The Pathway of US11-dependent Degradation of MHC Class I Heavy Chains Involves a Ubiquitin-conjugated Intermediate

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Abstract. The human cytomegalovirus protein, US11, initiates the destruction of MHC class I heavy chains by targeting them for dislocation from the ER to the cytosol and subsequent degradation by the proteasome. We report the development of a permeabilized cell system that recapitulates US11-dependent degradation of class I heavy chains. We have used this system, in combination with experiments in intact cells, to identify and order intermediates in the US11-dependent degradation pathway. We find that heavy chains are ubiquitinated before they are degraded. Ubiquitination of the cytosolic tail of heavy chain is not required for its dislocation and degradation, suggesting that ubiquitination occurs after at least part of the heavy chain has been dislocated from the ER. Thus, ubiquitination of the heavy chain does not appear to be the signal to start dislocation. Ubiquitinated heavy chains are associated with membrane fractions, suggesting that ubiquitination occurs while the heavy chain is still bound to the ER membrane. Our results support a model in which US11 co-opts the quality control process by which the cell destroys misfolded ER proteins in order to specifically degrade MHC class I heavy chains.

Key words: ubiquitin • US11 • dislocation • endoplasmic reticulum • quality control

MHC class I molecules display intracellularly derived peptides on the surface of almost all nucleated mammalian cells for recognition by the immune system. MHC class I heavy chain, which contains the peptide binding site, is a classical type I transmembrane protein with a large luminal/extracellular domain and a short cytosolic tail. The heavy chain enters the secretory pathway via the ER translocon, a channel whose major component is Sec61. The light chain β_2 microglobulin (β_2m)[1] and the peptide associate with the heavy chain after it has been inserted into the ER membrane, and the complex is then transported, via the Golgi, to the plasma membrane. In humans, the MHC class I heavy chain is a 43-kD protein that contains a single N-linked glycan.

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1. *Abbreviations used in this paper:* β_2m, β_2 microglobulin; Endo H, endoglycosidase H; HA, hemagglutinin; HCMV, human cytomegalovirus; HC, MHC class I heavy chain; IEF, isoelectric focusing; Staph A, fixed *Staphylococcus aureus* bacteria; TfR, transferrin receptor; Ub, ubiquitin; wt, wild-type; ZL_3VS, carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone.

Human cytomegalovirus (HCMV) evades detection by the immune system by targeting MHC class I heavy chains for destruction soon after they have been synthesized. The HCMV proteins responsible for MHC class I heavy chain destruction are US11 and US2 (Jones et al., 1995). Either protein, when stably expressed in human tissue culture cells, causes rapid transport of heavy chains from the ER to the cytosol, in a process called dislocation. The heavy chains are subsequently degraded by the proteasome (Wiertz et al., 1996a,b). US11 and US2 are both small, transmembrane glycoproteins proteins (<30 kD) that localize to the ER. They are 21% identical and 45% similar to each other (Ahn et al., 1996). Whereas US11 seems to be a typical type I membrane protein, the mode of membrane insertion of US2 remains to be determined. It is thought that each protein might affect the degradation of only a subset of the human MHC class I heavy chain alleles (Jones et al., 1995).

Specific inhibitors of the proteasome, such as lactacystin and carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone (ZL_3VS) (Bogyo et al., 1997), stabilize MHC class I heavy chains in cells expressing US11 or US2 (Wiertz et al., 1996a,b). In the presence of these inhibitors, soluble, deglycosylated heavy chains accumulate in the cytosol. Deglycosylation is carried out by an *N*-glycanase activity (Suzuki et al., 1994), which, in removing the glycans, converts the asparagine residue to which they were conjugated to an as-

partic acid. Thus, accumulation of deglycosylated heavy chains can be monitored both as a shift in molecular mass (43 kD to 40 kD, by SDS-PAGE) and as a shift in isoelectric point.

Some properties of US11/US2-dependent heavy chain degradation are also characteristic of ER quality control (Kopito, 1997; Sommer and Wolf, 1997). This process, conserved from yeast to human cells, functions to ensure that only properly folded and assembled proteins exit the ER for the Golgi on their way through the secretory pathway. Misfolded proteins or misassembled complexes are retained in the ER, at least in part because they are bound by ER-resident chaperones such as BiP and calnexin. Accumulated proteins that do not fold or assemble properly are eventually destroyed. Although the proteases responsible were long thought to reside in the ER, the emerging model is that misfolded proteins are actually dislocated from the ER and destroyed in the cytosol by the proteasome. Both US11/US2-dependent degradation of class I heavy chain and degradation of at least some misfolded secretory proteins require ATP and the maintenance of a proper redox potential across the ER membrane (McCracken and Brodsky, 1996; Qu et al., 1996; Tortorella et al., 1998). Furthermore, the membrane channel for dislocation may be the same as for translocation, the Sec61 complex (Wiertz et al., 1996b; Pilon et al., 1997; Plemper et al., 1997).

A major difference between the destruction of class I heavy chain via the US11/US2 pathway and the destruction of other ER proteins is the rate of degradation. The half-life of MHC class I heavy chain in US11- and US2-expressing cells is <3 min (Wiertz et al., 1996a,b), while the estimated half-lives of misfolded proteins range from 15 min to >1 h, depending on the protein examined (for example, see Finger et al., 1993; Yuk and Lodish, 1993; Ward et al., 1995; Biederer et al., 1996; Yu et al., 1997). This difference may simply reflect the role played by US11 or US2 in expediting dislocation of heavy chain from the ER. Once in the cytosol, all dislocated proteins might follow the same path to proteasomal destruction. However, differences in putative degradation intermediates have also been noted. In the quality control process, ubiquitinated degradation intermediates, or requirements for ubiquitin conjugation machinery, have been demonstrated in many cases (for example, see Ward et al., 1995; Hiller et al., 1996; Hampton and Bhakta, 1997; deVirgilio et al., 1998), while deglycosylated degradation substrates have been reported in the case of only three proteins, MHC class I heavy chain expressed in the absence of β_2m, CFTR, and T cell receptor α subunit (Hughes et al., 1997; Huppa and Ploegh, 1997; Yu et al., 1997; Johnston et al., 1998). In contrast, deglycosylated class I heavy chain is readily observed in the US11/US2 pathway, but no ubiquitinated heavy chain intermediates have been detected (Wiertz et al., 1996a,b). Thus, experiments to date leave open the possibility that the pathway by which US11 and US2 destroy class I heavy chain is distinct from the process by which misfolded secretory proteins are degraded.

To address the mechanisms of the HCMV-induced degradation of MHC class I heavy chains, we have focused on the US11-dependent dislocation pathway. We have developed a permeabilized cell system that recapitulates critical aspects of US11-dependent dislocation and degradation of the class I heavy chain that have been observed in intact cells. We have used this system, in combination with experiments carried out in intact cells, to demonstrate the existence of ubiquitinated heavy chain intermediates. We find that ubiquitination of the heavy chain cytosolic tail is not required for its US11-dependent dislocation or degradation. Moreover, ubiquitinated heavy chain is associated with membranes, suggesting that ubiquitination of heavy chain occurs after it has been dislocated from the ER but while it is still bound to the ER membrane. These observations have allowed us to order the basic steps of the pathway of MHC class I heavy chain destruction. They demonstrate that the HCMV pathway for heavy chain destruction is similar in major respects to ER quality control, and strongly support the hypothesis that US11 co-opts the cellular quality control process to destroy MHC class I heavy chains.

Materials and Methods

Cells and Cell Culture

Control and US11-expressing U373-MG astrocytoma cells (Jones et al., 1995) were cultured as described previously (Wiertz et al., 1996a).

Pulse–Chase Analysis: Intact Cells

Cells were detached from tissue culture flasks with trypsin and incubated in suspension in methionine- and cysteine-free DME for 1 h at 37°C. Cells at 1×10^7/ml were pulse-labeled for 10 min at 37°C in 290 μCi/ml [^{35}S]methionine and cysteine (^{35}S-Protein Express Labeling Mix; New England Nuclear). At the beginning of the chase period, 5 mM nonradioactive methionine and 1 mM cysteine were added. Samples were taken at various timepoints and lysates were made as described below. In all pulse–chase experiments, whether on intact or permeabilized cells (see below), when present, the proteasome inhibitor ZL_3VS (Bogyo et al., 1997) was at 50 μM throughout.

Pulse–Chase Analysis: Permeabilized Cells

Cells were labeled as described above, but for only 3 min, placed on ice, and washed once with PBS. They were then resuspended at 1.6×10^7 cells/ml in PB (25 mM Hepes 7.3, 115 mM potassium acetate, 5 mM sodium acetate, 2.5 mM $MgCl_2$, 0.5 mM EGTA) containing 0.025% digitonin (Merck, purified as described in Gorlich and Rapoport, 1993), an ATP-regenerating system (Feldman et al., 1997), and protease inhibitors (10 μg/ml leupeptin, 5 μg/ml chymostatin, 3 μg/ml elastatinal, and 1 μg/ml pepstatin). After a chase period at 37°C, lysates were made from samples taken at various time points and immunoprecipitations were carried out as described below. 5 min into the chase period, >95% of cells were permeable to Trypan blue and >90% of their lactate dehydrogenase activity was released (Tan et al., 1992; data not shown).

Mechanical Homogenization and Fractionation by Centrifugation

Permeabilized cells were resuspended at 6×10^6 cell equivalents/ml in homogenization buffer (PB with 250 mM sucrose, PMSF, aprotinin, and leupeptin). Homogenization was carried out using a ball bearing device (Balch and Rothman, 1985). The homogenates were fractionated by centrifuging sequentially at 1,000 *g* for 10 min, 10,000 *g* for 30 min, and 100,000 *g* for 1 h, and the resulting pellets were resuspended in homogenization buffer. The resuspended pellets and the 100,000 *g* supernatant were diluted with NP-40 lysis buffer and immunoprecipitations were carried out as described below. In the experiments shown in Fig. 12, the intact cells were resuspended at a concentration of 1.2×10^7 cells/ml in homogenization buffer that contained an ATP regenerating system (Feldman et al., 1997).

Fractionation by Squeeze-Out Centrifugation

Soluble, cytosolic proteins were squeezed out of permeabilized cells by centrifugation. At the indicated chase times, two samples were taken from each permeabilization reaction. Both were centrifuged in a microfuge at 14,000 rpm at 4°C for 10 min. The supernatant and pellet of one sample were remixed to represent the total starting material. The supernatant of the other sample was removed and saved. The pellet fraction was resuspended in PB containing digitonin and the ATP regenerating system. Lysates of each fraction (total, supernatant, and pellet) were made and immunoprecipitations were carried out, as described below.

Proteolysis Protection Experiments

Samples from the permeabilization reactions were added to ice-cold trypsin in PB, such that the final trypsin concentration in each reaction was as indicated (see Fig. 4). After 30 min on ice, trypsin digestion was stopped by the addition of the protease inhibitors PMSF and Nα–tosyl-lys chloromethyl ketone, hydrochloride (TLCK; Calbiochem-Novabiochem). Denaturing SDS lysates were made and heavy chains were immunoprecipitated as described below.

^{3}H-Mannose Labeling

US11 astrocytomas were harvested, washed once in DME containing calf serum and glucose at 0.45 g/liter, and resuspended at 2×10^6 cells/ml in the same media containing 1 mCi/ml ^{3}H-mannose (New England Nuclear) with or without 50 μM ZL_3VS. After labeling at 37°C for 1 h with frequent agitation, denaturing SDS lysates were made and sequential immunoprecipitations with αHC and αUb serum were carried out as described below and in the legend to Fig. 6 B.

Lysate Preparation and Immunoprecipitation

As noted in the figure legends, lysates were made in three different ways, depending on the experiment. In all cases, $1–2 \times 10^6$ cells (or cell equivalents) were used to make 1–1.5 ml of lysate for each immunoprecipitation. The type of lysate did not affect the overall outcome of any of the immunoprecipitation experiments (data not shown), although the yield of immunoprecipitated heavy chain was greater when SDS was present. Nondenaturing NP-40 lysates were made by resuspending cell pellets, permeabilized cells, or cell fractions so that the final buffer was 0.5% NP-40 (or Igepal CA-630; Sigma Chemical Co.), 50 mM Tris, pH 8, and 10 mM $MgCl_2$. Samples were agitated for 20 min at 4°C and then clarified by centrifuging in a microfuge at full speed for 10 min. The resulting supernatant was used for immunoprecipitation. Denaturing SDS lysates were made by resuspending cell pellets, permeabilized cells, or cell fractions in 100–150 μl of 1% SDS and 2 mM DTT. Samples were heated to 95°C for 5 min, cooled to room temperature, agitated vigorously, and diluted into NP-40 buffer so that the final lysate used for immunoprecipitation was 0.1% SDS, 0.2 mM DTT, 0.5% Igepal, 50 mM Tris, pH 8, and 10 mM $MgCl_2$. In some experiments (Figs. 7, 9, C and D, 11, and 12), nondenaturing NP-40 lysates were made and then SDS and DTT were added after the clarifying spin to final concentrations of 0.1% and 0.2 mM, respectively. All immune complexes were recovered by precipitation with fixed *Staphylococcus aureus* bacteria (Staph A).

Antibodies

Anti-heavy chain serum (αHC) was made by injecting rabbits with the luminal domains (amino acids 1-275) of both HLA-A2 and HLA-B27 that had been expressed in bacteria (kindly provided by D. Garboczi, Harvard University). This serum recognizes free MHC class I heavy chains, as did a similar antibody which was described previously (Neefjes et al., 1986). Antibodies were raised against purified bovine ubiquitin (Baboshina and Haas, 1996) exactly as described (Haas and Bright, 1985). The quality of the αUb serum that was obtained was evaluated in Western blots by its ability to bind Cdc34p-linked multi-ubiquitin conjugates (Banerjee et al., 1993; kindly provided by Seth Sadis, Harvard Medical School). US11 antiserum was raised in rabbits against a mixture of three US11 peptides (MPELSLTLFDEPPPLVETE, ESLVAKRYWLRDYRVPQRT, and FWGLYVKGWLHRHFPWMF) that were coupled to keyhole limpet hemocyanin. The monoclonal antibody 12CA5, which recognizes the influenza hemagglutinin (HA) epitope, was purified from tissue culture cell supernatants by standard methods (Harlow and Lane, 1988). The monoclonal antibody 66Ig10 was used to immunoprecipitate transferrin receptor (TfR; van de Rijn et al., 1983).

Con A Precipitations

Immunoprecipitated heavy chains were eluted from Staph A by heating to 95°C in 2% SDS and then diluted into Con A precipitation buffer (final conditions: 0.5 M NaCl, 50 mM Tris, pH 7.5, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5% Igepal, 0.1% SDS, and 0.1% BSA). Con A–Sepharose (Pharmacia Biotech) was added and samples were mixed gently for 15 h at 4°C. The precipitates were washed four times in Con A precipitation buffer before being analyzed by SDS PAGE.

Gel Electrophoresis

One-dimensional isoelectric focusing (IEF), and fluorography were carried out as described previously (Ploegh, 1995). Quantitation of proteins labeled with ^{35}S and ^{125}I was done with a Fuji PhosphorImager BAS1000. Densitometry on gels of ^{3}H-mannose–labeled proteins was done with a BioRad Fluor-S MultiImager.

HA/A2 Constructs and Cell Lines

HLA-A*0201 heavy chain (HLA-A2), with the mouse H-2 K^b signal sequence replacing its own (Story et al., 1999), was cloned into the pcDNA3.1 expression vector (Invitrogen Corp.). An HA epitope tag (Surdej and Jacobs-Lorena, 1994) was inserted in the α2 domain at amino acid 127, where amino acid 1 is the first amino acid of the protein after the signal sequence is removed. For the K→R HA/A2 construct, site-directed mutagenesis and standard cloning techniques were used to change the three lysine residues in the cytosolic tail (positions 311, 316, and 340) to arginines. The HA/A2 constructs were transfected into US11 and control astrocytoma cells using Superfect (Qiagen). Cell lines stably expressing wild-type (wt) HA/A2 or K→R HA/A2 astrocytomas were selected with G418-sulfate (GIBCO BRL).

^{125}I-Ubiquitin

Bovine ubiquitin (Sigma Chemical Co.) was iodinated using chloramine T (Ciechanover et al., 1980). Each 310-μl iodination reaction contained 2.6 mg/ml ubiquitin, 3.5 mCi/ml $Na^{125}I$ (NEN Life Science Products), and 0.33 mg/ml chloramineT in 0.32 M potassium phosphate, pH 7.6. Iodination was allowed to proceed for 1 min and was stopped by addition of sodium metabisulfite and nonradioactive NaI. Iodinated ubiquitin was separated from unincorporated $Na^{125}I$ by running Sephadex G-25 (Pharmacia Biotech) spin columns that had been equilibrated with PB. The final ubiquitin concentration was estimated to be 200 μM, labeled to 10,000–15,000 cpm/pmol. ^{125}I-ubiquitin was added to US11 cells in permeabilization reactions at a final concentration of 60 μM (see Fig. 6 A) or 15 μM (see Fig. 9 A).

Results

A Permeabilized Cell System for US11-dependent Dislocation and Degradation of MHC Class I Heavy Chains

To begin to dissect the mechanism of US11-dependent dislocation and degradation of MHC class I heavy chains, a permeabilized cell system was developed. We devised permeabilization conditions, using the mild detergent digitonin, such that cells appear microscopically intact, but are permeable to Trypan blue and ATP as well as to proteins such as hexokinase, trypsin, and lactate dehydrogenase (see below and Materials and Methods). In a typical experiment, human astrocytoma cells expressing US11 or control astrocytoma cells were pulse-labeled briefly at 37°C with [^{35}S]methionine to load the endoplasmic reticulum (ER) with radioactive MHC class I heavy chains. Labeled cells were permeabilized by incubation in a buffer containing a low concentration of digitonin and an ATP-regenerating system, and returned to 37°C for a chase pe-

riod. Lysates were made from samples taken at various timepoints and class I heavy chains were recovered by immunoprecipitation with rabbit anti-heavy chain serum (αHC).

As in intact cells (Fig. 1 A, lanes 1–3 and 7–9), in the permeabilized cell system, heavy chains were degraded only when US11 was present (Fig. 1 B compare lanes 1–6 with lanes 7–12). The half-life of MHC class I heavy chain in permeabilized US11 cells was ~10 min (Fig. 1 C), somewhat longer than its 2–3-min half-life in intact US11 cells (Wiertz et al., 1996a). In the presence of the proteasome inhibitors ZL_3VS (Fig. 1 D) or lactacystin (data not shown), degradation was largely prevented and a lower molecular mass heavy chain species accumulated in US11 cells. This species is endoglycosidase H (Endo H) resistant (data not shown) and its molecular mass by SDS-PAGE corresponds to that of the deglycosylated heavy chain species that accumulates in the cytosol of intact US11 cells treated with proteasome inhibitors (Fig. 1 A lanes 4–6). As was observed in intact cells, the change in molecular mass of the heavy chain in the permeabilized cells is accompanied by a change in isoelectric point upon hydrolysis of the glycoamide bond. This is best seen by comparison with bacterial *N*-glycanase (PNGase F) treated samples (Fig. 2 compare lane 3 with lanes 2 and 6). Thus, deglycosylated heavy chains also accumulate in permeabilized US11 cells in the presence of proteasome inhibitor.

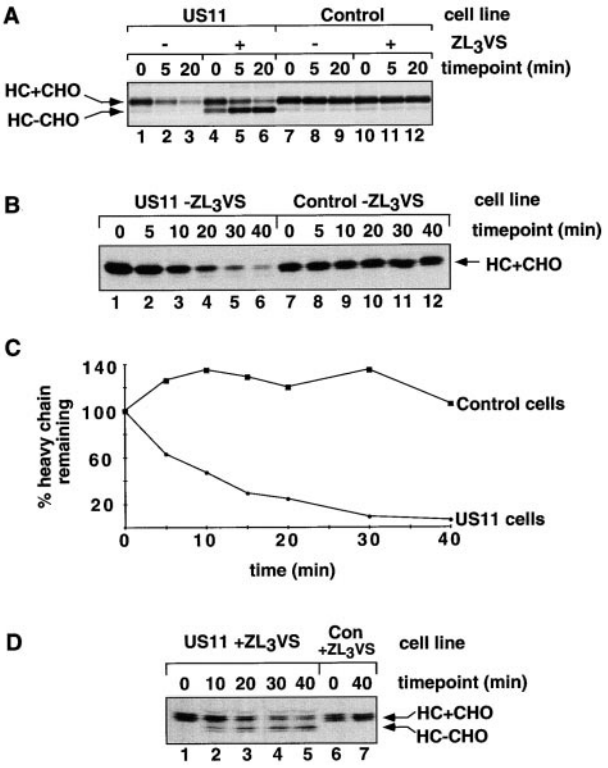

Figure 1. MHC class I heavy chain dislocation and degradation in intact cells and permeabilized cells. (A) The fate of class I heavy chain in intact control and US11 cells. Astrocytoma cells were pulse-labeled for 10 min with [^{35}S]methionine and chased, intact, at 37°C. Samples were taken at the indicated times and cells were lysed with NP-40 lysis buffer. Immunoprecipitations were carried out using rabbit anti–heavy chain serum (αHC). Where indicated, the cells were incubated with the proteasome inhibitor ZL_3VS. The bands corresponding to glycosylated heavy chain (HC+CHO) and deglycosylated heavy chain (HC-CHO) are labeled. (B) Heavy chain is degraded in permeabilized US11 cells. US11 cells and control cells were pulse-labeled for 3 min with [^{35}S]methionine, permeabilized, and chased at 37°C for the indicated times. Samples were lysed with NP-40 lysis buffer and class I heavy chain was recovered by immunoprecipitation with αHC serum. (C) The data in B, quantitated on a PhosphorImager. (D) Deglycosylated heavy chain accumulates in US11 cells permeabilized in the presence of the proteasome inhibitor ZL_3VS. Cells were labeled, permeabilized, and chased exactly as in B, but in the presence of ZL_3VS. Multiple closely spaced heavy chain bands that could often be separated by SDS-PAGE are likely the products of different alleles of MHC class I heavy chain present in the astrocytoma cells.

As in intact cells, the deglycosylated heavy chains in the permeabilized cells were soluble and cytosolic (Fig. 3 A). The glycosylated heavy chains from both permeabilized US11 and control cells fractionated mostly with the particulate fractions, as did the control membrane proteins transferrin receptor (TfR) and US11. The light chain β_2m, a soluble secretory protein, fractionated with membrane pellets in permeabilized control cells, as expected from its tight association with the MHC class I heavy chain. In US11 cells, because there is little heavy chain, most of the β_2m is soluble in the ER lumen (Wiertz et al., 1996a). We found β_2m in both the 10-K pellet and the 100-K supernatant fractions in permeabilized US11 cells (Fig. 3 A), indicating that a portion of the ER content was released during homogenization.

Because the mechanical homogenization was a rather harsh procedure, resulting in some disruption of vesicles, a squeeze-out fractionation technique was also applied. At each timepoint after permeabilization, samples were taken, subjected to centrifugation in a microfuge, and separated into pellet and supernatant fractions. Under these conditions, β_2m fractionated identically in US11 and con-

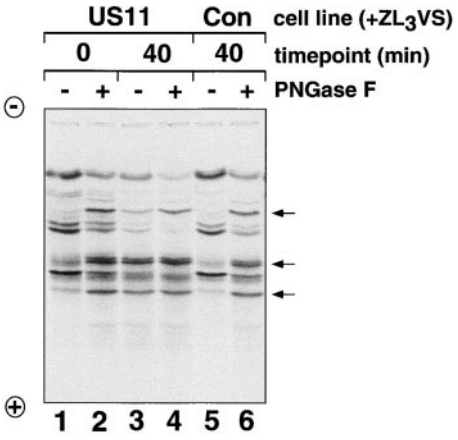

Figure 2. IEF demonstrates that deglycosylated heavy chains accumulate in permeabilized US11 cells. Samples from the experiment shown in Fig. 1 D were treated or mock-treated with PNGase F as indicated, and analyzed by one-dimensional IEF as described (Ploegh, 1995). Arrows point to bands corresponding to deglycosylated MHC class I heavy chains. The different HLA gene products migrate with disparate, though characteristic, isoelectric points.

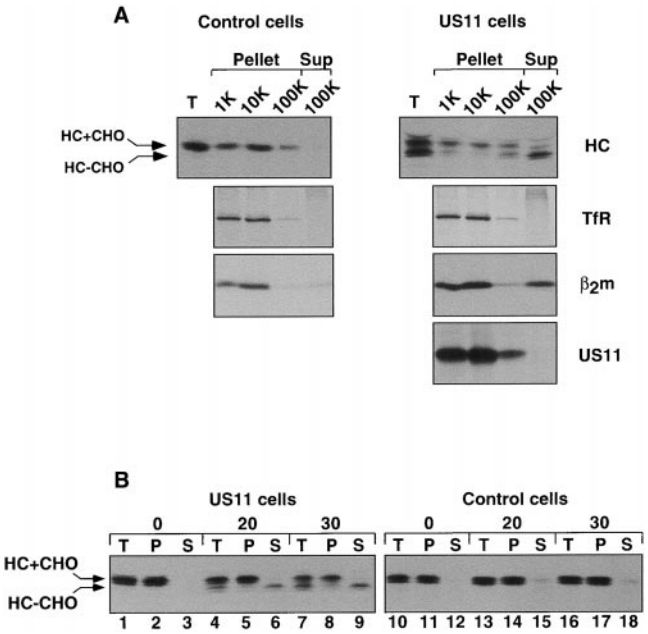

Figure 3. Deglycosylated heavy chain from permeabilized US11 cells is soluble. US11 and control cells were labeled, permeabilized, and chased in the presence of the proteasome inhibitor ZL_3VS as described for Fig. 1 D. (A) Fractionation after homogenization. After 30 min of chase at 37°C, the cells were homogenized mechanically and the homogenates were fractionated by centrifugation. Fractions were diluted into NP-40 lysis buffer and immunoprecipitation was carried out with antibodies to HC, transferrin receptor (TfR), β_2m, or US11. (B) Fractionation by squeeze-out centrifugation (see Materials and Methods). Total starting material (T), pellet (P), and supernatant (S) fractions are labeled. Denaturing SDS lysates were made and immunoprecipitations were carried out with αHC serum.

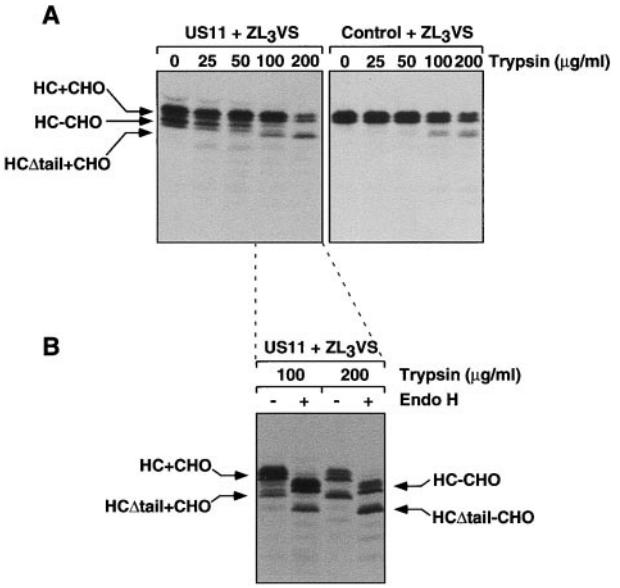

Figure 4. Deglycosylated heavy chain is preferentially accessible to protease in permeabilized cells. US11 and control cells were labeled, permeabilized, and chased in the presence of the proteasome inhibitor ZL_3VS as described for Fig. 1 D. After 30 min of chase at 37°C, samples from the permeabilization reactions were treated on ice with trypsin, at the final concentrations indicated. Denaturing SDS lysates were made and MHC class I heavy chains were immunoprecipitated with αHC serum. (A) Immunoprecipitation products were analyzed directly by SDS PAGE. Proteolysis produced a glycosylated heavy chain species that lacks its cytoplasmic tail (HCΔtail+CHO). (B) The indicated immunoprecipitation products from (A) were treated (+) or mock-treated (−) with Endo H (New England Biolabs) before SDS PAGE.

trol cells, >90% of the β_2m was found in the pellet fractions, confirming that little disruption of vesicles occurred. US11 was found only in pellet fractions (data not shown). The deglycosylated heavy chain was found mostly in the squeezed-out, soluble fractions (Fig. 3 B, lanes 6 and 9) whereas the glycosylated heavy chain was found in the membrane pellet fractions.

The cytosolic localization of the deglycosylated heavy chain in permeabilized cells was confirmed by proteolysis protection experiments. Samples of permeabilized cells were treated with increasing amounts of trypsin. Trypsin at 100 μg/ml degraded nearly all of the deglycosylated heavy chain present, while the glycosylated, membrane-bound heavy chain was largely protected (Fig. 4 A). Further experiments showed that the faster-migrating heavy chain species, seen at 100 μg/ml and 200 μg/ml trypsin, corresponds to glycosylated heavy chain lacking the 30–amino acid cytosolic tail. It was not immunoprecipitable with antibodies specific for the heavy chain cytosolic tail (data not shown) and it was sensitive to treatment with endo H (Fig. 4 B). Thus, deglycosylated heavy chain in permeabilized US11 cells accumulates in the cytosol and not in a membrane-bound compartment.

In intact astrocytomas expressing US11 or US2, accumulation of deglycosylated heavy chain requires ATP (Wiertz et al., 1996b; Tortorella, D., unpublished observation). To test whether this also applies to the permeabilized cell system, we depleted ATP from permeabilization reactions carried out in the presence of proteasome inhibitor. Simply omitting the ATP-regenerating system from the permeabilization reactions significantly reduced the amount of deglycosylated heavy chain that appeared (Fig. 5, lanes 5–8). When remaining ATP was depleted by the addition of hexokinase and glucose, no deglycosylated heavy chain was detectable (Fig. 5, lanes 9–12). Moreover, ATP could not be substituted with the nonhydrolyzable ATP analogue AMPPNP (Fig. 5, lanes 13–16). Thus, as in intact cells, the US11-dependent accumulation of deglycosylated heavy chain in permeabilized cells requires ATP. Taken together, these data demonstrate that the permeabilized cell system faithfully reproduces the US11-dependent degradation of MHC class I heavy chain seen in intact cells.

A Ubiquitinated MHC Class I Heavy Chain Intermediate

Although ubiquitin conjugates of ER proteins degraded in the course of quality control have been detected in both mammalian cells and in yeast (Ward et al., 1995; Hiller et al.,

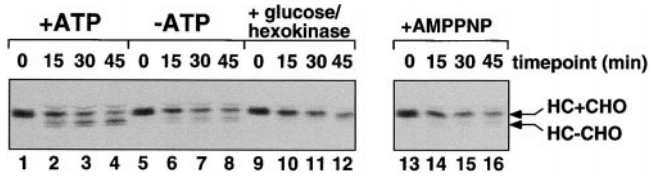

Figure 5. Appearance of deglycosylated heavy chain in permeabilized US11 cells requires ATP. (Lanes 1–4) US11 cells were labeled, permeabilized, and chased in the presence of the proteasome inhibitor ZL_3VS as described for Fig. 1 D. (Lanes 5–16) Prepared as in lanes 1–4, except that the ATP regenerating system was omitted from the permeabilization buffers and with the additions as indicated. Glucose was at 10 mM, hexokinase was at 0.1 unit/μl, and AMPPNP and magnesium acetate were each at 3 mM. Note that some degradation of heavy chain occurs in the absence of ATP. This degradation is not inhibitable by the addition of proteasome inhibitor (ZL_3VS) or standard protease inhibitors (aprotinin, leupeptin, pepstatin, chymostatin, or elastatinal) and has not been characterized further.

1996; Hampton and Bhakta, 1997; Mayer et al., 1998), ubiquitinated MHC class I heavy chains in cells infected with HCMV or in cells expressing US11 or US2 have not been reported. We used a direct and sensitive method to test for the presence of ubiquitinated heavy chain intermediates in permeabilized US11 cells. Instead of metabolically labeling cells before permeabilization, we permeabilized the cells in the presence of ^{125}I-ubiquitin. After a 10-min incubation at 37°C, lysates were made and heavy chains were isolated by immunoprecipitation. Iodinated, high molecular mass heavy chain species accumulated in permeabilized US11 cells, but only when proteasome inhibitor was present (Fig. 6 A, lanes 2 and 3). Much less of this material was seen in control cells, despite the presence of proteasome inhibitors (lane 5). The high molecular mass of the iodinated products is consistent with their being polyubiquitin-conjugated MHC class I heavy chains. They are not coimmunoprecipitating ubiquitinated proteins, because they are also immunoprecipitable with αHC antibody from permeabilized cells lysed under denaturing conditions (data not shown). Nor are they conjugates formed after lysis, because the appearance of ^{125}I-ubiquitin heavy chains requires incubation with permeabilized cells (lane 4).

To establish in intact cells that similar ubiquitin conjugates occur, anti-ubiquitin antibodies were raised and used in immunoprecipitation experiments. US11 and control cells were pulse-labeled with [^{35}S]methionine for 10 min and chased at 37°C in the absence or presence of protea-

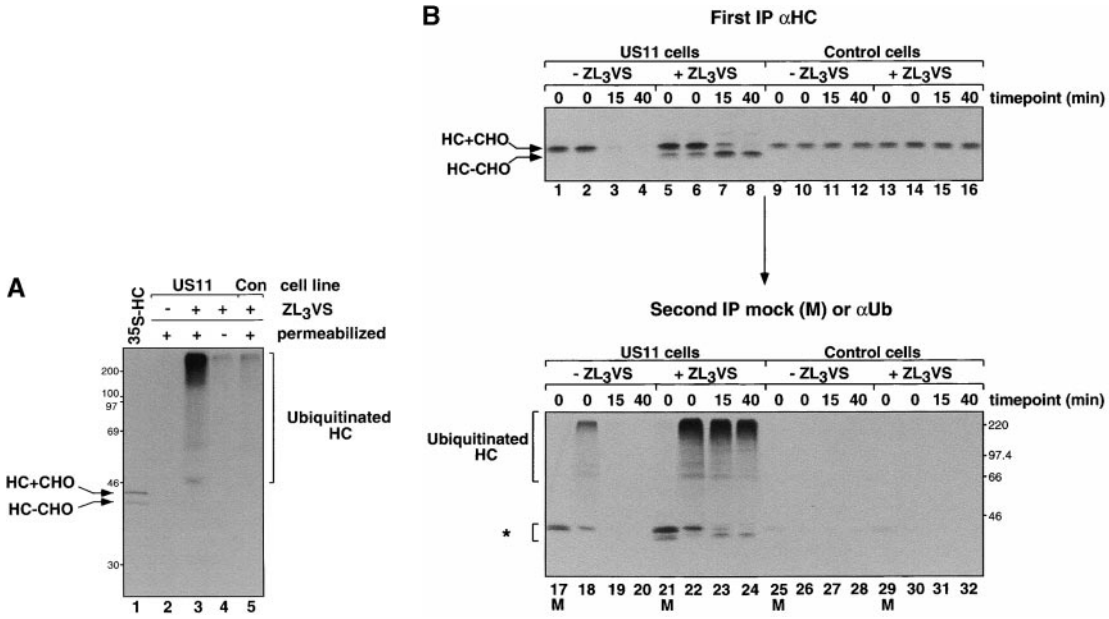

Figure 6. Identification of a ubiquitinated heavy chain intermediate. (A) ^{125}I-ubiquitin labels heavy chains in permeabilized US11 cells (lanes 2–5). US11 and control cells were incubated in solution in the presence or absence of ZL_3VS for 1 h. Cells were then incubated with permeabilization buffer containing ^{125}I-labeled ubiquitin. After incubation at 37°C for 10 min, NP-40 lysates were made and subjected to immunoprecipitation with αHC serum followed by SDS PAGE analysis. The mock permeabilization reaction (lane 4) contained no digitonin. [^{35}S]methionine-labeled heavy chain immunoprecipitates from another experiment were run alongside the ^{125}I-labeled samples (lane 1). The migration of molecular mass markers is indicated at the left of the gel. This figure is a composite of nonconsecutive lanes from a single exposure of one gel. (B) Ubiquitinated heavy chain intermediates in ^{35}S-labeled US11 cells in vivo. US11 and control cells were pulse-labeled with [^{35}S]methionine and chased intact at 37°C in the absence or presence of ZL_3VS as described for Fig. 1 A. Denaturing SDS lysates were made from samples taken at each timepoint and these were diluted with NP-40 buffer. A first immunoprecipitation was carried out with αHC serum, followed by incubation with Staph A. Bound material was eluted with SDS and one-third of each sample was analyzed directly by SDS PAGE and autoradiography (lanes 1–16). The remaining two thirds of each sample was diluted into NP-40 buffer and reimmunoprecipitated with anti-ubiquitin serum (αUb) or mock-immunoprecipitated (no serum added, M) before SDS PAGE (lanes 17–32). Background bands that precipitate with Staph A alone are identified by the mock immunoprecipitations (*). The migration of molecular mass markers is indicated at the right of the lower gel. Exposure time of the gel for lanes 1–16 is 20 h, and for lanes 17–32 is 30 d.

some inhibitor. MHC class I heavy chains were immunoprecipitated and the material bound to the αHC antibodies was dissociated and denatured with SDS. One aliquot was analyzed directly (Fig. 6 B, lanes 1–16), and the rest was diluted into immunoprecipitation buffer and subjected to a second round of immunoprecipitation with anti-ubiquitin serum (αUb). High molecular mass, ubiquitin-conjugated heavy chains were detected in US11 cells but not in control cells (Fig. 6 B, lanes 17–24 vs. lanes 25–32). Some nonubiquitinated heavy chains were also recovered, probably due to nonspecific interactions (lanes 17, 21, 25, and 29). Thus, MHC class I heavy chain is ubiquitinated in intact US11 cells just as it is in permeabilized cells.

In the absence of proteasome inhibitor, a small amount of heavy chain was found ubiquitinated immediately after labeling (Fig. 6 B, lane 18), but subsequently disappeared during the chase period, presumably because it was degraded. In the presence of proteasome inhibitor, the amount of ubiquitinated heavy chain observed remained essentially constant throughout the chase period (lanes 22–24). This probably reflects a balance in the action of ubiquitinating and deubiquitinating enzymes in the absence of appreciable degradation of the heavy chain.

The majority of heavy chain in US11 cells is not ubiquitinated. Given the short labeling time in these experiments and the stability of ubiquitin in cells, we believe that most of the radioactivity in the ubiquitinated heavy chains is incorporated into the heavy chains themselves and not the ubiquitin molecules. With this assumption, we estimate that, at most, 10–20% of the MHC class I heavy chains are ubiquitinated in intact US11 cells treated with proteasome inhibitor. In the absence of proteasome inhibitor, at most 5% of the heavy chains were ubiquitinated.

Consistent with the observation that dislocation is slower in permeabilized cells than in intact cells, we found that accumulation of ubiquitinated heavy chain is also slower under those conditions (Fig. 7 A). Interestingly, both deglycosylated heavy chains and ubiquitinated heavy chains accumulate at approximately the same rate (Fig. 7 B), suggesting that a common, preceding step is rate-limiting.

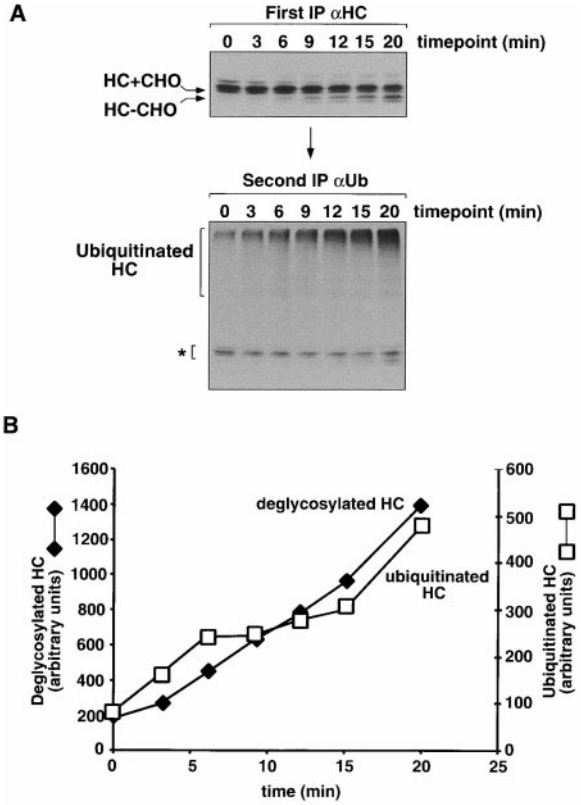

Figure 7. Ubiquitin-conjugated and deglycosylated heavy chains accumulate at the same rate. (A) US11 cells were labeled, permeabilized, and chased in the presence of the proteasome inhibitor ZL_3VS as described for Fig. 1 D. αHC immunoprecipitates of each lysate were split and part was reimmunoprecipitated with αUb antibodies, as described for the experiments shown in Fig. 6 B. Nonspecific bands that precipitate with Staph A alone are identified by the asterisk. Note that the exposure of the αHC gel is 12 h whereas the exposure of the αUb gel is 3 wk. (B) The data in A were quantitated on a PhosphorImager and graphed. Note the different scales on the two y axes.

Ubiquitinated Heavy Chains Are Largely Not Glycosylated

Having demonstrated the occurrence of ubiquitinated heavy chains, we wished to know whether ubiquitination precedes deglycosylation or vice versa. Two independent approaches were taken to determine the fraction of ubiquitinated heavy chains that are glycosylated. First, intact US11 cells were treated with proteasome inhibitors and labeled with ^{3}H-mannose. Very little ^{3}H-mannose-labeled, ubiquitinated heavy chain was detected. On very long (2 mo) exposures of gels from three independent experiments, the amount of ^{3}H-mannose–labeled heavy chain precipitated with αUb serum was barely above background levels (data not shown). Furthermore, in αHC immunoprecipitations, ^{3}H-mannose–labeled, nonubiquitinated heavy chains were efficiently recovered, but very little mannose label was detectable in the area of the high molecular mass, ubiquitinated HC species (Fig. 8 A, lanes 3 and 4). In contrast, such species were readily detectable in ^{35}S-labeled cells (Fig. 8 A, lanes 1 and 2). Ubiquitinated glycosylated heavy chains account, at most, for only 1–2% of all glycosylated heavy chains in these experiments. Thus, it appears that the majority of the ubiquitinated heavy chains in US11 cells are deglycosylated.

This finding is supported by a second set of experiments, in which glycosylated heavy chains were isolated by their ability to bind the lectin Con A. ^{35}S-labeled ubiquitinated heavy chains were isolated by immunoprecipitation with αHC serum, followed by reimmunoprecipitation with αUb serum. Heavy chains were eluted from the αUb antibodies with SDS and each sample was divided in two. Half was analyzed directly by SDS-PAGE and the other half was precipitated with Con A–Sepharose. Only a very small amount of ubiquitinated HC precipitated with con A (Fig. 8 B, compare lanes 1 and 2 with lanes 3 and 4). The precipitation was specific for glycosylated material because, in control experiments carried out in parallel, very little deglycosylated, nonubiquitinated HC was precipitated with Con A (Fig. 8 C, lanes 1 and 2). No heavy chain was recov-

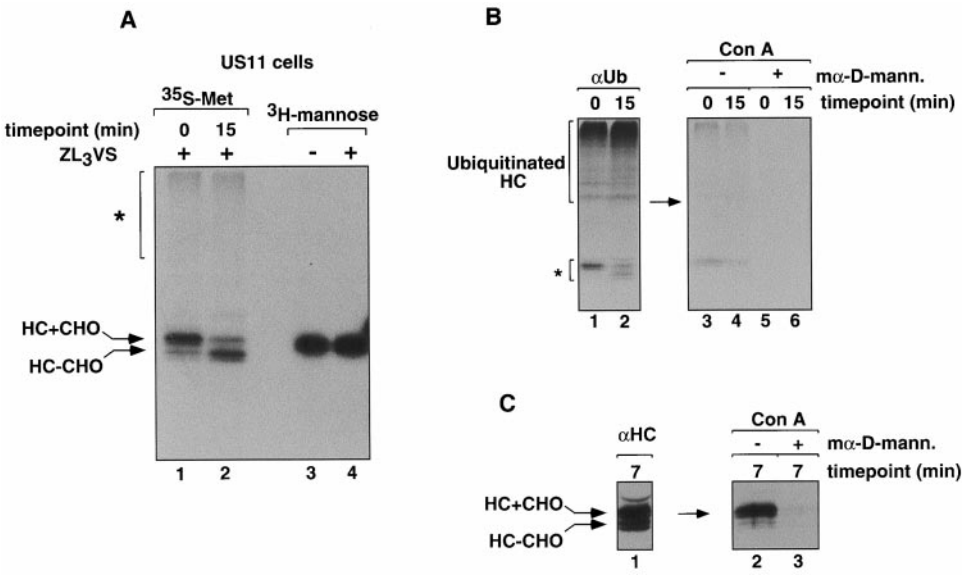

Figure 8. The majority of ubiquitinated heavy chain is not glycosylated. (A) US11 cells were labeled with ^{3}H-mannose in the presence (lane 4) or absence (lane 3) of ZL_3VS, denaturing SDS lysates were made, and heavy chains were immunoprecipitated with αHC serum. [^{35}S]methionine-labeled heavy chain immunoprecipitates from another experiment (US11 cells treated with ZL_3VS) were run alongside the ^{3}H-mannose–labeled samples (lanes 1 and 2). The asterisk indicates high molecular mass species, most likely ubiquitinated heavy chains, that are immunoprecipitated with αHC serum. The exposure time of this gel is 24 d. (B) US11 cells treated with ZL_3VS were labeled with [^{35}S]methionine and chased intact for 0 or 15 min. Denaturing SDS lysates were made and subjected to sequential immunoprecipitation with αHC and αUb serum, as described for Fig. 6 B. One-half of each αUb precipitate was analyzed directly by SDS PAGE (lanes 1 and 2) and the other half was precipitated with Con A–Sepharose, either in the presence (+) or absence (−) of 0.5 M methyl α-D-mannopyranoside. Note that the two panels are from a single exposure (4 wk) of the same gel; the lanes were separated to help clarify the experimental procedure. Nonspecific bands that precipitate with Staph A alone are identified by the asterisk. (C) US11 cells from the experiment shown in B but chased intact for 7 min. Con A precipitations were done as in B after immunoprecipitation with αHC serum. The two panels are from a single exposure (3 d) of the same gel.

ered in samples incubated with Con A in the presence of the competitor methyl α-D-mannopyranoside (Fig. 8 B, lanes 5 and 6, Fig. 8 C, lane 3) or in samples incubated only with Sepharose beads (data not shown). We calculated that only ~10% of the ubiquitinated heavy chains bound to Con A. Taken together, these data suggest that either deglycosylation precedes ubiquitination or that ubiquitinated heavy chains are rapidly deglycosylated.

Membrane Association of Ubiquitinated Heavy Chain Intermediates

Does ubiquitination occur while heavy chains are still associated with membranes? We first examined permeabilized US11 cells that had been treated with proteasome inhibitors because ubiquitinated heavy chains are most abundant under those conditions. In fractionation experiments, after mechanical homogenization of permeabilized cells that had been incubated in the presence of ^{125}I-ubiquitin, 50% of the ubiquitinated heavy chain was found in the 100-K supernatant and 30% in the 100-K pellet (Fig. 9 A). In squeeze-out fractionation experiments on permeabilized US11 cells labeled with ^{125}I-ubiquitin in the same way, ~75% of ^{125}I-ubiquitinated heavy chains fractionated with the cytosol (data not shown). A similar result was obtained in ^{35}S-labeled permeabilized US11 cells, where ~80% of ubiquitinated heavy chains fractionated with the cytosol after 20 min of chase (Fig. 9 B, lanes 10–12). Thus, most of the ubiquitinated heavy chains in US11 cells treated with proteasome inhibitors are released from the ER, although a small percentage seems to be membrane-associated.

We next examined the localization of ubiquitinated heavy chain in cells not treated with proteasome inhibitors. To follow the small amount of ubiquitinated heavy chain that accumulates in intact cells under these conditions, we permeabilized the cells after the chase period and fractionated their contents using the squeeze-out technique (see Materials and Methods). We found that almost all of the ubiquitinated heavy chain fractionated with the membrane pellets in US11 cells not treated with proteasome inhibitor (Fig. 9 C, lanes 5–7). This result was not simply due to the failure to achieve permeabilization because, under the same conditions, proteasomes and other cytosolic proteins can be squeezed out of these cells (data not shown). Moreover, in an experiment carried out in parallel using cells treated with proteasome inhibitor, deglycosylated heavy chain (Fig. 9 D, lanes 2 and 5) and ubiquitinated heavy chains (lanes 8 and 11) were squeezed into the cytosolic supernatant. Note that in the presence of proteasome inhibitor, the amount of soluble, ubiquitinated heavy chain increases with time (Fig. 9 D, compare lanes 8 and 9 with lanes 11 and 12). Thus, these results are consistent with those shown in Figs. 9, A and B. They suggest that, both in the presence and absence of proteasome inhibitor, heavy chain is ubiquitinated while it is still associated with the ER membrane. Over time, in the absence of proteasome inhibitor, the ubiquitinated heavy chain is degraded, while, in the presence of proteasome inhibitor, it is released into the cytosol.

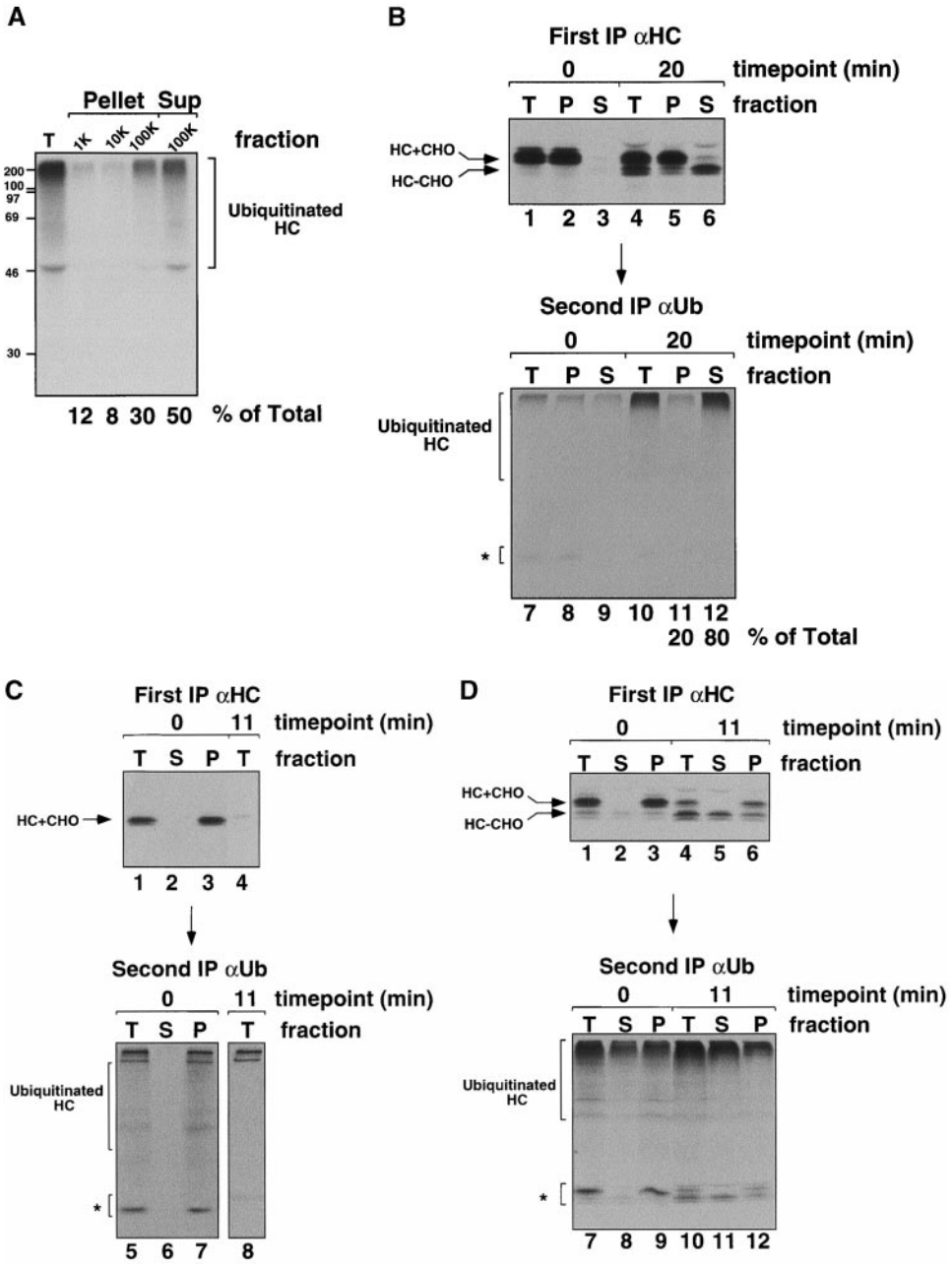

Figure 9. Fractionation of ubiquitinated heavy chains. (A) US11 cells treated with ZL_3VS were permeabilized and chased in the presence of ^{125}I-ubiquitin as in Fig. 6 A. The cells were then homogenized and fractionated by centrifugation as in Fig. 3 A. αHC immunoprecipitates are shown. US11 cells labeled with [^{35}S]methionine but permeabilized without ^{125}I-ubiquitin were fractionated in a parallel experiment; ^{35}S-labeled HC, US11, TfR, and β_2m fractionated exactly as in Fig. 3 A (data not shown). The ubiquitinated heavy chain in each fraction was quantitated by PhosphorImager and the results, expressed as a percent of the total amount of ubiquitinated heavy chain, are shown at the bottom of each lane. (B) Squeeze-out fractionation was carried out on ^{35}S-labeled, permeabilized US11 cells treated with ZL_3VS as described for Fig. 3 B. MHC class I heavy chains were immunoprecipitated with αHC serum followed by αUb serum, as in Fig 6 B. The ubiquitinated heavy chain in the pellet and supernatant fractions from the 20-min time point was quantitated by PhosphorImager and the results are shown at the bottom of each lane. Nonspecific bands that precipitate with Staph A alone are identified by the asterisk. Note that the exposure of the αHC immunoprecipitate gel is 56 h, while that of the αUb immunoprecipitate gel is 3 wk. (C and D) Squeeze-out fractionation was carried out on US11 cells in the absence (C) or presence (D) of ZL_3VS. In this case, cells were labeled with [^{35}S]methionine and chased intact, and then permeabilized for 10 min on ice in the presence of 0.025% digitonin and the ATP regenerating system before squeeze-out centrifugation. Proteins in each fraction were solubilized with buffer containing 0.5% NP-40 and lysates were prepared for immunoprecipitation as described in Materials and Methods. Sequential immunoprecipitations with αHC serum followed by αUb serum were as in Fig 6 B. Nonspecific bands that precipitate with Staph A alone are identified by the asterisk. Note that the exposure of the αHC gels in C and D is 16.5 h, while the exposures of the αUb gels are 6 wk (C) and 3 wk (D).

Ubiquitination of the MHC Class I Heavy Chain Tail Is Not Required for US11-dependent Dislocation and Degradation

With heavy chain ubiquitination occurring at the ER membrane, it seemed possible that ubiquitination might be the initiating signal for heavy chain dislocation. To determine whether ubiquitination of the heavy chain is required for its dislocation, we prevented ubiquitination of the heavy chain cytosolic tail by removing all lysines from the tail domain. Two different HA epitope-tagged heavy chain constructs, based on the HLA allele A2, were made, one with a wild-type (wt) cytosolic tail and one with the three cytosolic tail lysines mutated to arginine (K→R). Cell lines stably expressing the HA-tagged heavy chains (HA/A2) were selected. We found that K→R HA/A2 is degraded at approximately the same rate as wt HA/A2 in US11 cell lines, while both HA/A2 proteins are stable in control cells (Figs. 10, A and B, and data not shown). When pulse–chase experiments were conducted in the presence of proteasome inhibitor, deglycosylated HA-

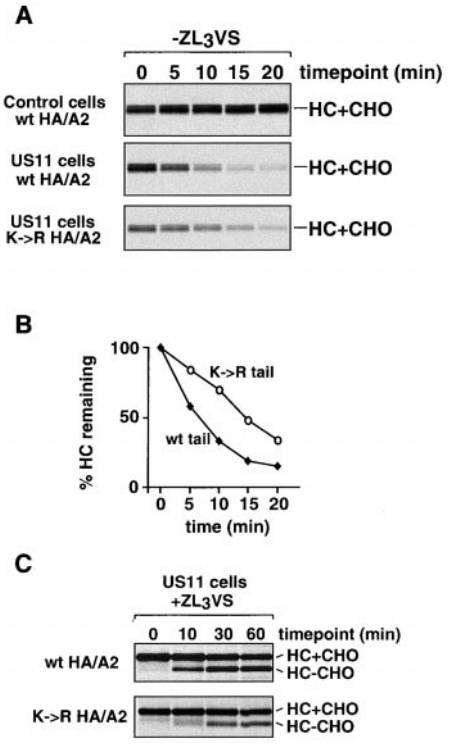

Figure 10. Ubiquitination of the heavy chain cytoplasmic tail is not required for its dislocation from the ER. The stability of HA-tagged MHC class I allele A2 (HA/A2), with either a wt cytoplasmic tail (wt) or with a mutant cytoplasmic tail (K→R), was analyzed in US11 and control cells. Astrocytoma cells stably expressing the HA/A2 constructs were pulse-labeled and chased in the absence (A) or in the presence (C) of ZL_3VS, and NP-40 lysates were made as described for Fig. 1 A. HA-tagged heavy chains were immunoprecipitated specifically using monoclonal antibody 12CA5. (B) The data in A were quantitated by PhosphorImager.

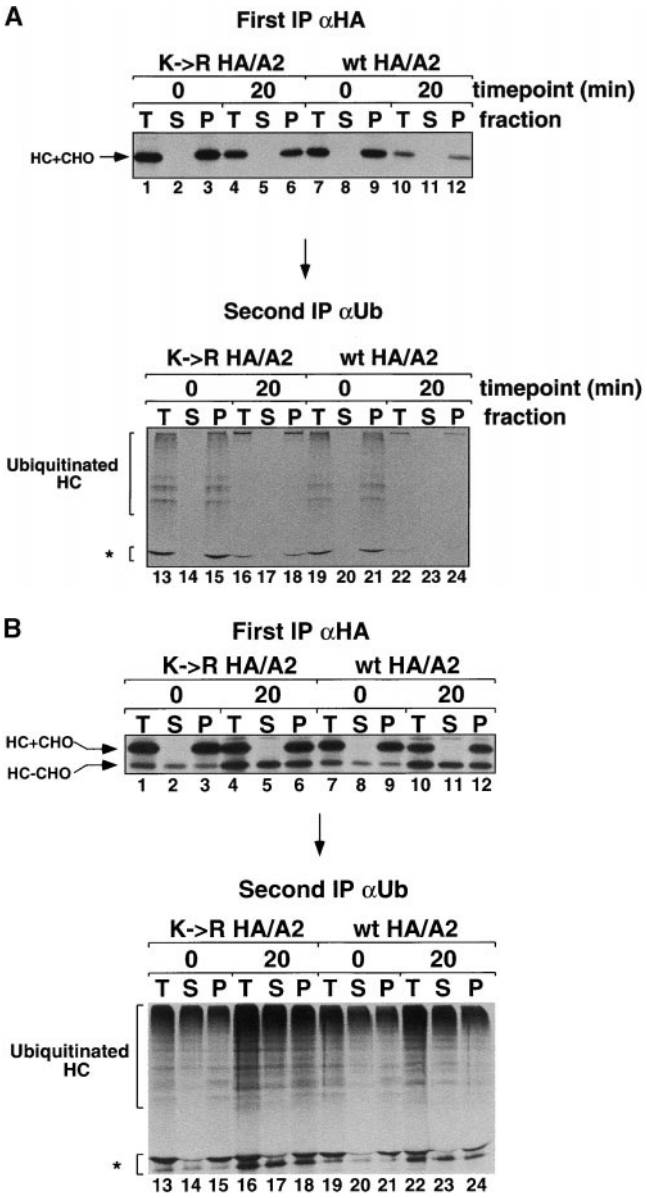

Figure 11. HA-tagged heavy chains with either wt or K→R mutant cytoplasmic tails are ubiquitinated. Squeeze-out fractionation, as described for Fig. 9, C and D, was carried out on US11 cells expressing K→R HA/A2 or wt HA/A2. Sequential immunoprecipitation was done on the fractions as in Fig. 6 B, except that the monoclonal antibody 12CA5 was used in the first immunoprecipitation to isolate HA-tagged heavy chains. Experiments were carried out in the absence (A) or presence (B) of ZL_3VS. Note that the exposure time of the αHA gels in these panels is 7.5 h and of the αUb gels is 1 wk.

tagged wt and K→R heavy chain intermediates accumulated at roughly the same rate (Fig. 10 C). In all cases, the HA/A2 heavy chains were dislocated and degraded more slowly than endogenous heavy chain in cells not expressing HA/A2 constructs. We attribute this to the overexpression of the HA/A2 degradation substrates, which may saturate the degradation machinery (Story et al., 1999). These results were reproducible in multiple, independently derived cell lines. Ubiquitination of the heavy chain cytosolic tail, therefore, is not required to initiate US11-dependent destruction of the protein.

Next, we asked whether the K→R heavy chain is ubiquitinated. We isolated ubiquitinated HA/A2 from [^{35}S]methionine-labeled cell lines by sequential immunoprecipitation with αHA and αUb antibodies. Ubiquitin-conjugated K→R HA/A2 was detected in US11 cells both in the absence (Fig. 11 A, lane 13) or presence (Fig. 11 B, lanes 13 and 16) of proteasome inhibitor. In fact, K→R HA/A2 was ubiquitinated to approximately the same extent as wt HA/A2. The finding that dislocated heavy chains are not ubiquitinated exclusively on residues in the cytosolic tail implies that dislocation must start before ubiquitination of the heavy chain.

In squeeze-out fractionation experiments, the majority of ubiquitinated K→R HA/A2 and wt HA/A2 fractionated with membrane pellets in the absence of proteasome inhibitors (Fig. 11 A, lanes 13–21). In the presence of proteasome inhibitors, slightly more than half of the ubiquitinated HA/A2 in each cell line fractionated with the cytosol (Fig. 11 B, lanes 13–24). These results were confirmed in experiments in which the cell lines expressing the two different HA/A2 alleles were homogenized me-

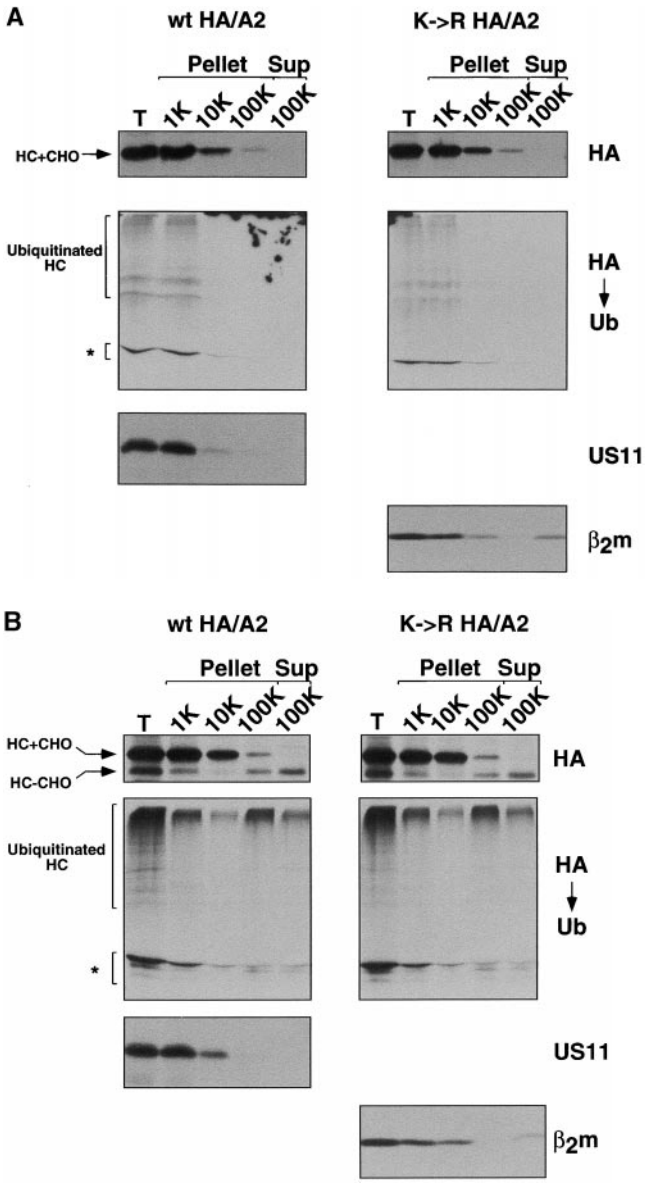

Figure 12. Membrane association of ubiquitinated K→R HA/A2. US11 cells expressing wt or K→R HA/A2 were labeled for 10 min and then homogenized mechanically, without an intervening chase period. Homogenates were fractionated by centrifugation as in Fig. 3 A. Proteins in each fraction were solubilized with buffer containing 0.5% NP-40 and lysates were prepared for immunoprecipitation as described in Materials and Methods. Ubiquitinated, HA-tagged heavy chains were isolated by immunoprecipitating first with the 12CA5 monoclonal antibody, followed by reimmunoprecipitation with αUb serum. Different fractions were also immunoprecipitated for US11 and β_2m. Experiments were carried out in the absence (A) or presence (B) of ZL_3VS. Nonspecific bands that precipitate with Staph A alone are identified by the asterisk. Note that the exposure of the αHA gel in each panel is 27 h whereas the exposure of the αUb gels is 3 wk (A) or 10 d (B).

chanically and fractionated by differential centrifugation. Both ubiquitinated HA/A2 species showed identical fractionation behavior. In the absence of proteasome inhibitors, they were clearly membrane-associated, with most found in the 1-K pellet (Fig. 12 A). In the presence of proteasome inhibitors, the majority of the ubiquitinated HA/A2 was in the 100-K pellet and supernatant (Fig. 12 B). Although we have not characterized the heavy chain in the 100-K pellet fraction further, we have found proteasome subunits in this fraction as well (data not shown), raising the possibility that some ubiquitinated heavy chain is tightly associated with proteasomes in the presence of proteasome inhibitor. Taken together, these data support the idea that the ER luminal domain of heavy chain is ubiquitinated while the protein is still associated with the membrane.

Discussion

We have developed a permeabilized cell system that recapitulates many of the important aspects of US11-dependent dislocation and degradation of MHC class I heavy chains that have been observed in intact cells. In the presence of proteasome inhibitors, deglycosylated heavy chain accumulates in the cytosolic fraction of permeabilized cells. As in intact cells, appearance of this deglycosylated species is ATP-dependent.

We have used this permeabilized cell system, in combination with experiments carried out in intact cells, to identify and order intermediates in US11-dependent dislocation. In so doing, we have provided strong evidence that US11-dependent degradation of class I heavy chain follows a sequence of events similar to that described for other proteins that exit the ER for degradation in the cytosol. First, using two independent methods, we have shown that ubiquitinated MHC class I heavy chains accumulate in US11 cells. Experiments involving the addition of ^{125}I-ubiquitin to permeabilized cells followed by immunoprecipitation with αHC serum provide direct evidence that heavy chain is ubiquitinated. Double immunoprecipitation experiments on lysates of ^{35}S-labeled cells, using antibodies against MHC class I heavy chain and against ubiquitin, confirmed the identity of the ubiquitinated heavy chain. Importantly, ubiquitinated heavy chains were detected in US11 cells regardless of whether they had been treated with proteasome inhibitors, indicating that they are not an artifact of proteasome inhibitor addition.

The majority of the ubiquitinated heavy chain that accumulates in US11 cells treated with proteasome inhibitor is not glycosylated, suggesting that deglycosylation may precede ubiquitin conjugation. However, the presence of a small population of glycosylated, ubiquitinated heavy chains and the observation that, at least in permeabilized US11 cells, ubiquitinated heavy chains seem to accumulate simultaneously with deglycosylated heavy chains (Fig. 7), suggest that there might be no obligatory order to the deglycosylation and ubiquitination steps.

We addressed the role of heavy chain ubiquitination in US11-dependent export from the ER by mutating all of the lysine residues in the heavy chain cytosolic tail to arginine, creating a K→R heavy chain mutant that lacks potential ubiquitination sites in its cytosolic domain. This mutant is still dislocated and degraded at approximately the same rate as wt heavy chain. It is also ubiquitinated. Thus, ubiquitination of the cytosolic tail of MHC class I heavy

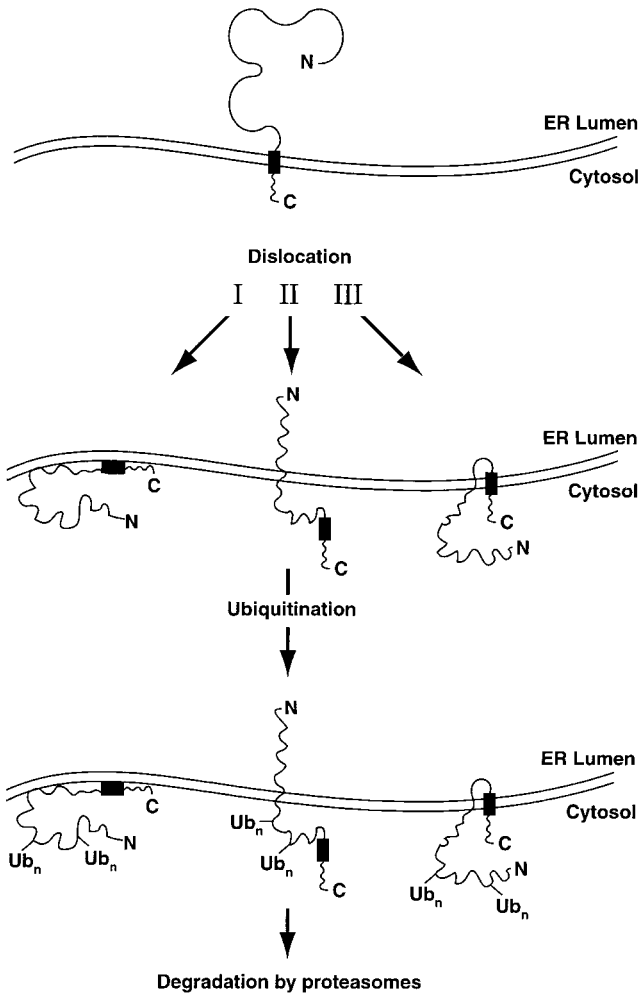

Figure 13. A tentative model for US11-dependent MHC class I heavy chain dislocation and degradation. Arrows I, II, and III indicate three possibilities for how the heavy chain might be associated with the membrane while it is ubiquitinated. Ub_n indicates poly-ubiquitin chains and the black box indicates the transmembrane domain of MHC class I heavy chain molecule. See text for details.

chain is not required to initiate heavy chain dislocation and degradation.

Our data lead to the following model for US11-dependent degradation of MHC class I heavy chain (Fig. 13). The first step is dislocation, in which all or part of the luminal domain of heavy chain enters the cytosol. We do not know the source of the force that pulls or pushes the heavy chain from the ER, nor do we know which segment of the protein exits the ER first. However, once the luminal domain of the heavy chain has been dislocated, the protein seems to undergo ubiquitination while still associated with the ER membrane. We find ubiquitinated heavy chains, including the K→R heavy chain mutants, in cell membrane fractions. Thus, at this stage, the ubiquitinated heavy chains are either completely dislocated from the ER but still tightly associated with the membrane on the cytosolic side (Fig. 13, arrow I), or they are partially dislocated from the ER, with part of the protein integrated in the ER membrane or held in the dislocation channel. Partially dislocated heavy chain could be in two different orientations: with a portion of the luminal domain situated in the ER membrane (Fig. 13, arrow II), or, more likely for energetic reasons, with the transmembrane domain in the ER membrane and with both NH_2 and COOH termini in the cytosol (Fig. 13, arrow III). ER membrane-associated ubiquitin-conjugating enzymes have been identified in yeast and they are required for ER-associated degradation (Sommer and Jentsch, 1993; Biederer, 1996; Hiller et al., 1996; Biederer et al., 1997). Our results suggest that similar enzymes may play a role in US11-dependent heavy chain degradation.

Ultimately, the heavy chain is degraded. It is not clear how the heavy chain is brought into contact with the proteasome, nor where degradation takes place. If we assume that the proteasome inhibitor simply causes a backup of intermediates of the normal degradation pathway, then both soluble deglycosylated and soluble ubiquitinated heavy chains must also be present in the absence of inhibitor, but as very short-lived species. Thus, degradation would occur on cytosolic proteasomes. Alternatively, it is possible that the proteasome inhibitor actually alters the degradation pathway and that the soluble heavy chain species are an artifactual consequence of proteasome inhibition. If this is the case, then heavy chain degradation would occur at the ER membrane, carried out by a population of proteasomes that localizes there (Rivett, 1998).

The work presented here suggests that US11 accelerates a process that normally occurs with misfolded proteins in uninfected cells. By inference, US2 probably operates similarly. In fact, MHC class I heavy chains are also ubiquitinated in cells expressing US2 (Shamu, C.E., unpublished observation). Thus, understanding the exact mechanism by which US11 and US2 induce rapid MHC class I heavy chain degradation will be important not only for understanding viral immune evasion, but also for understanding ER quality control. The speed and specificity with which heavy chain is degraded in the presence of US11 will be particularly useful for further characterization of the dislocation pathway using our permeabilized system. It should be possible to dissect the cytosolic requirements of the pathway and to carry out more detailed mechanistic studies.

We thank Domenic Tortorella, Seth Sadis, Michael Glickman, Margo Furman, Johannes Huppa, Alexei Kisselev, and Maurits Kleijnen for reagents, protocols, and helpful discussions. We thank Dan Finley, Melissa Rolls, and Peter Sorger for comments on the manuscript.

This work was supported by the National Institutes of Health (grant 5R37-A133456 to H.L. Ploegh) and Boehringer Ingelheim Inc. (Ridgefield, CT). C.E. Shamu was supported by the Jane Coffin Childs Memorial Fund for Medical Research and by a Special Fellowship from the Leukemia Society of America. C.M. Story was supported by the Cancer Research Institute of New York. T.A. Rapoport is an Investigator of the Howard Hughes Medical Institute.

Submitted: 22 July 1999
Revised: 24 August 1999
Accepted: 25 August 1999

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