Membrane-specific, Host-derived Factors Are Required for US2- and US11-mediated Degradation of Major Histocompatibility Complex Class I Molecules*

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The mammalian immune system relies on the presentation of cell surface glycoproteins to alert lymphocytes to the presence of intracellular pathogens. One class of these glycoproteins, MHC class I molecules, is assembled in the endoplasmic reticulum (ER) as a trimeric complex consisting of a 43-kDa heavy chain in association with a 12-kDa light chain ($\beta_2$-microglobulin ($\beta_2$-m)) and an antigenic peptide of 8–10 amino acids. Presentation of viral peptides complexed with MHC class I molecules on the surface of infected cells leads to recognition and destruction by cytotoxic T lymphocytes. Human cytomegalovirus (HCMV) encodes two proteins, the products of the US2 and US11 genes, which interfere with MHC class I expression by inducing rapid degradation of newly synthesized heavy chains (1–3). The destruction of heavy chains by US2 and US11 presumably enables evasion of cytotoxic T lymphocyte recognition early after infection or reactivation from latency, allowing the virus more time to replicate unnoticed. The degradation of MHC class I heavy chains by US2 and US11 is similar to the destruction of misfolded proteins that arises as a consequence of imperfections in the normal translation and folding reactions. US2 and US11 recognize the class I molecules within the lumen of the ER and dislocate them into the cytosol where they are destroyed by the proteasome (2, 3).

The ER is a major site of quality control for secretory proteins in all eukaryotes. Many glycoproteins that lack the ability to fold properly or associate with partner polypeptides are retained in the ER and are eventually targeted for degradation by the proteasome (4, 5). The involvement of the proteasome in the destruction of ER proteins necessitates their access to the cytosol where proteasomes are located. The mechanism by which aberrant proteins are targeted for destruction and dislocated from the ER remains unresolved and there may in fact be multiple pathways (6–8). US2 and US11 have co-opted this degradation process to interfere with the expression of MHC class I molecules and therefore provide a model system for the study of ER-to-cytosol transport.

Genetic and cell-free systems in yeast have identified molecules that participate in the recognition, dislocation, and degradation steps of the process. Chaperones such as BiP and calnexin participate in recognition of some substrates (9, 10). Several mutations in the Sec61p translocon compromise degradation and suggest that this pore may mediate dislocation into the cytosol, in addition to the import of nascent polypeptides (11). Ubiquitin-conjugating enzymes also play a role in the degradation of most ER-to-cytosol substrates in yeast, consistent with ubiquitination as a major signal for proteasomal destruction (4).

Mammalian cell-free systems have confirmed some of the observations made in yeast and intact cells. Ubiquitination and degradation of the cystic fibrosis transmembrane conductance regulator has been observed in canine microsomes (12, 13). The degradation of CD4 by HIV Vpu has also been observed in canine microsomes (14). Export of a misprocessed glycosylphosphatidylinositol-anchored protein from microsomes was shown to be cytosol dependent (15). The release of $\beta_2$m from microsomes is suppressed by export of short (8–10 residues) peptides, suggesting that such peptides and glycoproteins may both exit through the Sec61p translocon (16, 17).

Our objective was to recapitulate the steps of early MHC
class I synthesis and recognition by US2/US11 in vitro. Dislo-
cation and degradation in a cell-free environment would facil-
itate dissection of the components required for these events,
but no such integrated system has been reported. Synthesis
and assembly of MHC class I molecules within microsomes has
been demonstrated (18). Furthermore, interactions between in
vitro translated assembled class I molecules with US2 and
US11 in canine pancreas-derived microsomes have likewise
been shown, but in vitro dislocation by US2 or US11 has not
been demonstrated (19, 20).

The use of the system presented here reveals a heterogeneity
in the membrane-bound components required for dislocation
of heavy chains by US2 and US11. The intermediates that occur
in the course of degradation by US2 and US11 in vitro are
biochemically indistinguishable from the deglycosylated, cyto-
solic intermediates observed in intact cells. However, US2 and
US11 do not rely equally on cytosolic domains to trigger degra-
dation. Further, the mechanism for US2/US11 and class I
appears to be distinct from that used by Vpu for the degrada-
tion of CD4. Here we also show that microsomes of human
origin support US2/US11 degradation, whereas canine pan-
creas microsomes do not. Our data are consistent with wide
divergence in the manner by which proteins are exported from
the ER for cytosolic degradation.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cell Culture—** U373-MG astrocytoma cells (control), US2 transfectants (US2 ×), and US11 transfectants (US11 ×) cell lines were maintained as described (21).

**DNA Constructs and Transfection—** The HLA-A2, US2, US11, β2m, CD4, and HLA-C constructs used to generate mRNA have been de-
scribed previously (19, 20, 22). The US2–186 and US11–201 were ampli-
fied from full-length US2 and full-length US11 cDNA using PCR. The 3′ primer introduced a stop codon after amino acid 186 in US2 and 201 in US11 and contained a restriction site for subcloning into pCDNA3.1 (Invitrogen). The pSP9-Vpu and pSP9-226 plasmids encoding Vpu and a phosphorylation mutant of Vpu, respectively, were kindly provided by Klaus Strebel (23). Transfection of US2 and US11 in canine pancreas-derived microsomes have likewise
been demonstrated (19, 20).

**Antibodies and Reagents—** Polyclonal rabbit anti-HC, which recog-

nizes unfolded heavy chains, has been described (24). Western, a mouse
monoclonal antibody that recognizes properly folded MHC class I molecules in association with β2m, has been described (25). MG132 was synthesized in the laboratory as described (26).

**Preparation of Microsomes—** Canine pancreas microsomes were pre-
pared as described (28). Astrocytoma microsomes were prepared from U373-MG control cells, US2, and US11 transfectants. Astrocytoma cells were detached by trypan
treatment, resuspended in homogenization buffer (10 mM HEPES, pH 7.4, 1 mM EDTA, 0.25 mM sucrose, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 0.25 mM phenylmethylsulfonyl fluoride) at 25–35 × 10^6 cells/ml, and homogenized by repeated passage through 25-, 27-, and 30-
gauge syringe needles. Unbroken cells and nuclei were pelleted by centrifugation (1,500 × g for 10 min at 4 °C). The 1,500 × g supernatant was then centrifuged at 75,000 × g for 60 min at 4 °C. The membrane pellet was resuspended in homogenization buffer (approximately 100 × 10^6 cells/ml of homogenization buffer) and added to in vitro transla-
tions as a source of microsomes. The supernatant was discarded.

**In Vitro Degradation Assay—** In vitro translations of HLA-A2 and/or other polypeptides were performed in the presence of microsomes (0.5 μl of astrocytoma microsomes/21.5 μl of translation reaction, 1 μl of canine pancreas microsomes/21.5 μl of translation reaction). Transla-
tion reactions were centrifuged at 20,000 × g for 15 min. The superna-
tant was removed, and microsomes were resuspended in 5 μl of homog-

enization buffer and 15 μl of Flexi RRL containing 1 mg/ml RNase
(Roche Molecular Biochemicals) and 50 μg/ml GM132 where indicated. Cytosol-containing an ATP-regenerating system (29) was
added where indicated in place of RRL. Reactions were incubated at
30 °C, and aliquots were withdrawn at indicated time points. Micros-
omal pellet was separated from RRL supernatant by centrifugation at
20,000 × g for 15 min. Reducing sample buffer was added to superna-
tant and pellet fractions followed by separation by SDS-PAGE, or
samples were mixed with lysis buffer (see below) for immunoprecipita-
tion and were indicated.

**Pulse-Chase Analysis, Immunoprecipitation, and Endoglycosidase Digestion—** Pulse-chase analysis, immunoprecipitation, and endoglyco-
sidase H digestion was performed as described (24). Peptide N-gly-
canase (PNG) (New England Biolabs) digestion was performed as

**RESULTS**

**Microsomes Derived from Human Astrocytoma Cells Support Proper Insertion, Signal Sequence Cleavage, Glycosylation, and Folding of in Vitro Translated Membrane Glycoproteins—** The HCMV US2 and US11 gene products induce proteasomal
degradation of MHC class I heavy chains. To generate a cell-free
system with which to study US2- and US11-mediated degrada-
tion, microsomes were isolated from U373-MG cells, a human
astrocytoma cell line that supports rapid degradation when transfected with US2 or US11. We first characterized the ability of these microsomes to insert and process glycoproteins in the course of in vitro translation reactions (Fig. 1). The addition of membranes to an in vitro translation reaction of HLA-A2 yielded a polypeptide with a slower mobility than the
product translated in the absence of membranes (Fig. 1A, com-
pare lanes 1 and 3). This product was sensitive to digestion
with endoglycosidase H (EH), indicating the presence of an
N-linked glycan (Fig. 1A, lane 2). The faster mobility of the
EH-treated microsomal species relative to the HLA-A2 polypeptide translated in the absence of microsomes indicates that the signal sequence had been cleaved from the microsomal
HLA-A2. This HLA-A2 polypeptide was identical in molecular
size to HLA-A2 translated into canine pancreas microsomes
(Fig. 7). To ascertain proper assembly of HLA-A2 molecules in
astrocytoma microsomes, HLA-A2 was co-translated with
mRNA that encodes the light chain, β2m (Fig. 1B). Immunopre-
cipitation with a rabbit polyclonal antiserum that recog-
nizes unfolded MHC class I heavy chains, or with W6/32, a mouse
monoclonal antibody that specifically recognizes properly
folded MHC class I molecules in association with β2m (Fig. 1B,
lanes 2 and 3) showed that proper assembly of translated
HLA-A2 and β2m readily occurred. Therefore folded and un-
folded species can be immunoprecipitated from the translation
reactions, indicating that these microsomes fully support pro-
tein folding.

**Microsomes Derived from Human Astrocytoma Cells Support US2- and US11-mediated Degradation of Class I Molecules—** We next examined the ability of the membranes to in-
duce US2/US11-mediated degradation of MHC class I heavy
chains. HLA-A2 was translated into microsomes derived from
control, US2-expressing, or US11-expressing astrocytoma cells
(Fig. 2A). RNase was used, and after 45 min to terminate ongoing
translation and reactions were sampled at 0, 30, and 90
min after addition of RNase. Microsomal fractions from each
time point were isolated by centrifugation and analyzed di-
rectly by SDS-PAGE. The amount of the HLA-A2 polypeptide
recovered decreased over time in the US2 and US11 micro-
somes (Fig. 2A, lanes 5–12), whereas it was stable in control
microsomes over 90 min (Fig. 2A, lanes 1–4). Amounts of
HLA-A2 were quantitated and plotted as the percentage remaining relative to time 0. Approximately 60% of HLA-A2 molecules were degraded in US2 and US11 microsomes after 90 min, with a more rapid decline in HLA-A2 levels in US2 microsomes at earlier time points. Additionally, less HLA-A2 was visible in US2 microsomes at time 0 relative to control and US11 microsomes (Fig. 2A, compare lanes 1, 5, and 9). These results have been observed consistently in three experiments and indicate that US2-mediated degradation of HLA-A2 was active during the translation period. US2 targets HLA-A2 for degradation at an earlier step during HLA-A2 maturation than US11.

Next, we modified the assay by performing translations in the presence of microsomes, followed by isolation of the microsomes by centrifugation, removal of the translation supernatant, and resuspension of microsomes in a fresh batch of cytosol containing RNase to terminate translation and start the chase. HLA-A2 was translated into control or US2 microsomes, and CD4 was co-translated to serve as a control (Fig. 2B). CD4 is a protein of a size comparable with that of MHC class I products, and likewise a member of the Ig superfamily, yet not a substrate for US2 or US11. After translation for 45 min at 30°C, the microsomes were pelleted by centrifugation and resuspended in a small volume of homogenization buffer. RRL containing an ATP-regenerating system and RNase was added to resuspended microsomes as chase cytosol. The mixture was then incubated at 30°C and sampled at 0, 30, and 60 min after the addition of cytosol. The membrane fractions were analyzed directly by SDS-PAGE (Fig. 2B). Whereas the amount of radiolabeled HLA-A2 polypeptide present in the US2 microsomes decreased over the chase period as compared with the control microsomes, CD4 was equally stable over 60 min in US2 and control microsomes (Fig. 2B, lanes 1–3 and 7–9). No significant loss of HLA-A2 or CD4 occurred in control microsomes (Fig. 2B, lanes 1–6). Therefore, US2 specifically destabilized HLA-A2. US2-11-mediated degradation is sensitive to agents that alter redox potential (21). Similar to what is observed in intact cells, no dislocation of HLA-A2 was observed from US2 microsomes when microsomes were resuspended in RRL containing 10 mM N-ethylmaleimide (NEM), to alkylate free sulfhydryls (Fig. 2B, lanes 4–6 and 10–12). The addition of 1 mM NEM or another oxidant, diamide, blocked dislocation by more than 50% in RRL as opposed to 100% in intact cells. The lower sensitivity to NEM and diamide in the in vitro reactions may be attributable to the high (100–200 mg/ml) protein concentration of RRL.

Proteasome Inhibition Reveals Accumulation of Soluble Heavy Chain Intermediates Released from US2 Microsomes—When US2-expressing cells are treated with proteasome inhibitors, deglycosylated MHC class I heavy chains accumulate in the cytosol (3). To determine whether the same intermediates can be observed in vitro, the dislocation assay was performed with proteasome inhibitors added to the RRL chase mix. Membrane-associated and soluble class I products were analyzed separately (Fig. 3). When the supernatant fraction was analyzed, two distinct polypeptide species were observed in inhibitor-treated reactions, but at later chase points only (Fig. 3A, lane 8). These species were detected in incubations only in the presence of the proteasome inhibitor, MG132, and when US2 was present in the microsomes. Identical species were observed in supernatants recovered from US11-containing microsomes (Fig. 6A, lanes 9 and 12) but not from control microsomes. Other specificity controls confirm that these intermediates were a product of functional US2/US11-mediated degradation only (see below). The loss of HLA-A2 from microsomes was unaffected by the inclusion of MG132 in the chase cytosol (Fig. 3A, lanes 1–4). The amount of soluble intermediates that can be recovered was always less than the amount lost from the microsomes, indicating incomplete inhibition of proteasome activity or the involvement of other proteases present in rabbit reticulocyte lysate. Release of glycosylated CD4 into the supernatants was not observed in the presence of US2 (Fig. 3A, lanes 5–8). Immunoblotting of membrane and supernatant fractions for Sec61β, an integral ER protein, shows that supernatants did not contain ER-membrane proteins, and therefore the release of HC into the supernatant cannot be attributed to a failure to retrieve small microsomal vesicles (see below; Fig. 5A).

To establish the identity of the class I-derived polypeptides and to verify that they represent the deglycosylated species,
immunoprecipitations were performed and analyzed by both SDS-PAGE and IEF (Fig. 3B). The polypeptides found in the supernatants of the in vitro reactions in the presence of MG132 (Fig. 3B, lane 6) were immunoprecipitable with anti-heavy chain antiserum and co-migrate with HLA-A2 recovered from US2 cells treated with MG132 (Fig. 3B, lane 2). Furthermore, the mobility of the faster-migrating species was identical to the mobility of HLA-A2 treated with PNG both on SDS-PAGE and on IEF gels (Fig. 3B, lanes 4 and 8).

The heavy chain intermediate that accumulates in US2- and US11-expressing cells in the presence of proteasome inhibitors is generated by the removal of the N-linked glycan by an N-glycanase and therefore has a faster mobility on SDS-PAGE (2, 3). Deglycosylated heavy chains acquire an additional negative charge, because of the conversion of asparagine to aspartic acid upon hydrolysis of the glycosamide linkage by N-glycanase, and therefore have a distinct mobility on IEF gels (3). This identification was confirmed by digestions of immunoprecipitates with EH and recombinant PNG (Fig. 3B, IEF gel). The latter is particularly relevant, as the time-dependent conversion of glycosylated HLA-A2 in the in vitro reactions to the more acidic species identifiable by IEF paralleled that seen in US2 cells (except that less of the material was recovered from the in vitro chase supernatants (lane 6) than from intact cells (lane 2)) (Fig. 3B). Thus, biochemical analysis confirmed that the intermediates generated in this system were identical to the HLA-A2 intermediates produced in intact cells. In the absence of a source of the reportedly cytosolic mammalian N-glycanase that could be added to the in vitro system described here, the role of N-glycanase in the reaction remains to be explored.

**In Vitro Degradation of MHC Class I Molecules by US2 Is Allele-specific**—For intact cells, the recognition of heavy chains by US2 and US11 is to a large extent specific for particular MHC locus products. To establish the specificity of the in vitro reactions, degradation assays were performed in US2 microsomes with another MHC class I allele, HLA-C. Unlike HLA-A2, HLA-C was stable in US2 microsomes and no intermediates were detected in the US11-expressing cells. This suggests that US2 is allele-specific in its degradation of MHC class I molecules.
US2 catalyze the degradation of heavy chains? If so, this would facilitate the analysis of US2 and US11 mutants by translation. Detection of radiolabeled viral protein would assist in future identification of interacting proteins in the system.

We translated HLA-A2 into control microsomes, divided the translation mixture in half, and added either wild type US2 mRNA or US2–186 mRNA, which lacks the putative cytosolic domain. Translation was continued for 30 min, after which the assay was continued as above with membrane and supernatant fraction sampled at the indicated times. HLA-A2 was unstable in the presence of co-translated US2 but not US2–186 (Fig. 5A, lanes 1–6), indicating that the cytosolic portion of US2 is critical for inducing heavy chain degradation. Additionally, deglycosylated HC intermediates appeared in the supernatants of reactions that contained wild type US2 but not US2–186 (Fig. 5A, lanes 7–12). A portion of each reaction was immunoblotted for Sec61β (Fig. 5A). The Sec61β polypeptide was observed only in pellet fractions, indicating that the appearance of HLA-A2 in the supernatant fractions was not because of an inability to retrieve membrane proteins. The luminal domain of US2 is sufficient for interaction with HLA-A2 (33), and therefore the lack of dislocation by US2–186 is not attributable to an inability to bind to HLA-A2 molecules. Pulse-chase analysis of proteasome-inhibitor treated US2–186 astrocytoma transfectants confirmed the inactivity of this mutant also in intact cells, as no conversion of HC from glycosylated to deglycosylated species was observed (Fig. 5B, lanes 1 and 2). A corresponding set of experiments was done with US11. When wild type US11 or US11–201, which lacks the putative cytosolic domain, were translated into microsomes which contain radiolabeled HLA-A2 and chased (as described for Fig. 5) (Fig. 6A), deglycosylated intermediates accumulated in the supernatant from both US11- and US11–201-containing reactions (Fig. 6A, lanes 7–12). These HLA-A2 intermediates migrated at positions identical to intermediates released from US2-containing microsomes.2 Similarly, MHC class I intermediates found in US11- and US2-expressing cells are biochemically indistinguishable. Significant loss of HLA-A2 was not apparent from microsome pellets (Fig. 6A, lanes 1–6), as US11 degradation is less efficient in vitro and quantities of in vitro translated US11 were less than the amounts present in microsomes derived from US11 transfectants. Again, the ability of both molecules to degrade HLA-A2 was identical to the result observed in intact US11/US11–201 transfectants (Fig. 6B). HC immunoprecipitations indicated that rapid conversion to deglycosylated HC was observed in both wild type US11 and US11–201 transfectants (Fig. 6B, lanes 1–4). The apparent increase of wild type US11/US11–201 over the chase was caused by delayed cleavage of the US11 signal sequence (Fig. 6B, lanes 1–4) (24). Both wild type US11 and US11–201 are stable in intact cells and in microsomes. Therefore the stability of these molecules does not correlate with their ability to degrade MHC class I. We conclude that US2 requires its cytosolic domain for function whereas US11 does not, suggesting that the two polypeptides utilize distinct mechanisms to facilitate degradation of the MHC class I molecules.

Canine Pancreas Microsomes Do Not Support Degradation of HLA-A2 in the Presence of Cotranslated US2—We were able to observe US2/US11-specific destabilization of HLA-A2 in the astrocytoma-derived microsomes. We examined canine pancreas as a more readily available source of microsomes to support US2-mediated degradation. Degradation assays were performed with HLA-A2 and US2 co-translated in control astrocytoma or canine pancreas membranes (Fig. 7). Whereas the HLA-A2 polypeptide was unstable in the presence of co-

![Image](http://www.jbc.org/)

**Fig. 3.** Deglycosylated intermediates are released from US2 microsomes in the presence of proteasome inhibitors. A, CD4 and HLA-A2 mRNAs were translated into US2 microsomes for 45 min, followed by the degradation assay in the presence (lanes 3, 4, 7, and 8) or absence (lanes 1, 2, 5, and 6) of 50 μM MG132. Reactions were sampled at 0 and 60 min. Microsomes (US2+ ms. pellet) (lanes 1–4) and supernatant (lanes 5–8) were separated by centrifugation and analyzed by SDS-PAGE (12.5%). B, to compare intermediates generated in vitro with intermediates found in intact cells, US2 cells preincubated with 50 μM MG132 for 60 min were metabolically labeled with [35S]methionine for 5 min and chased up to 20 min. MHC class I molecules were recovered from cell lysates with anti-HC serum (lanes 1–4).HC immunoprecipitations were digested with EH (lane 3) or PNG (lane 4) prior to analysis. In vitro degradation reactions were performed in US2 microsomes (as described above, with HLA-A2 mRNA, chased in the presence of MG132), and heavy chains were recovered from the microsomal fraction of the reactions (M) at time 0 (lanes 5, 7, and 8) or supernatant fraction (S) recovered after 60 min (lane 6) with anti-HC serum. Microsomal HC immunoprecipitated were digested with EH (lane 7) or PNG (lane 8). All samples were analyzed by SDS-PAGE (10%) and one-dimensional IEF. +CHO and –CHO refer to the presence or absence of an N-linked glycan, respectively; * indicates the more acidic species generated by N-glycanase and PNG digestion.
transferred US2 in the astrocytoma microsomes (Fig. 7, lanes 1–3), we were unable to observe destabilization of HLA-A2 in the canine microsomes (Fig. 7, lanes 4–6). No deglycosylated species were released from the canine microsomes in the presence of US2. One possible explanation would be the inability of preexisting canine /H252 to support assembly of HLA-A2. However, addition of human /H252mRNA to translation reactions did not enable HLA-A2 degradation from canine membranes even though complete assembly of the HLA-A2 molecules was readily demonstrable.

To examine whether canine-derived membranes were competent to dislocate another ER-resident protein susceptible to degradation, we performed in vitro assays with the CD4 and Vpu polypeptides. Vpu is an accessory protein encoded by HIV that causes ER-resident CD4 to be destroyed by the proteasome in a manner similar to MHC class I by US2, although many of the details of Vpu- and US2-US11-mediated degradation remain to be worked out (34, 35). CD4 was translated into control or canine pancreas membranes and mRNA encoding Vpu or a mutant of Vpu lacking the ability to catalyze CD4 degradation (23) was added (Fig. 8). CD4 was destabilized by wild type Vpu in both types of microsomes (Fig. 8, lanes 1–3 and 7–9), although the destabilization was more pronounced in the human microsomes. However, CD4 was stable in the presence of mutant Vpu in both sets of microsomes, indicating that the instability of CD4 was not the result of a nonspecific property of the membranes or the presence of an additional in vitro translated polypeptide (Fig. 8, lanes 4–6 and 10–12). Degradation of CD4
did not result in the accumulation of soluble intermediates in this system, consistent with what has been observed in vitro and in intact cells (14, 36). We conclude that the inability of canine microsomes to support US2-mediated degradation is specific to US2. This observation points to the existence of multiple mechanisms for recognition and/or dislocation of type I membrane proteins, with a species-specific component to the host membrane protein involved.

Degradation of Heavy Chains in Vitro Is Dependent on Cytosol—To examine the requirements for in vitro degradation,
HCMV-mediated Degradation of MHC Class I Molecules in Vitro

Fig. 9. Degradation of heavy chains in vitro is dependent on cytosol. HLA-A2 mRNA was translated into US2 microsomes for 45 min. The degradation assay was performed with homogenization buffer (lanes 1, 2, 7, and 9), RRL (lanes 3, 4, 9, and 10), or cow liver cytosol (lanes 5, 6, 11, and 12) as chase cytosol. (Buffer and cow liver cytosols had an ATP-regenerating system added; all included RNase and MG132). Reactions were sampled at 0 and 60 min, and pellet and supernatant fractions were analyzed by SDS-PAGE (10%). Lanes 1–6 were exposed for 1 day, and lanes 7–12 were exposed for 4 days.

Polypeptides were translated into US2 microsomes and these microsomes were then further incubated in either RRL, cow liver cytosol with an ATP-regenerating system, or homogenization buffer with an ATP-regenerating system. Cytosol is required to induce the loss of heavy chains from the microsomes as well as the appearance of the deglycosylated intermediate in the cytosolic supernatant (Fig. 9). Both rabbit and cow liver cytosol supported heavy chain degradation from US2 microsomes (Fig. 9, lanes 3–6). Cytosol prepared from astrocytoma cells also supported degradation and formation of the deglycosylated intermediate (data not shown). No disappearance of HLA-A2 was seen in US2 microsomes chased in buffer (+ATP regenerating system) (Fig. 9, lanes 1 and 2). Therefore, although species-specific membrane factors may be required to interact with US2 and US11 in the membrane, the cytosolic factors required for dislocation are less species-specific. RRL was more effective than cow liver cytosol in extraction of HLA-A2 from membranes but equal amounts of intermediate were observed in the supernatant (Fig. 9, lanes 10 and 12). Either proteasome inhibition was more effective in the less concentrated cow liver cytosol, or RRL contained proteases not inhibited by proteasome inhibitors, that also destroy HLA-A2.

DISCUSSION

Here we describe a cell-free system that duplicates the ER-to-cytosol dislocation and degradation of MHC class I molecules triggered by the HCMV US2 and US11 proteins in a specific manner. We use this in vitro system to dissect the dislocation process. First, we demonstrate that membranes prepared from a human cell line perform comparably to canine microsomal membranes in the insertion and processing of glycoproteins. Next, we show that class I heavy chains are unstable in astrocytoma-derived membranes that contain US2 or US11. The half-life of heavy chains in US2 and US11 microsomes is ~40–60 min compared with less than 5 min in intact cells. This is comparable with the delayed kinetics for the degradation of CD4 by Vpu in microsomes (14). For the Vpu-CD4 co-translational degradation, the degradation in vitro occurred three times more slowly than in intact cells. The US2-mediated degradation in vitro is ~8–10 times slower than in intact cells. The relative amounts of US2 present in microsomes prepared from US2 cells is high, as the cell line overexpresses US2, and therefore a shortage of US2 is unlikely to be the reason for the delay. Additionally, supplementation of US2 microsomes with additional US2 by in vitro translation does not enhance degradation. Many factors may explain the reduced rate of degradation in the in vitro system. A specialized subcompartment of the ER in which US2 rapidly triggers dislocation may be disrupted in the microsomes used. The microsomal vesicles most proficient in import of in vitro translated polyepitopes may not support efficient dislocation. Local concentrations or assembly of membrane or cytosolic factors that drive extraction may be limiting. Although energy in the form of ATP is required, it remains to be established that ATP is in fact the high energy intermediate directly powering the reaction and therefore metabolic steps that may occur in vitro may be rate-limiting.

Proteasome inhibition permits the accumulation of a breakdown intermediate that is identical to the breakdown intermediate generated in intact US2/US11 cells. It is sufficient to treat the cytosol with proteasome inhibitors to observe this effect in vitro. The appearance of glycosylated and non-glycosylated heavy chains released from the microsomes suggests that fully glycosylated material is exported through the ER membrane and that this material is released from the membrane prior to exposure to N-glycanase. This observation required the use of the in vitro system described here. In US2/US11 cells, only deglycosylated material is detected in cytosolic fractions. N-Glycanase may be significantly more active in intact cells than in RRL. Alternatively, glycosylated material that has exited may not be immediately accessible to N-glycanase and the steps that render it accessible may be less efficient in RRL. The molecular identity of the N-glycanase involved in this reaction in mammalian cells remains to be established.

The ER-luminal portion of US2 contains an immunoglobulin-like fold that mediates the interaction with folded MHC class I molecules but is not itself sufficient to induce degradation. Therefore elements in the transmembrane and cytoplasmic tail regions of US2 must be important for its function (37). Alignment of the US11 sequence with US2 suggests that US11 contains a similar Ig-like fold, which is also likely to dictate association with MHC class I (37). Surprisingly, deletion of the cytosolic tail ablated US2 function but did not ablate US11 function, a result verified both in vitro and in intact cells. Therefore, the two molecules may use different machinery to target heavy chains, or may depend to a different degree on regions of the heavy chain cytosolic tail to stimulate extraction. Despite the proposed Ig fold for both molecules, there is only 25% amino acid similarity between US2 and US11, very little of which maps to the transmembrane and tail regions. A difference in mechanism may also explain different kinetics of degradation between US2 and US11 in vitro.

The ability of newly translated US2 to target class I molecules for degradation further strengthens the hypothesis that US2 recruits, rather than induces, factors that trigger dislocation. The mammalian ER thus contains all of the factors required for US2 to function. Several studies in yeast show that the degradation process is assisted by the induction of an unfolded protein response (38, 39). Up-regulation of chaperones during an unfolded protein response is thought to assist protein folding and/or facilitate recognition of misfolded proteins for dislocation. The mechanism used by US2/US11 may bypass this requirement by recognizing the target soon after synthesis. An important distinction between US2/US11–mediated degradation and the degradation of misfolded proteins is the difference in kinetics; class I molecules are rapidly degraded in US2/US11-expressing cells, whereas misfolded proteins may be retained in the ER for hours before being recognized and disposed of. Additionally, the ability of newly translated US2 to target pre-existing heavy chains suggests that US2 recruits factors to stimulate dislocation rather than triggering dislocation of heavy chains while they are still in the Sec61 pore.

The difference between human astrocytoma microsomes and canine pancreas microsomes to enable US2/US11 degradation...
indicates that membrane-associated factors play a role in steps subsequent to recognition of class I heavy chains. Whether this is the result of a species-specific or tissue-specific factor is unclear at present. Degradation of HLA molecules by vaccinia virus-expressed US2/US11 in a porcine endothelial cell line has been observed (20). Vpu activity may not be as dependent on membrane components because it directly interacts with a component of an ubiquitin-protein isopeptide ligase complex, β-TRCP, which is proposed to recruit Skp1 and direct ubiquitination of CD4 (40). This model does not address the mechanism by which CD4 is physically extracted from the ER, but it is notable that no cytosolic intermediates of CD4 are detected in vitro or in cells in the presence of proteasome inhibitors (14, 36). The interactions between translocating heavy chains or CD4 and the Sec61 complex need to be examined. The soluble heavy chain intermediates generated in vitro are found in a compartment in which no Sec61βγδ is detected (Fig. 5A). It appears that fully glycosylated material is released from the microsomes. Therefore, a fraction of the glycosylated membrane-associated heavy chains in US2 microsomes may be associated with translocation channels. The delayed kinetics of dislocation in this system and the ability to manipulate heavy chains after translation and before dislocation may allow us to trap molecules in association with these channels in future experiments.

Another observation from this work addresses the potential heterogeneity of the ER. Canine pancreas is often used as a convenient and reliable source of microsomes that will support all manner of ER-associated reactions. Generally, the results obtained by in vitro translation using canine pancreas have compared well with results obtained in living cells, where relevant. However, the exocrine portion of the pancreas is a tissue that may well display highly tissue-specific specializations and limitations. Not necessarily all pancreas is a tissue that may well display highly tissue-specific specializations and limitations. Not necessarily all

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