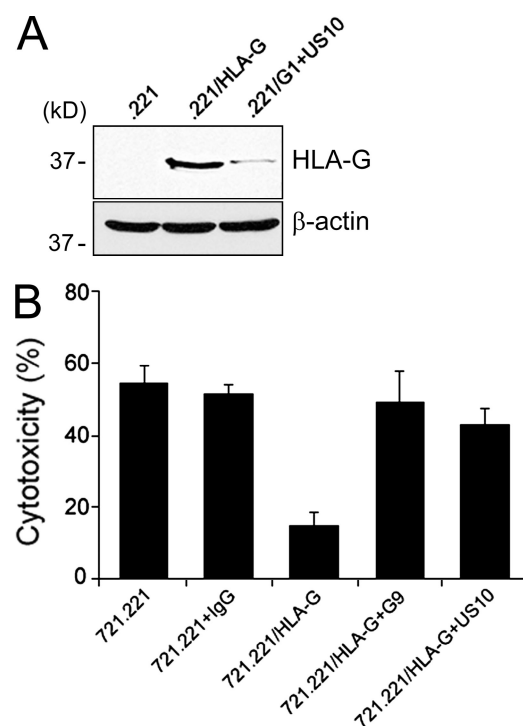
Figure 6. US10 interferes with HLA-G–mediated Natural Killer cell cytotoxic activity. (A) Expression level of HLA-G by US10 in 721.221 cells was analyzed by immunoblotting with MEM-G/1 antibodies. (B) Target cells were stained with DiOC$_{18}$ (3) for 30 min and then pNK cells were coincubated with different target cells at a 10:1 ratio (Effect: Target) for 5 h. After incubation time, cells were stained with propidium iodide for dead cells for 10 min and analyzed by cytofluorimetry. Data are representative of three experiments.

The functional consequences of HLA-G down-regulation include, yet may not be limited to interference with HLA-G–mediated NK cell inhibition. This is consistent with previous studies that documented a role of HLA-G in the control of feto-maternal interactions (Carosella et al., 2000) and the more recently described involvement of HLA-G in the regulation of both DC differentiation (Romani et al., 1994) and proliferation of T cells (Le Gal et al., 1999; Lila et al., 2001). Therefore, we must consider the possibility that HCMV targets these aspects of immune recognition no less attentively than it perturbs antigen presentation via classical class I MHC molecules. The ability to exploit US10 to achieve selective elimination of HLA-G may be useful as a tool to establish in greater detail the immunological function of HLA-G. Our experiments do not address the impact of US10 on the generation of soluble HLA-G, the function of which remains to be established at physiological concentrations.

Why HCMV should use several different gene products to target a common set of substrates—class I MHC products—remains a mystery. Earlier work has shown that inhibition of TAP via US6 compromises HLA-G expression (Jun et al., 2000). Variable effects of US2, US3, and US11 on expression of HLA-G have been reported as well, but these experiments often rely on overexpression US gene products, mostly using vaccinia virus vectors, and do not examine their effects in the context of HCMV-infected cells (Jun et al., 2000; Barel et al., 2003). Because HCMV can infect many different cell types, it is possible that each cell type prefers a distinct mode of dislocation of class I MHC products, depending on the dislocation or ER retention machinery available. The mere presence of the HCMV gene products is sufficient to mediate accelerated destruction, and consequently these small viral membrane proteins must co-opt host machinery to achieve their goals. HLA-G shows a pattern of expression markedly different from that of classical class I MHC products. Although HLA-G was first described as a molecule whose expression is restricted to extravillous trophoblasts, it is now appreciated that dendritic cells and other cell types can express HLA-G and may do so to regulate the activation of regulatory T cells through interaction with ILT4 (Ristich et al., 2007; Liang et al., 2008). The ability of CMV to target this nonclassical class I molecule for degradation suggests that HCMV maintains tight control over the expression not only of classical class I molecules, but also of those whose functions may be more specialized. What could be the selective advantage that accrues to HCMV through expression of US10, and hence down-modulation of HLA-G? Obviously, relieving inhibition of NK cells and inviting cytolytic attack on HCMV-infected, US10-positive cells would seem counterintuitive, unless one assumes that cytolysis may assist in the release of already assembled intracellular virus particles to facilitate further spreading of the infection. In many instances, activation of NK cells need not lead to cytolysis, but rather results in altered cytokine production instead (Pazmany et al., 1996; Li et al., 2009; Ponte et al., 1999). Any action that would alter the activation status of NK cells, more specifically of those that express several inhibitory receptors, might create an environment

more favorable for HCMV, and herein might provide a rationale for why HCMV has retained the US10 gene as a possible immunoevasin. The ability of HCMV to be passed from mother to child in the course of pregnancy may well be related to the unique mechanisms by which HCMV abrogates both classical and nonclassical MHC products.

MATERIALS AND METHODS

Cells and virus. The JEG3 choriocarcinoma cell line, HeLa cells, and human foreskin fibroblast cells were cultured in complete medium: DME supplemented with 10% fetal bovine serum and 50 U/ml penicillin (Invitrogen). YTS and 721.221 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. Cells were grown at 37°C in humidified air with 5% CO_2. HCMV strain AD169 was obtained from the American Type Culture Collection. HCMV mutant virus AD169ΔUS2–US11 (deletion of US2 to US11) and RV670 (deletion of IRS to US11, but contains only US10) were provided by A.E. Campbell (Eastern Virginia Medical School, Norfolk, Virginia; Jones et al., 1995).

Isolation of pNK cells. pNK cells were isolated from peripheral blood as previously described (Koopman et al., 2003). For cytotoxicity assay, pNK were further purified by the subsequent use of anti-CD56–coated magnetic beads.

DNA constructs and antibodies. HLA-G, HLA-E, and HLA-A2.1 cDNAs and all chimeric mutants were subcloned in the pcDNA3.1 vector (Invitrogen, San Diego, CA). HA-US10 and the chimeras of US10 were constructed as previously described (Lilley and Ploegh, 2004). For site-directed mutagenesis and the truncation mutants, respective DNA fragments were amplified by PCR with oligonucleotides that introduced the desired, unique restriction site. Derlin1GFP, Derlin2GFP, and shRNA for Sel1L constructs were generated as described (Lilley and Ploegh, 2004; Mueller et al., 2006). The monoclonal antibody W6/32 recognizes only MHC class I heavy chains associated with β_2m. The MEM-G/9, MEM-G/1, and BB7.2 recognize β_2m-associated HLA-G (Abcam), denatured HLA-G (AbD Serotech), and HLA-A2.1 (Abcam). Antibody against HLA-E was purchased from Abcam.

Flow cytometry. Surface expression of MHC class I molecules and HLA-G were determined by flow cytometry (FACSCalibur; BD) as previously described (Park et al., 2003).

Pulse-chase analysis and immunoprecipitation. Cells (10^7) were starved for 50 min in medium lacking methionine and cysteine; labeled with 0.1 mCi/ml [35S]methionine and cysteine (TranS-label; NEN Life Science); and chased in complete medium with or without added inhibitor for the indicated times. Cells were lysed with 1% NP-40 (Sigma-Aldrich) in PBS for 30 min at 4°C. After preclearing lysates with protein G–Sepharose (Sigma-Aldrich), primary antibodies, and protein G–Sepharose were added to supernatants and incubated at 4°C. The protein G–Sepharose beads were washed four times with 0.1% NP-40 in PBS. Proteins were eluted from the beads by boiling in SDS sample buffer and separated by SDS-PAGE. For endo H treatment, immunoprecipitates were digested with 3 mU endo H (NEB) at 37°C overnight in 50 mM sodium acetate, pH 5.6, 0.3% SDS.

Coimmunoprecipitation and Western blot analysis. Cells were lysed in 1% digitonin (Calbiochem) in buffer containing 25 mM Hepes, 100 mM NaCl, 10 mM $CaCl_2$, and 5 mM $MgCl_2$, pH 7.6, supplemented with 0.5 mM PMSF, leupeptin, and 10 mM NEM. Lysates were precleared with protein G–Sepharose for 1 h at 4°C. For immunoprecipitation, samples were incubated with the appropriate antibodies for 12 h at 4°C before protein G beads were added. Beads were washed four times with 0.1% digitonin, and bound proteins were eluted by boiling in SDS sample buffer. Proteins were separated by 12% SDS-PAGE, transferred into a nitrocellulose membrane, blocked with 5% skim milk in PBS with 0.1% Tween-20 for 2 h, and probed with the appropriate antibodies for 4 h. Membranes were washed three times with PBS with 0.1% Tween-20 and incubated with horseradish peroxidase–conjugated streptavidin for 1 h. The immunoblots were visualized with ECL detection reagent.

Cytotoxicity assay. Target cells were stained with $DiOC_{18}$ (3) (Invitrogen) for 30 min, and then washed with PBS 3 times. Effect cells were co-incubated with different target cells for 5 h. Effect cells alone and target cells alone were prepared for data analysis. After the incubation period, propidium iodide was added immediately into the co-cultured cells and analyzed by Flow cytometry.

Online supplemental material. Fig. S1 shows that expression of HLA-G is slightly reduced by US10. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20091793/DC1.

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