Ubiquitinylation of the Cytosolic Domain of a Type I Membrane Protein Is Not Required to Initiate Its Dislocation from the Endoplasmic Reticulum

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Human cytomegalovirus US2 and US11 target newly synthesized class I major histocompatibility complex (MHC) heavy chains for rapid degradation by the proteasome through a process termed dislocation. The presence of US2 induces the formation of class I MHC heavy chain conjugates of increased molecular weight that are recognized by a conformation-specific monoclonal antibody, W6/32, suggesting that these class I MHC molecules retain their proper tertiary structure. These conjugates are properly folded glycosylated heavy chains modified by attachment of an estimated one, two, and three ubiquitin molecules. The folded ubiquitinated class I MHC heavy chains are not observed in control cells or in cells transfected with US11, suggesting that US2 targets class I MHC heavy chains for dislocation in a manner distinct from that used by US11. This is further supported by the fact that US2 and US11 show different requirements in terms of the conformation of the heavy chain molecule. Although ubiquitin conjugation may occur on the cytosolic tail of the class I MHC molecule, replacement of lysines in the cytosolic tail of heavy chains with arginine does not prevent their degradation by US2. In an in vitro system that recapitulates US2-mediated dislocation, heavy chains that lack these lysines still occur in an ubiquitin-modified form, but in the soluble (cytoplasmic) fraction. Such ubiquitin conjugation can only occur on the class I MHC luminal domain and is likely to take place once class I MHC heavy chains have been discharged from the endoplasmic reticulum. We conclude that ubiquitinylation of class I MHC heavy chain is not required during the initial step of the US2-mediated dislocation reaction.

Glycoproteins undergo quality control in the endoplasmic reticulum (ER)† before progressing along the secretory path-

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§ The on-line version of this article (available at http://www.jbc.org) contains a supplemental Fig. 1A showing a darker exposure of the autoradiogram shown in Fig. 5A (A), a lighter exposure of autoradiogram shown in Fig. 6 (C).

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2 The abbreviations used are: ER, endoplasmic reticulum; MHC, major histocompatibility complex; HC, heavy chain; HA, hemagglutinin; MG132, carboxybenzyl-leucyl-leucyl-leucinal; ZL3VS, carboxybenzyl-leucyl-leucyl-lysylvinylsulfone; RRL, rabbit reticulocyte lysate; NEM, N-ethylmaleimide; IP, immunoprecipitates; Ub, ubiquitin; Vpu, viral protein u; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase.
in See61p compromise dislocation in yeast (9–11), and dislocation substrates such as class I MHC heavy chains and cystic fibrosis transmembrane regulator have been detected in association with See61 in mammalian cells (5, 12). The ubiquitin system is involved in this process. Most ER degradation substrates are modified by the attachment of ubiquitin. Ubiquitin-conjugating enzymes are required for the dislocation of many ER proteins (13, 14). The ODC45-NPL4-UFD1 complex, which recognizes ubiquitinated proteins, is also required for the dislocation of mutant carboxypeptidase Y, murine class I MHC heavy chain, H-2Kβ, and OLE1 (Δ-9 fatty acid desaturase) from the yeast ER (15–17). The details of recognition, timing, and post-translational modification of dislocation substrates remain to be clarified.

The ubiquitin system plays a role in the extraction of class I MHC heavy chains in US11-expressing cells. Polyubiquitinated heavy chains have been detected in US11-expressing cells (18), and ubiquitination is required for the dislocation of heavy chains in US11 cells (19, 20). Polyubiquitinated heavy chains are also present in US2-expressing cells (18).2 Here we show that US2 triggers the ubiquitination of apparently folded class I MHC molecules while still in the ER, but in a manner distinct from that required for US11. These modified class I MHC heavy chain intermediates then disappear from the ER. Although ubiquitin can potentially be added to the lysine residues in the cytosolic tail of class I MHC molecules, ubiquitin conjugation within this domain is not required for dislocation and degradation of the class I MHC molecules.

MATERIALS AND METHODS

DNA Constructs and Cell Lines—Class I MHC heavy chains (HLA-A2) containing an N-terminal influenza hemagglutinin (HA) epitope tag (HA-A2), and the HA-A2 molecule in which the lysine residues at positions 335, 340, and 364 were replaced with arginine (HA-A2K-R) have been described (18, 21). HLA-A2 containing an N-terminal HA tag in which the cysteine residues at positions 227 and 283 within the α3 domain of the HLA-A2 molecule were replaced with serine residues (HA-A2C227S,C283S) was generated by site-directed mutagenesis (QuickChange mutagenesis kit, Stratagene, La Jolla, CA). The CD4 construct has been described (22). The Vpu construct was kindly provided by Klaus Strebel. The cell lines used were wild type (U373 cells), US2 transfectants (US2 cells), or US11 transfectants (US11 cells) of U373-MG human astrocytoma cells and were maintained as described (26). U373 cells that do not express US2 or US11 do not support class I MHC degradation and are therefore used and referred to as control cells. HA-A2, HA-A2K-R, and HA-A2C227S,C283S constructs were transduced into U373, US2, and US11 cells using the pLH CX and pLN CX retrovector vectors (Clontech).

Antibodies and Reagents—Rabbit anti-class I MHC heavy chain serum (HC) recognizes free heavy chains (24), and the monoclonal antibody W6/32 recognizes assembled trimeric class I MHC complexes (25). The anti-US2 polyclonal antiserum has been described (26). The anti-ubiquitin monoclonal antibody was obtained from Zymed Laboratories Inc. (San Francisco, CA). The monoclonal antibody 12CA5, which recognizes the influenza HA epitope, was purified from tissue culture fluids and preincubated in methionine-/cysteine-free Dulbecco’s modified Eagle’s medium with or without proteasome inhibitor. The proteasome inhibitor ZL VS (50 μM) was added where indicated. Metabolic labeling, immunoprecipitation, and immunoreplication were performed as described (8). SDS-PAGE and fluorography were performed as described (31).

In Vitro Transcription/Translation—In vitro transcription was performed as described (32) on 5 μg of HLA-A2, HA-A2, HA-A2K-R cDNAs cloned into pCDNA3.1 (Invitrogen). In vitro translations were performed for 45 min at 30 °C in a reaction mixture containing 17.5 μl of Flexi rabbit reticulocyte lysate (RLR) (Promega, Madison, WI), 0.8 μl of KCl (2.5 M), 0.5 μl of amino acid mixture minus methionine (1 mm, Promega), 2.5 μl of [35S]methionine (10 μCi/ml, translation grade; PerkinElmer Life Sciences), and 0.5 μl of human astroctomy (U373-MG) microsomal membranes and not supplemented with diithiothreitol. Microsomes were prepared from U373 cells and US2 cells and used in the in vitro dislocation assay. In vitro dislocation assays were performed with human astrocytoma microsomes as described (26). Where indicated, reactions were incubated in cow liver cytosol in place of rabbit reticulocyte lysate.

RESULTS

US2 and US11 Differ in Their Ability to Target Partially Folded Class I MHC Molecules for Degradation—We had indirect evidence suggesting that US2 and US11 target newly synthesized class I MHC heavy chains for dislocation and proteasomal degradation by distinct strategies. This difference may have its origin either in the initial stages of class I MHC recognition or at later steps during the dislocation and degradation processes. We first addressed whether US2 and US11 target specific conformational states of class I MHC molecules. We therefore examined whether US2 and US11 can target a partially folded class I MHC molecule for destruction. We generated a retroviral vector encoding a partially folded HLA-A2 molecule by eliminating the disulfide bridge within the α3 domain of the class I MHC heavy chain. This disulfide bridge helps maintain the proper fold of class I MHC molecules. Its elimination should therefore yield a class I MHC molecule with a more flexible conformation than that of wild type. We replaced the two cysteine residues at positions 227 and 283 with serine residues (A2C227S,C283S). An N-terminal HA-tagged version of this molecule, HA-A2C227S,C283S, was then transduced using a retroviral vector into control (U373), US2, and US11 cells. The N-terminal HA epitope tag allows us to distinguish the mutant from the endogenous heavy chains. The inclusion of the HA tag in the class I MHC heavy chains prevents properly folded (W6/32-reactive) class I MHC molecules from reaching the cell surface for reasons as of yet unknown. However, the HA-tagged class I MHC heavy chains continue to be targeted for proteasomal degradation in an US2- and US11-dependent manner (see Figs. 1 and 5B) (18). Cells stably expressing HA-A2 and HA-A2C227S,C283S were metabolically labeled for 15 min in the absence of proteasome inhibitors and chased for up to 30 min (Fig. 1). The HA-A2 and HA-A2C227S,C283S molecules were recovered with anti-HA antibody, and the immunoprecipitates were analyzed by SDS-PAGE (Fig. 1). In control cells, both the HA-A2 and the HA-A2C227S,C283S molecules are stable over the course of the chase (Fig. 1, lanes 1 and 2 and lanes 3 and 4). The HA-A2 molecule is unstable in the presence of both US2 and US11 (Fig. 1, lanes 5 and 6) (18), respectively. Strikingly, HA-A2C227S,C283S molecules are unstable in US11-expressing cells but are completely stable in US2-expressing cells (Fig. 1, compare lanes 9 and 10 and lanes 7 and 8). This suggests that class I MHC molecules must acquire a properly folded conformation to be targeted for degradation by US2. On the other hand, US11-mediated degradation is not dependent on the tertiary structure of class I MHC molecules. Moreover, US2 and US11 have markedly different length requirements for the cytoplasmic tail of class I MHC molecules to mediate dislocation.3 These results suggest the involvement of different pathways utilized by US2 and US11 to target class I MHC molecules for degradation.

Class I MHC Conjugates Are Stabilized by N-ethylmaleimide (NEM) in US2-expressing Cells—A protein covalently tagged...
with ubiquitin is usually degraded in the cytoplasm by the proteasome. In the presence of US11, a small fraction of class I MHC heavy chains is polyubiquitinated (18), and a functional ubiquitination system is necessary for the dislocation of class I MHC heavy chains (19, 20). These results suggest that ubiquitin plays a role in US11-mediated dislocation of class I MHC heavy chains. The different conformational requirements for class I MHC degradation mediated by US2 and that mediated by US11 (see above) prompted us to investigate whether US2 and US11 also induce the formation of ubiquitin-modified class I MHC heavy chains differently. We examined whether in the presence of US2 the class I MHC heavy chains are modified with ubiquitin. The addition and removal of ubiquitin, by ubiquitin ligases and ubiquitin-specific isopeptidases, respectively, are reactions that depend on a cysteine residue in their active sites. To block these activities, the alkylating agent NEM was included in a pulse-chase experiment. US2-expressing astrocytoma cells were pulsed for 15 min with $^{35}$S-methionine in the presence of proteasome inhibitor (ZL3VS) and chased for 30 min. HA-A2 and HA-A2 C227S,C283S molecules were recovered with anti-HA antibody, and the immunoprecipitates were analyzed by SDS-PAGE (12.5%).

Control, US2-, and US11-expressing U373 cells that express HA-A2 and HA-A2 C227S,C283S were metabolically labeled for 15 min in the absence of proteasome inhibitors and chased for up to 30 min. HA-A2 and HA-A2 C227S,C283S molecules were recovered with anti-HA antibody, and the immunoprecipitates were analyzed by SDS-PAGE (12.5%).

A diagnostic trait of US2-mediated degradation of class I MHC heavy chains is the removal of the single N-linked glycan on class I MHC heavy chains (HC + CHO) by a cytoplasmic N-glycanase. This deglycosylated intermediate (HC − CHO) is seen only in the presence of proteasome inhibitors (5). In anti-heavy chain immunoprecipitates from US2-expressing cells treated with ZL3VS, the cytosolic deglycosylated heavy chains (HC − CHO) are present at both the 0- and 30-min chase period (Fig. 2, lanes 1 and 2). In US2-expressing cells treated with NEM at the onset of the chase, dislocation of the unfolded class I MHC heavy chains is inhibited, and no conversion of the heavy chains to deglycosylated species occurs (Fig. 2, lane 3) (33). Because until recently we had assumed that dislocation would require full dissociation and unfolding of the class I MHC substrate, we had not examined in any detail the possible modification of the folded class I MHC molecule. In W6/32 immunoprecipitations from US2-expressing cells, we now detect a series of polypeptides with a slower mobility than the glycosylated heavy chain backbone (Fig. 2, lanes 4–6, asterisk). These conjugates are recovered exclusively with W6/32 (Fig. 2, lanes 4 and 6). In proteasome inhibitor-treated cells, the conjugates disappear over the chase period but are stabilized when cells are chased in the presence of NEM (Fig. 2, compare lanes 5 and 6). The composition of the conjugates visualized in the presence of NEM appears to be somewhat different from those seen in the absence of NEM. Although the underlying cause of this difference was not identified, differential attack by ubiquitin-specific proteases could contribute. We have so far been unable to explore this issue further.

W6/32 recognizes a conformation-dependent epitope on the luminal domain of class I MHC molecules. Therefore, we considered it unlikely that the slower migrating class I MHC conjugates are modified on the luminal domain, but rather reflect modification of the cytoplasmic tail. As we will show below (see Fig. 5), the appearance of these modified class I MHC heavy chain species requires at least in vitro the presence of the lysine residues in the cytoplasmic tail. This suggests that the slower migrating species may be ubiquitinated class I MHC polypeptides.

HLA-A2 Molecules Are Mono-, Di-, and Triubiquitinated in the Presence of US2 and Not US11—We identified these higher molecular weight species as modified class I MHC heavy chains by a reimmunoprecipitation experiment, considering that $^{35}$S-methionine cells contain many polypeptides that may co-purify with W6/32-reactive material (Fig. 3A). Control, US2-, and US11-expressing cells were metabolically labeled for 15 min in the presence of proteasome inhibitor (ZL3VS). NEM was added to the cells 1 min prior to lysis. Folded class I MHC heavy chains were recovered with W6/32 (1° IP). Immunoprecipitates were then fully denatured by boiling in 1% SDS and reimmunoprecipitated with anti-heavy chain serum (2° IP) (Fig. 3A, lanes 1–3). The higher molecular weight class I MHC heavy chain conjugates are recognized by the anti-heavy chain serum and are present in samples derived from US2-expressing cells but not in samples from control or US11-expressing US2 cells.
cells (Fig. 3A, compare lane 2 with lanes 1 and 3). Therefore, these higher molecular weight polypeptides correspond to modified class I MHC heavy chains and not to co-precipitating proteins. To examine whether these polypeptides are ubiquitinated heavy chains, a portion of the W6/32 immunoprecipitations was denatured and reimmunoprecipitated with an anti-ubiquitin antibody (2° IP) (Fig. 3A, lanes 4–6). This immunoprecipitation recovered a subset of the polypeptide species seen in the anti- HC immunoprecipitates (Fig. 3A, lane 5). The fact that not all of the higher molecular weight class I MHC conjugates are recognized in this reimmunoprecipitation experiment may be related to the inability of the anti-Ub antibody to recognize all oligoubiquitinylated species equally well. Based on this result, we cannot formally exclude the modification of class I MHC heavy chains with a polypeptide(s) other than ubiquitin. A trace amount of heavy chain polypeptides is observed in anti-Ub immunoprecipitations from all three cell lines (Fig. 3A, lanes 4–6), presumably due to the relatively high level of unmodified heavy chains present in the initial immunoprecipitates.

The slower migrating heavy chain polypeptides have molecular masses of ~52, 60, and 68 kDa. The molecular mass of ubiquitin is ~8 kDa, and that of class I MHC heavy chain is 43 kDa. The heavy chain conjugates are therefore predicted to be modified with one, two, and three ubiquitins. For heavy chains that contain two or three ubiquitin moieties, attachment at two or three sites, as well as the possible occurrence of di- and tri-Ub chains could generate a large number of different isoforms, each with distinct electrophoretic properties, and thus contribute to the electrophoretic heterogeneity observed. Moreover, the U373 cell line expresses multiple HLA-A, -B, and -C locus products, further contributing to heterogeneity.

To verify that ubiquitin is indeed conjugated to class I MHC heavy chains in a US2-dependent manner, we generated US2- and US11-expressing cells transfected with an influenza HA epitope-tagged ubiquitin (HA-Ub) expression construct. Control, US2HA-Ub, and US11HA-Ub cells were metabolically labeled for 15 min in the presence of proteasome inhibitor (ZL3VS) (Fig. 3B). Folded class I MHC heavy chains were recovered with W6/32 (1° IP). One-third of the W6/32 immunoprecipitates was analyzed directly by SDS-PAGE (Fig. 3B, lanes 1–3). Consistent with the experiment in Figs. 2 and 3A, the slower migrating class I MHC heavy chain conjugates were recovered from US2-expressing cells (Fig. 3B, lane 2) and not from control and US11-expressing cells. The remaining two-thirds of the W6/32 reactive material was fully denatured by boiling in 1% SDS and reimmunoprecipitated with anti-HA antibody.

## Figure 3

**Fig. 3.** Class I MHC heavy chains are ubiquitinated in a US2-dependent manner. As shown in A, control, US2-, and US11-expressing U373 cells were metabolically labeled for 15 min. NEM (5 mM) was added for 1 min prior to the lysis. Properly folded class I MHC heavy chains were recovered from cell lysates by immunoprecipitation with W6/32. W6/32 immunoprecipitates were denatured and reimmunoprecipitated with anti-heavy chain serum or anti-ubiquitin antibody. Samples were analyzed by SDS-PAGE (10%). Molecular mass markers are indicated on the right in kDa. As shown in B, control, US2, and US11 cells that express HA-tagged ubiquitin were metabolically labeled for 15 min in the presence of proteasome inhibitor (ZL3VS) (Fig. 3B). Folded class I MHC heavy chains were recovered with W6/32 (1° IP). One-third of the W6/32 immunoprecipitates was analyzed directly by SDS-PAGE (Fig. 3B, lanes 1–3). Consistent with the experiment in Figs. 2 and 3A, the slower migrating class I MHC heavy chain conjugates were recovered from US2-expressing cells (Fig. 3B, lane 2) and not from control and US11-expressing cells. The remaining two-thirds of the W6/32 reactive material was fully denatured by boiling in 1% SDS and reimmunoprecipitated with anti-HA antibody.
antibody (2° IP) followed by SDS-PAGE (Fig. 3B, lanes 4–6). Only slower migrating class I MHC polypeptides were recovered from the W6/32 immunoprecipitates that originated from US2HA-Ub cells, confirming that properly folded molecules are modified with ubiquitin in an US2-dependent manner. Again, the set of polypeptides recovered in the secondary immunoprecipitation with anti-HA antibody represents a subset of the polypeptides present in the primary immunoprecipitation. No unmodified class I MHC heavy chains were recovered.

We established the identity of the modified heavy chains as ubiquitin conjugates by a third method. We utilized a cell-free in vitro system in which microsomal membranes prepared from U373 astrocytoma cells that express US2 (or US11) support the export and degradation of in vitro translated class I MHC heavy chain mRNA (26). Microsomes derived from U373 cells that do not express US2 do not support class I MHC degradation in vitro and were used as a control in all the experiments involving US2-containing microsomes (data not shown). In the in vitro degradation system, when US2-containing microsomes are added, slower migrating class I MHC heavy chain conjugates are also observed (Fig. 3C). The relative sizes of ubiquitin-modified heavy chains are similar in vitro and in US2 cells.4 To show directly that these species are ubiquitylated, we translated HLA-A2 (class I MHC heavy chain) mRNA into US2-containing microsomes in the presence and absence of purified recombinant HA-Ub (Fig. 3C, lanes 1 and 2). The HA-ubiquitin cannot access the lumen of microsomes when added to the in vitro translation. As a control, CD4 was translated in the presence of Vpu, an accessory protein encoded by human immunodeficiency virus that induces the ubiquitination of CD4 (34) (Fig. 3C, lanes 5 and 6). Microsomal samples were analyzed directly (Fig. 3C, direct load, lanes 1 and 2 and lanes 5 and 6) or immunoprecipitated with anti-HA antibody (Fig. 3C, lanes 3 and 4 and lanes 7 and 8). In the absence of HA-Ub, conjugates are observed for both the HLA-A2 heavy chains and CD4 (Fig. 3C, lanes 1 and 5). In the presence of HA-Ub, conjugates with a slightly slower mobility (asterisk) appeared, slightly offset when compared with their endogenous counterparts. Due to the presence of the HA tag, modification with HA-ubiquitin results in a polypeptide that migrates slightly more slowly than that produced by modification with wild-type ubiquitin (Fig. 3C, lanes 2 and 6). Immunoprecipitation with anti-HA antibody recovered these conjugates in samples that included HA-Ub (Fig. 3C, lanes 4 and 8) but not from control (no HA-Ub added) samples. Unmodified HC and CD4 are present in the anti-HA immunoprecipitates but in relative amounts far less than those seen in the direct loads. Again, their presence may reflect non-specific binding of the unmodified polypeptides to the antibody. Based on the three independent criteria applied here, we conclude that the class I MHC heavy chain conjugates observed in US2-expressing cells and in vitro correspond to ubiquitinated species.

Ubiquitinated Class I MHC Heavy Chains Appear in the Absence of Proteasome Inhibitors—The molecular weight of the ubiquitinated species suggests the addition of one, two, or three ubiquitin molecules to the heavy chain polypeptide (see above). To determine whether these ubiquitinated species observed in US2-expressing cells require inclusion of a proteasome inhibitor, W6/32-reactive material from proteasome inhibitor (ZL3VS)-treated US2-expressing cells was compared with W6/32 immunoprecipitates from untreated cells (Fig. 4). US2 cells treated with and without ZL3VS were metabolically labeled for 10 min and chased for 0 and 25 min. NEM was added 1 min prior to lysis. The properly folded class I MHC molecules were recovered from cell lysates using the monoclonal antibody W6/32. Ubiquitin-conjugated heavy chains are observed in both untreated and ZL3VS-treated cells at the 0-min chase period (Fig. 4, lanes 1 and 3). A significant fraction of these conjugates disappear over the course of the 25-min chase, a result that is indicative of US2-mediated class I MHC degradation (Fig. 4, lanes 1 and 2 and lanes 3 and 4; also Fig. 2). Inclusion of proteasome inhibitor only marginally stabilizes the ubiquitinated heavy chains. Ubiquitination of properly folded class I MHC molecules is therefore not dependent on proteasomal inhibition.

Class I MHC Heavy Chain Molecules Lacking Lysines in the Cytoplasmic Tail Are Targeted for Degradation by US2—To further characterize the site of ubiquitination of the heavy chain molecules, we analyzed class I MHC mutants in which all three cytoplasmic lysines were replaced with arginines (21). These mutants contain an N-terminal HA epitope tag. First we examined the degradation of HA-tagged HLA-A2 (HA-A2) and HA-tagged HLA-A2 in which the three cytoplasmic lysines were replaced with arginines (HA-A2K,R) in our in vitro system (26). mRNA encoding HA-A2 or HA-A2K,R was translated into US2-containing microsomes. After termination of translation, the incubation mixtures were chased in RRL treated with proteasome inhibitor (MG132) (Fig. 5A). We observed slower migrating ubiquitin-modified heavy chain polypeptides in the HA-A2 translation, and if anything, less then 10% of this ubiquitin conjugated material in the HA-A2K,R translations (Fig. 5A, compare lanes 1 and 3, and supplemental Fig. 1A, a darker exposure of Fig. 5A), confirming that ubiquitination must occur on the lysines of the cytoplasmic tail. The loss of class I MHC heavy chains from the microsome pellet at the 60-min chase

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4 M. Furman and H. Ploegh, unpublished observations.
period may be observed on a lighter exposure of the autoradiogram (supplemental Fig. 1B).

Both HA-A2 and HA-A2K-R mRNAs were *in vitro* translated in US2-containing microsomes for 45 min followed by the *in vitro* degradation assay in the presence of MG132. Microsomes (lanes 1–4) and supernatant (sup., lanes 5–8) fractions were analyzed separately by SDS-PAGE (10%). As shown in B, US2 cells stably expressing HA-A2 or HA-A2K-R were metabolically labeled and chased up to 40 min in the absence of proteasome inhibitor. Cells were lysed in 1% SDS to allow recovery of the total population of HA-tagged molecules followed by immunoprecipitation with anti-HA antibody (lanes 1–6) and anti-heavy chain and anti-US2 serum, respectively (lanes 7–12). Immunoprecipitates were analyzed by SDS-PAGE (12.5%).

The stability of HA-A2 and HA-A2K-R in US2-expressing cells was examined by pulse-chase analysis (Fig. 5B). Both HA-A2 and HA-A2K-R were unstable in US2 cells as observed by the decreased recovery of the HA-containing class I MHC molecules over the chase period (Fig. 5B, lanes 1–3 and lanes 4–6). Endogenous heavy chains are also unstable (Fig. 5B, lanes 7–9 and lanes 10–12). We were unable to recover high molecular weight conjugates of the HA-containing class I MHC molecules in living cells.5 Both *in vitro* and *in vivo*, heavy chain molecules whose cytosolic tails are deficient in lysines are targeted for degradation in an US2-dependent fashion. Therefore, these results confirm that ubiquitination of the class I MHC cytoplasmic tail is not required for US2-mediated degradation.

Although ubiquitination of the cytosolic tail of heavy chain is not required, we considered the possibility that ubiquitination of HA-A2K-R may occur in the course of dislocation. *In vitro* degradation assays were performed with a cytosol source that supports the dislocation reaction but is not as concentrated and efficient at extraction as RRL (26). mRNA encoding HA-A2 or HA-A2K-R was translated into US2-containing microsomes. After termination of translation, the incubation mixtures were chased in cow liver cytosol treated with proteasome inhibitor (MG132) (Fig. 6). The loss of heavy chains from the microsome pellet may be observed on a lighter exposure of the autoradiogram (supplemental Fig. 1C). Extraction of the heavy chains from the microsomal membrane and delivery to the supernatant was observed for both HA-A2 and HA-A2K-R (Figs. 5A and 6, lanes 1 and 2). Interestingly, polypeptides with a migration similar to that of the ubiquitinated species in the microsome fraction are observed in the supernatants of both HA-A2 and HA-A2K-R-containing reactions (Fig. 6, lanes 6 and 8). Thus HA-A2K-R is ubiquitinated in the course of dislocation and soluble ubiquitinated (*n* = 1, 2, or 3 Ub) species accumulate outside of the microsomes in a time-dependent manner. It is important to note that the relative intensity of the polypeptides containing 1, 2, or 3 ubiquitin moieties are not the same for the class I MHC heavy chains recovered from the microsomal pellet and from the supernatant. This suggests that ubiquitin modification of the cytoplasmic tail of class I MHC heavy chains in the microsomes and modification of a lumenal lysine residue with ubiquitin once extracted from the ER.

5 J. Loureiro and H. Ploegh, unpublished observations.
DISCUSSION

We demonstrate that US2 induces the ubiquitination of class I MHC heavy chains in the course of degradation. The characterization of these intermediates allows us to construct a more complete model for US2-mediated and -related degradation processes. Also, our results clearly distinguish the pathway used by US2 from that used by US11.

In US2-expressing cells, modified class I MHC heavy chains are observed at the onset of the chase in a pulse-chase experiment (Fig. 2) and are stabilized by inclusion of N-ethylmaleimide (Fig. 2, lanes 4 and 6). We estimate that the modified class I MHC heavy chains contain from one to three molecules of ubiquitin. At present, we cannot distinguish between single and multiple sites of ubiquitin attachment. For wild-type heavy chains, ubiquitination precedes dislocation, and the kinetics with which the ubiquitinated species disappear suggests that they are precursors to the deglycosylated molecules that accumulate outside of the ER (Fig. 2, lanes 1 and 2). The US2-specific modifications are distinct from the polyubiquitination described for US11 (18) as they occur on heavy chains that are recovered with a conformation-specific antibody (W6/32). Further, the pattern of US2-dependent ubiquitin conjugation is distinct from the high molecular weight smear seen for US11-expressing cells (18).

The quantity of ubiquitinated heavy chains observed is not significantly increased by treatment with proteasome inhibitors (Fig. 4), suggesting that ubiquitinated heavy chains are not direct substrates for the proteasome. It is of course possible that further addition of ubiquitin is blocked when the ubiquitination is inhibited, yielding similar overall levels of ubiquitin-conjugated class I MHC heavy chains in cells untreated and treated with proteasome inhibitor. In contrast, the amount of ubiquitinated class I MHC heavy chains in semipermeabilized US11 cells increases from 5 to ~10–20% of total heavy chains in the presence of proteasome inhibitor (18). These differences between the type and amount of ubiquitin-conjugated class I MHC heavy chain intermediates detected in US2- and US11-expressing cells suggest that distinct mechanisms can trigger dislocation. Indeed, US2 and US11 show different structural requirements in targeting class I MHC molecules for degradation (Fig. 1). US2 triggers degradation of properly folded MHC class I heavy chains, whereas US11 can mediate the degradation of class I MHC molecules regardless of their tertiary structure. Furthermore, US2 and US11 require the recognition of different lengths of the cytoplasmic tail of class I MHC in the process of degradation. Combined, our data support a model in which US2 and US11 engage different cellular pathways (different adaptors?) to achieve proteasomal degradation of a common substrate, class I MHC heavy chains. This US2- and US11-mediated ER disposal mechanism provides us with the opportunity to study subtle details of the dislocation reaction not easily tractable in other systems.

Surprisingly, mutation of the lysine residues in the class I MHC cytoplasmic tail to arginine does not prevent degradation by US2. Ubiquitination of the cytosolic tail is therefore not required for US2-mediated dislocation. The same mutant has been examined in US11-expressing cells and is also susceptible to degradation (18). Therefore, if ubiquitination is required, as it is for US11-mediated degradation (19, 20), then this can occur on luminal portions of class I MHC heavy chains, or it occurs on another, non-MHC heavy chain polypeptide that stimulates dislocation in trans. Although ubiquitination of the cytosolic tail is not required for degradation, the complete removal of the class I MHC cytoplasmic tail renders heavy chains resistant to US2- and US11-mediated dislocation (21).

How does the ubiquitination machinery access luminal portions of the target molecule? It is intriguing that we observe ubiquitination of HA-A2 in the supernatants of in vitro degradation reactions (Fig. 6, lane 8), whereas no ubiquitination is observed on membrane-associated molecules (Fig. 6, lanes 3 and 4). The fact that the pattern of ubiquitination is very similar to that of HA-A2 suggests that this type of modification may be a feature of the US2-mediated process. Indeed, if luminal portions of class I MHC molecules remain inaccessible to ubiquitin-conjugating enzymes while in the ER, then a ubiquitin-independent step must initiate dislocation.

Similar mono-, di-, and triubiquitin conjugates have been reported for murine class I MHC molecules in the presence of murine herpesvirus 68-encoded MK3 (35). MK3 contains a RING finger-like plant homeodomain that is required to trigger degradation of murine class I MHC molecules from the ER (35). In contrast to our observations for US2, MK3 does contain the presence of lysines in the cytosolic domain of the heavy chain, yet replacement of the lysines does not abolish ubiquitination (35). US2 does not contain a plant homeodomain, and the cytosolic region of US2 contains only 14 amino acids. This region is critical for US2 function (26). Although itself devoid of E2 or E3 activity, it may recruit E2 and E3 ubiquitin-conjugating enzymes to the site of dislocation.

A model that is consistent with the data presented here suggests the following sequence of events: US2 binds to an epitope on a folded heavy chain molecule (36, 37). The US2-HC complex is recruited to a larger protein complex that mediates accessibility to the cytosol, perhaps by interacting with an aqueous channel. US2 may promote interaction with a subset of ubiquitin-conjugating enzymes in this dislocation complex that adds ubiquitin molecules (n < 3) to accessible lysines on class I MHC heavy chains in the course of US2-mediated dislocation. HA-A2 or HA-A2K-R mRNAs were in vitro translated for 45 min in the presence of US2-containing microsomes followed by the in vitro degradation assay. Microsomes were chased in cow liver cytosol in the presence of proteasome inhibitor (MG132). Microsome (lanes 1–4) and supernatant (lanes 5–8) fractions were analyzed separately by SDS-PAGE (10%). Exposure time of the gel was of several days.

Fig. 6. Class I MHC heavy chains are ubiquitinated on the luminal domain in the course of US2-mediated dislocation. HA-A2 or HA-A2K-R mRNAs were in vitro translated for 45 min in the presence of US2-containing microsomes followed by the in vitro degradation assay. Microsomes were chased in cow liver cytosol in the presence of proteasome inhibitor (MG132). Microsome (lanes 1–4) and supernatant (lanes 5–8) fractions were analyzed separately by SDS-PAGE (10%). Exposure time of the gel was of several days.

[Image 263x579 to 563x738]
the heavy chain molecule. In the case of the HA-A2K-R mutant, these lysines map to the lumenal domain of the heavy chains. For a lumenal portion of the heavy chain to be available for dislocation, the transmembrane segment of the protein may already be extracted from the ER membrane, or the addition of ubiquitin stimulates rapid extraction, as we do not observe membrane-associated, ubiquitin-conjugated HA-A2K-R molecules. In the class I MHC heavy chains, lysine residues in the tail are readily accessible and therefore are targeted for Ub conjugation prior to extraction of the molecules from the membrane. Following the recruitment of the heavy chains to the site of dislocation and their extraction from the membrane, the proteasome destroys them. In the presence of proteasome inhibitors, cytosolic ubiquitin-isopeptidases remove the ubiquitin molecules from the heavy chains.

Conjugation of ubiquitin onto a polypeptide usually results in proteasomal destruction of that polypeptide. However, ubiquitin can play a role in membrane and intracellular trafficking of proteins (38). In these cases, ubiquitin acts as a sorting signal, or for US2, as a dislocation signal, and not as a degradation signal. A further possibility is that the addition of ubiquitin onto a substrate may act as a “barb” and simply prevent the polypeptide from reintegration into the ER membrane after recruitment to the dislocation complex. A degradation substrate modified with ubiquitin may promote recognition by cytosolic factors, possibly the CDC48-NPL4-UFD1 complex, reported to facilitate extraction from the ER membrane (17), or other factors. The failure to detect polyubiquitinated heavy chain intermediates in US2-expressing cells suggests that mono-, di-, and trinubiquitination does not serve as a signal for further ubiquitination because such polyubiquitin conjugates are readily observed in U373 cells transfected with US11 (19) and should therefore have been detected in US2-expressing cells as well.

The interplay of ubiquitin with the dislocation machinery is only now beginning to be elucidated. The US2-mediated class I MHC heavy chain-ubiquitin conjugates may provide an excellent substrate to further explore the role of ubiquitin in the dislocation reaction.

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