Dislocation of an Endoplasmic Reticulum Membrane Glycoprotein Involves the Formation of Partially Dislocated Ubiquitinated Polypeptides*

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Accumulation of improperly folded polypeptides in the endoplasmic reticulum (ER) can trigger a stress response that leads to the export of aberrant proteins into the cytosol and their ultimate proteasomal degradation. Human cytomegalovirus encodes a type I glycoprotein, US11, that binds to nascent MHC class I heavy chain molecules and causes their dislocation from the ER to the cytosol where they are degraded by the proteasome. Examination of US11-mediated class I degradation has identified a host of cellular proteins involved in the dislocation reaction, including the cytosolic AAA ATPase p97, the membrane protein Derlin-1, and the E3 ubiquitin ligase Sel1L. However, the intermediate steps occurring between the initiation of dislocation and full extraction of the misfolded substrate into the cytosol are not known. We demonstrate that US11 itself undergoes ER export and proteasomal degradation and utilize this system to define multiple steps of US11 dislocation. Treatment of US11-expressing cells with proteasome inhibitor resulted in the accumulation of glycosylated and ubiquitinated species as well as a deglycosylated US11 intermediate. Subcellular fractionation of proteasome-inhibited US11 cells demonstrated that deglycosylated intermediates continued to be integrated within the ER membrane, suggesting that the proteasome functions in the latter steps of dislocation. The data supports a model in which US11 is modified with ubiquitin, whereas the transmembrane region is integrated in the ER membrane, and deglycosylation occurs before complete dislocation.

The endoplasmic reticulum (ER)3 of eukaryotic cells is the site of synthesis of membrane and secretory proteins. Polypeptides are cotranslationally inserted into the ER lumen where they can be modified by the addition of asparagine-linked glycan precursors. Proteins achieve proper tertiary structure with the aid of ER resident molecules such as calnexin, calreticulin, ERp57, and protein disulfide isomerase (1–4). Around 30% of newly synthesized proteins are unable to assemble into their final functional conformation or complex and are degraded (5). Improperly assembled proteins are recognized by the ER quality control machinery and exported from the ER in a process called dislocation and are degraded by the proteasome. The ability of the cell to dispose of misfolded proteins is critical for cell survival (6). The accumulation of misfolded proteins within the ER can result in the activation of an ER stress signaling pathway that aims to restore cellular homeostasis through transcriptional and translational alterations. This may result in up-regulation of ER folding chaperones and inhibition of eIF2α-dependent translation (7, 8).

The removal and degradation of aberrant proteins in the ER is a multifaceted process that likely occurs through several steps. First, degradation substrates are recognized by ER sensors and targeted for removal via an aqueous membrane pore referred to as the “dislocon.” Ubiquitination of ER degradation substrates is carried out by cytosolic or ER membrane bound E3 ubiquitin ligases. Once exposed to the cytosolic environment, N-linked glycans can be cleaved by the cytosolic enzyme peptidase N-glycanase. Finally, the ER degradation substrates are delivered to the proteasome and destroyed.

Model systems have been established in mammalian cells to study the export of misfolded ER substrates. For example, a mutant form of the cystic fibrosis transmembrane conductance regulator, a multimembrane-spanning protein, is targeted for proteasomal destruction (9). Degradation of type I membrane proteins have been investigated using the human cytomegalovirus (HCMV)-encoded type I glycoproteins US2 and US11 that bind directly to nascent MHC class I heavy chains in the ER and catalyze their export and subsequent destruction by the proteasome (10–12). Unassembled α subunits of the T cell antigen receptor are also exported and degraded (13, 14). Proteasome degradation of soluble proteins in the ER has been studied via the export of mutant forms of the α1-antitrypsin protein (15–17). These are only some examples of ER proteins marked for disposal. In fact, ER quality control is important in the folding and maturation of many proteins that could give rise to disease states (18, 19).

Dislocation of type I glycoproteins targeted for degradation in mammalian cells has been partially defined as a multistep process using HCMV US2 and US11. Class I heavy chains are highly unstable in US2- and US11-expressing cells, displaying a

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3 The abbreviations used are: ER, endoplasmic reticulum; MHC, major histocompatibility complex; US, unique short; HCMV, human cytomegalovirus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TRAM1, translocating chain-associated membrane protein-1; ZL,VS, carboxybenzyl-leucyl-leucyl-leucyl vinyl sulfone; IEF, isoelectric focusing; WT, wild type.
Proteasome Degradation of an ER Membrane Protein

half-life of 1–2 min, and degradation can be attenuated through the use of proteasome inhibitors. Deglycosylation of class I heavy chains by cytosolic peptide N-glycanase occurs promptly upon exposure of the polypeptide to the cytoplasmic environment in cells that have been treated with proteasome inhibitor. MHC class I heavy chains accumulate as deglycosylated, polyubiquitinated intermediates before degradation. In this context class I heavy chains exist as distinct populations after treatment with proteasome inhibitor: glycosylated, ER resident molecules, ubiquitinated membrane-bound species, and deglycosylated cytosolic intermediates. However, the role of ubiquitination in substrate extraction is not fully understood, and at what point during the dislocation reaction ubiquitination occurs remains an outstanding question. The accumulation of dislocation intermediates during proteasome inhibition provides a window through which the steps of dislocation can be delineated.

US11 co-opts the host cellular dislocation machinery to degrade MHC class I heavy chains. US11 activates a mild ER stress response to facilitate class I heavy chain degradation. The mammalian ER transmembrane protein Derlin-1, a homologue of yeast Der1p, has been implicated in the US11-mediated dislocation of class I heavy chains, and forms a complex with p97 (the mammalian homologue of yeast Cdc48p) in association with Ufd1-Npl4. A requirement for Sel1L, the mammalian ortholog of yeast Hrd3p, has been demonstrated for class I degradation in US11-expressing cells. The addition of ubiquitin to dislocation substrates is a critical step in ER dislocation, and ubiquitin-conjugated MHC class I heavy chains accumulate in US11-expressing cells. p97 binds to ubiquitinated MHC class I heavy chains and is thought to use its ATP hydrolysis activity to extract membrane-bound substrates. Additionally, these cellular components of dislocation may be important in CFTR degradation as well, implying that the pathways utilized by US11 likely reflect those employed by the cell to export endogenous misfolded ER substrates.

The study presented here identifies HCMV US11 as an uncharacterized ER dislocation and proteasome degradation substrate and explores the impact of proteasome inhibition on the dislocation reaction. A population of glycosylated US11 molecules that are modified with ubiquitin accumulate under conditions of proteasome inhibition. Notably, deglycosylated membrane-bound US11 intermediates accumulate after treatment with a proteasome inhibitor, suggesting that proteasome function impacts the final steps of US11 removal from the ER membrane. The findings presented here further define the dislocation reaction and demonstrate that US11 ubiquitination and deglycosylation occur before its extraction from the ER membrane. Ubiquitination is followed by deglycosylation and complete dislocation.

EXPERIMENTAL PROCEDURES

Cells and Antibodies—Human U373-MG and U373-MG stably expressing HCMV US11 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 8% fetal bovine serum, 1 mM HEPES, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a humidified atmosphere (95% air/5% CO2). gp2-293 cells (Clontech, Palo Alto, CA) were utilized to generate retroviruses (see below) and were cultured in media similar to U373 cells. Rabbit polyclonal anti-US11, anti-MHC class I heavy chains, and anti-p97 antibodies were kind gifts from H. Ploegh (Massachusetts Institute of Technology). Anti-TRAM1 antibodies were raised against peptides that correspond to amino acids 278–291 and 361–374 of TRAM1. The monoclonal antibody W6/32 was purified from hybridoma cultured supernantant. Anti-glyceraldehyde-3-phosphate dehydrogenase GAPDH polyclonal antibody was obtained from Chemicon International (Temecula, CA). Mouse monoclonal anti-ubiquitin (P4D1) and rabbit polyclonal anti-ubiquitin antibodies were obtained from Cell Signaling Technology (Danvers, MA) and NeoMarkers (Fremont, CA), respectively.

cDNA Constructs—The US11 site-directed cDNA constructs (US11K200R, US11Q192L, US11Q192L,K200R) were generated from the ligation of two PCR fragments in which one fragment contained the mutated codon. All of the constructs were engineered with restriction sites permissive for insertion into pcDNA3.1(+) and subsequent subcloning into the retrovirus vector pLgPW, which contained EGFP as a selection marker.

Retrovirus Transduction—Retrovirus vectors encoding US11 wild-type or mutant constructs were transiently transfected simultaneously with vector containing the VSV-G envelope into gp2-293 cells via a lipid-based protocol to generate pseudotyped Moloney Murine Leukemia Virus amphotropic retrovirus as described previously. Enhanced green fluorescent protein-expressing cells were sorted using a Vantage high speed cell sorter (Mount Sinai Flow Cytometry Facility) 3–5 days post-infection.

Cell Lysis and Immunoprecipitation—Cells were recovered by trypsin digestion and resuspended in 1 ml of 1× phosphate-buffered saline containing 1 mM N-ethylmaleimide followed by incubation on ice for 2 min. Cells were lysed in Nonidet P-40 lysis mix (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% (v/v) Nonidet P-40, supplemented with 1.5 mg/ml aprotinin, 1 µM leupeptin, and 2 mM phenylmethylsulfonyl fluoride). Usually, 1 × 106 cells were suspended in 1 ml of Nonidet P-40 lysis mix. Nuclei and insoluble material were removed by centrifugation at 15,000 × g at 4 °C for 10 min. US11 was immunoprecipitated with anti-US11 antibody and recovered using Pansorbin cells (Calbiochem) for 1 h at 4 °C. Pansorbin cells were pelleted and washed 3 times with NET buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% (v/v) Nonidet P-40). The polypeptides were removed from the Pansorbin cells with 1× SDS sample buffer (50 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 0.02% bromphenol blue, 50 mM dithiothreitol) and separated by SDS-PAGE (12.5%). Proteins were visualized by immunoblot analysis. Alternatively, U373 and US11 cells were lysed using 1% SDS followed by extreme agitation and incubation at 95 °C for 3 min (cycle repeated 3 times). After lysis, the SDS concentration was diluted to 0.06% using 1× Nonidet P-40 lysis mix, and immunoprecipitations were carried out as described above.

Direct cell lysis was carried out by resuspending pelleted cells in 1× SDS sample buffer (0.5 × 106/50 µl of SDS sample buffer) followed by cycle of extreme agitation and incubation at 95 °C (repeated 3 times).
Endoglycosidase H and peptide N-glycanase sensitivity was determined as per the manufacturer’s protocol (New England Biolabs). In brief, polypeptide samples were incubated in 1× denaturing buffer (0.5% SDS, 0.04 M dithiothreitol) followed by the addition of 10× G5 buffer (0.5 M sodium citrate, pH 5.5, for endoglycosidase H) or 10× G7 buffer (0.5 M sodium citrate, pH 7.5, 10% Nonidet P-40, for peptide N-glycanase) and 1000 units of endoglycosidase H or 500 units of peptide N-glycanase. Enzymatic reactions were carried out at 37 °C for 1.5 h.

Metabolic Labeling of Cells and Pulse-chase Analysis—U373 and US11 cells were starved in methionine/cysteine-free Dulbecco’s modified Eagle’s medium for 45 min at 37 °C. Metabolic labeling was carried out with 500 mcCi of [35S] methionine/cysteine (1200 Ci/mM; PerkinElmer Life Sciences)/ml at 37 °C for 10 min. For experiments performed in the presence of proteasome inhibitor, cells were incubated with 50 μM ZL3VS for 1 h at 37 °C. After labeling, the chase was performed in Dulbecco’s modified Eagle’s medium containing an excess of 25 mM cold methionine and 50 μg/ml cyclohexamide for the indicated times. Cells were pelleted at each chase point by centrifugation at 12,000 × g for 15 s at 4 °C and lysed in 1.5 ml of 1× Nonidet P-40 lysis buffer on ice for 15 min with intermittent vortexing. The cell lysates were preclarified by the addition of 3 μl of normal mouse serum/ml, 3 μl of normal rabbit serum/ml and subsequently incubated with 50 μl of Pansorbin cells/ml for 1 h at 4 °C. Pansorbin cells were removed through centrifugation at 15,000 × g for 2 min, and immunoprecipitations for US11 polypeptides were performed as described above. The immunoprecipitates were separated by SDS-PAGE (12.5%), and the polypeptides were performed as described above. The immunoprecipitates were separated by SDS-PAGE (12.5%), and the radioactive signal in the polyacrylamide gel was enhanced using Autofluor (National Diagnostics). The dried polyacrylamide gel was exposed to autoradiography film for 3 (Fig. 1A) or 11 (Fig. 1C) days at −80 °C. Radiolabeled polypeptides were quantified using GE Healthcare Storm 860.

Flow Cytometry Analysis—Quantitative flow cytometry analysis of surface expressed MHC class I molecules in US11 and US11<sub>c200R</sub> cells was performed using the monoclonal antibody W6/32. The cells were then incubated with Alexa647-conjugated anti-mouse IgG (Molecular Probes), and fluorescence was measured using a Cytomics FC 500 Flow Cytometer (Beckman Coulter). The data were analyzed using FlowJo software (Tree Star, Inc). The levels of surface class I molecules are represented by plots of normalized cell number (% of cells) versus fluorescence signal. The % of cells was calculated by converting the maximum cell number at the respective peak fluorescence value to 100%.

Isoelectric Focusing—US11 membrane fractions (subcellular fractionation) were subjected to endoglycosidase H or peptide N-glycanase digestion for 2.5 h at 37 °C. 100-μl reaction mixtures were diluted to 1.5 ml with 1× Nonidet P-40 lysis buffer, and US11 polypeptides were recovered using anti-US11 serum as described above. The precipitates were incubated with isoelectric focusing sample buffer (57% (w/v) urea, 2% (v/v) Nonidet P-40, 0.02% amphoteric phosphates pH 3.5–10 (Amersham Biosciences), 0.025% 2-mercaptoethanol), and resolved on a 17-cm IEF gel (57% (w/v) urea, 2% (v/v) Nonidet P-40, 15.5% acrylamide (30/1.6 acrylamide/bis- acrylamide), 4% amphoteries pH 5–7, 1% amphoteries pH 3.5–10, 0.4% amphoteries pH 7–9) for 14 h (33). After electrophoresis, the gel was washed 5 times for 10 min each in 50% methanol, 1% (w/v) SDS/5 mM Tris Cl, pH 8.0, transferred to polyvinylidene fluoride membrane, and subjected to immunoblot analysis.

Subcellular Fractionation—US11 cells were untreated or treated with 5 μM ZL3VS for 14 h. Cells were detached using trypsin and resuspended in 1× homogenization buffer (100 mM Tris, 150 mM NaCl, 250 mM sucrose, 1.5 mg/ml aprotinin, 1 μM leupeptin, and 2 mM phenylmethylsulfonyl fluoride) and mechanically homogenized using a 12-μm ball bearing homogenizer (Isobiotec, Hiedelberg, Germany) with 12 passes. Unbroken cells and other debris were pelleted at 15,000 × g for 10 min at 4 °C, and the supernatants were treated with 4.5 M urea (final) for 10 min on ice. Supernatants were centrifuged at 120,000 × g for 1 h at 4 °C. Pellets from both centrifugation steps were lysed directly in 1× SDS sample buffer, and the 12,000 × g supernatant was concentrated using an Amicon Ultra centrifugal filter (10,000 M<sub>r</sub> cut-off) at 15,000 × g at 4 °C until a final volume of around 100 μl. Supernatants and pellets were lysed directly in 1× SDS sample buffer and resolved using SDS-PAGE (12.5%).

RESULTS

US11 Is Degraded in a Proteasomal-dependent Manner—The degradation of type I membrane glycoproteins is a multistep process that involves both membrane and cytosolic protein complexes. The relationship of the proteasome to the extraction of degradation substrates is not well defined. The current study addresses how proteasome inhibition impacts dislocation of the type I ER membrane protein HCMV US11.

Our initial experiment examined the degradation kinetics of US11 using pulse-chase analysis to determine the half-life of US11 protein. U373 cells and cells that stably express US11 were metabolically labeled with [35S]methionine for 10 min followed by a chase of up to 120 min. US11 was immunoprecipitated from cell lysates using anti-US11 serum at the 0, 30, 60, and 120 min chase points. The immunoprecipitates were resolved using SDS-PAGE and visualized by fluorography. US11 is a 215-amino acid glycoprotein with a predicted molecular mass of ~25 kDa. As expected, US11 molecules were not recovered from U373 lysates (Fig. 1A, lanes 1–4). US11 polypeptides recovered from US11 cells decreased throughout the course of the chase (Fig. 1A, lanes 5–8) and were largely absent from the 120-min chase point (Fig. 1A, lane 8). Quantification of the recovered US11 reveals that it has a half-life of around 60 min (Fig. 1B). The instability of US11 molecules throughout the course of the chase demonstrates that US11 was targeted for proteolysis.

The instability of US11 molecules is reminiscent of that observed for other ER degradation targets (10, 13) suggesting that US11 may also be targeted for proteasomal degradation. To address this hypothesis, U373 and US11 cells treated with or without the proteasome inhibitor ZL3VS (50 μM, 1 h) were pulsed with [35S]methionine for 10 min and chased for up to 60 min (Fig. 1C). US11 was recovered from cell lysates at the 0-, 30-, and 60-min chase points using anti-US11 serum. Immunoprecipitates were subjected to SDS-PAGE and fluorography (Fig. 1C). US11 was not recovered from U373
Proteasome Degradation of an ER Membrane Protein

A.

| Cells | U373 | US11 |
|-------|------|------|
| Chase (min) | 0 | 30 | 60 | 120 | 0 | 30 | 60 | 120 |

Lane 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8

Pulse: 10 min
Immunoprecipitation: anti-US11

B.

![Graph showing US11 degradation kinetics](image)

C.

| Cells | U373 | US11 |
|-------|------|------|
| Chase (min) | 0 | 30 | 60 |

Lane 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8

Pulse: 10 min
Immunoprecipitation: anti-US11

US11 Degradation Intermediates Accumulate upon Proteasome Inhibition—A major difference between US11 degradation and US11-mediated class I degradation is the kinetics of the reaction. In US11-expressing cells, class I heavy chains are degraded with a half-life of 1–2 min (10), whereas the half-life of US11 is around 60 min (Fig. 1). The appearance of degradation intermediates is likely dependent upon the efficiency of proteasome inhibition and the rate of dislocation. To determine conditions that allow for accumulation of US11 degradation intermediates, US11 was immunoprecipitated from US11 cells that had been treated with ZL3VS for up to 12 h. Treatment with a minute amount of proteasome inhibitor for a prolonged period of time was not toxic to cells (data not shown). Immunoprecipitates were subsequently separated using SDS-PAGE and visualized by immunoblot analysis using anti-US11 serum (Fig. 2A). As expected, US11 was not recovered from U373 cells (Fig. 2A, lanes 1–4). An increasing amount of glycosylated US11 was recovered from US11 cells treated with proteasome inhibitor, with nearly equal amounts of glycosylated US11 recovered from cells treated for 6 and 12 h (Fig. 2A, lanes 6–8). Interestingly, a faster migrating polypeptide was also recovered from US11 cells treated with ZL3VS for 6 and 12 h (Fig. 2A, lanes 7 and 8). Therefore, accumulation of glycosylated and deglycosylated US11 species after long periods of proteasome inhibition is representative of US11 degradation intermediates.

Notably, high molecular weight US11-reactive polypeptides were observed after 3 h of proteasomal inhibition (Fig. 2A, lane 6, denoted by arrows). These molecules increased in molecular weight during the time course (Fig. 2A, lanes 6–8, denoted by arrow) and are likely US11 molecules modified with ubiquitin. Indeed, slower migrating US11 species are modified with ubiquitin, as evidenced by immunoblot of US11 immunoprecipitates using anti-ubiquitin monoclonal antibody (Fig. 2B, compare lanes 3 and 4). In addition, US11 recovered from ZL3VS-treated cells followed by immunoblot using polyclonal anti-ubiquitin antibody shows a discrete pattern of ubiquitin-reactive bands similar to that observed in the anti-US11 immunoblot (Fig. 2C). It could be the case that the polyclonal antibody recognizes polyubiquitin chains more readily than monoubiquitin modifications and, thus, gives rise to the...
observed distinct high molecular weight US11 species (Fig. 2C, lane 2). Collectively, these data suggest that longer periods of proteasome inhibition are required to observe US11 degradation intermediates.

Proteasome Inhibition Induces US11-deglycosylated Intermediates—US11 is an ER resident glycoprotein that possesses a single N-linked glycan at amino acid 73 (34, 35). Cleavage of the N-linked glycan by the cytosolic enzyme peptide N-glycanase during dislocation would result in a faster migrating polypeptide. Is the faster-migrating US11 species observed during proteasome inhibition (Fig. 2A) a deglycosylated degradation intermediate? To address this question, US11 molecules were treated with endoglycosidase H, a bacterial enzyme that cleaves high mannose asparagine-linked residues from glycoproteins (36). U373 and US11 cells untreated or treated with ZL3VS (5 μM, 14 h) were lysed in 2% SDS. Half of the lysates were subjected to endoglycosidase H digestion. Cell lysates were then resolved via SDS-PAGE and US11, class I heavy chains, and GAPDH proteins were visualized by an immunoblot using their cognate antibodies (Fig. 3A). Immunoblot using anti-GAPDH antibody confirms that equal levels of protein were loaded onto the gel (Fig. 3A, lanes 17–24). US11 was not observed in U373 cell lysates (Fig. 3A, lanes 1–4). In lysates from US11-expressing cells, a glycosylated form of US11 (Fig. 3A, lane 5) was sensitive to endoglycosidase H digestion resulting in faster migrating US11-reactive polypeptides (Fig. 3A, lane 6). Both the glycosylated form of US11 and the faster-migrating species were observed in US11 cells treated with ZL3VS (Fig. 3A, lane 7). After incubation with endoglycosidase H, glycosylated US11 has a mobility similar to the faster migrating species (Fig. 3A, lane 8), suggesting that the faster migrating species was most likely a deglycosylated US11 intermediate. These data demonstrate that the accumulation of a faster migrating US11 species can be used as a diagnostic marker for the dislocation reaction.

To verify the effectiveness of proteasome inhibition, MHC class I heavy chains were visualized by immunoblot using anti-heavy chain serum (Fig. 3A, lanes 9–16). Class I heavy chains traffic through the ER and Golgi, where their N-linked glycans become further modified by Golgi-resident glycosylating enzymes (37, 38). Thus, class I heavy chains were unaffected by endoglycosidase H (Fig. 3A, lanes 9 and 10). When U373 cells were treated with ZL3VS, a small fraction of class I heavy chains were retained in the ER due to the inhibition of proteasomal peptide processing. This is evidenced by the susceptibility of a minute amount of class I heavy chains to endoglycosidase H (Fig. 3A, lane 12). However, the majority of class I heavy chains observed during steady state in U373 cells has trafficked past the ER, rendering them resistant to endoglycosidase H treatment.
Proteasome Degradation of an ER Membrane Protein

A. 

| Cells  | U373 | US11 |
|--------|------|------|
| ZL3VS  | -    | +    |
| endo H | -    | +    |
| 25 kDa | Lane 1, 2, 3, 4, 5, 6, 7, 8 |

Immublot: anti-US11

| US11 (+CHO) | US11 (+CHO) |
|-------------|-------------|
| US11 (-CHO) | US11 (-CHO) |

B. US11 cells

| N-glycanase | (-)ZL3VS | (+)ZL3VS |
|-------------|----------|----------|
| endo H      | -        | -        |
| 1D IEF      | Lane 1, 2, 3, 4, 5, 6, 7 |

Immublot: anti-US11

C. 

| Cells | U373 | US11 | US11 |
|-------|------|------|------|
| ZL3VS | -    | +    | -    |
| endo H | -    | +    | -    |
| 25 kDa | Lane 1, 2, 3, 4, 5, 6, 7 |

Immublot: anti-US11

US11 molecules accumulate during proteasomal inhibition. A, SDS lysates from US11 and US11 WT cells treated with ZL3VS (5 μM, 14 h) were incubated without or with endoglycosidase H (endo H) and separated by SDS-PAGE. The proteins were subjected to immunoblot analysis using anti-US11 (lanes 1–8), anti-heavy chain (lanes 9–16), and GAPDH (lanes 17–24). Glycosylated (+CHO) and deglycosylated (−CHO) US11 and class I species are noted as are GAPDH proteins. B, US11 proteins were immunoprecipitated from ER-derived microsomes generated from US11 cells treated without (lanes 1–3) or with (lanes 4–6) ZL3VS (2.5 μM, 14 h). Precipitates were untreated (lanes 1 and 4), treated with endoglycosidase H (lanes 2 and 5) or peptide N-glycanase (lanes 3 and 6), and resolved on a one-dimensional isoelectric focusing gel. The glycosylated (+CHO) and deglycosylated (−CHO) US11 polypeptides were visualized by anti-US11 immunoblot. C, US11 was recovered from US373 and US11 WT cells untreated with ZL3VS, and the recovered polypeptides were untreated (lane 5) or treated with endoglycosidase H (lane 6) or peptide N-glycanase (lane 7). The immunoprecipitates and SDS cell lysates were resolved by SDS-PAGE, and proteins were visualized by anti-US11 immunoblot.

FIGURE 3. Deglycosylated US11 molecules accumulate during proteasomal inhibition. A, SDS lysates from U373 and US11 cells treated with ZL3VS (5 μM, 14 h) were incubated without or with endoglycosidase H (endo H) and separated by SDS-PAGE. The proteins were subjected to immunoblot analysis using anti-US11 (lanes 1–8), anti-heavy chain (lanes 9–16), and GAPDH (lanes 17–24). Glycosylated (+CHO) and deglycosylated (−CHO) US11 and class I species are noted as are GAPDH proteins. B, US11 proteins were immunoprecipitated from ER-derived microsomes generated from US11 cells treated without (lanes 1–3) or with (lanes 4–6) ZL3VS (2.5 μM, 14 h). Precipitates were untreated (lanes 1 and 4), treated with endoglycosidase H (lanes 2 and 5) or peptide N-glycanase (lanes 3 and 6), and resolved on a one-dimensional isoelectric focusing gel. The glycosylated (+CHO) and deglycosylated (−CHO) US11 polypeptides were visualized by anti-US11 immunoblot. C, US11 was recovered from U373 and US11 cells untreated with ZL3VS, and the recovered polypeptides were untreated (lane 5) or treated with endoglycosidase H (lane 6) or peptide N-glycanase (lane 7). The immunoprecipitates and SDS cell lysates were resolved by SDS-PAGE, and proteins were visualized by anti-US11 immunoblot.

In conclusion, US11 is degraded in a proteasome-dependent manner upon treatment with ZL3VS, and its stability is analyzed in the presence of proteasome inhibitor. US373, US11 WT, and US11 Q192L cells were treated up to 12 h with ZL3VS, lysed in 1% SDS, and immuno-
precipitated with anti-US11 serum. The precipitates were subsequently analyzed by immunoblot analysis using anti-US11 serum. As expected, US11 was not recovered from U373 cells (Fig. 4, lanes 1 and 2). Increased amounts of glycosylated US11WT and deglycosylated intermediate were recovered from lysates of cells treated with ZL3VS (Fig. 4, lanes 3–5). Similarly, increased amounts of glycosylated US11Q192L molecules were recovered from cells at the 0-, 6-, and 12-h time points (Fig. 4, lanes 6–8). Deglycosylated US11Q192L was recovered at 6 and 12 h of inhibition (Fig. 4, lanes 7–8), similar to deglycosylated US11WT (Fig. 4, lanes 4–5). Slightly less amounts of deglycosylated US11Q192L were recovered as compared with US11WT (Fig. 5C, lanes 7–8 and 4–5). This suggests that the kinetics of US11Q192L degradation may be delayed compared with wild-type US11, perhaps due to prolonged association with class I heavy chains in the ER (data not shown (39)).

These data indicate that class I heavy chain degradation is not a prerequisite for US11 degradation.

**Lysine Residue in the Cytoplasmic Tail of US11 Is Not Required to Initiate Its Degradation**

Is ubiquitination of US11 required to initiate its extraction from the ER membrane? US11 possesses a single lysine residue in its cytoplasmic region (amino acids 200) that could be a target for the cytosolic ubiquitin-conjugating machinery. To investigate whether ubiquitination of lysine 200 is critical for the initiation of US11 degradation, we generated a site-directed mutant where lysine 200 was mutated to arginine (US11K200R). Before examining US11K200R stability, its ability to down-regulate the surface expression of class I heavy chains was examined by flow cytometry using an antibody directed against properly folded class I molecules (W6/32, Fig. 5A). US11K200R-expressing cells (Fig. 5A, thin solid line) reduced cell surface class I molecules to levels similar to wild-type US11 (Fig. 5A, dashed line) when compared with class I expression in U373 cells (Fig. 5A, solid thick line). A further confirmation of class I degradation in US11K200R cells was observed by immunoblot analysis, where deglycosylated class I heavy chains accumulated in ZL3VS-treated US11K200R cells (data not shown). These results demonstrate that US11K200R is functionally active.
Proteasome Degradation of an ER Membrane Protein

and can down-regulate class I molecules similar to its wild-type counterpart.

We next examined whether US11<sub>K200R</sub> itself was targeted for proteasomal degradation. U373, US11<sub>WT</sub>, and US11<sub>K200R</sub> cells were treated with ZL<sub>3VS</sub> for up to 6 h. US11 was recovered from SDS cell lysates using anti-US11 serum, and the precipitates were analyzed by an immunoblot using anti-US11 serum (Fig. 5B). As expected, US11 was not recovered from U373 cells (Fig. 5B, lanes 1 and 2), whereas increasing amounts of glycosylated and deglycosylated US11<sub>WT</sub> molecules were recovered from cells treated with proteasome inhibitor (Fig. 5B, lanes 3–5). Significantly, US11<sub>K200R</sub> was also immunoprecipitated at increasing levels at 3 and 6 h after ZL<sub>3VS</sub> treatment (Fig. 5B, lanes 7 and 8). In addition, a deglycosylated US11<sub>K200R</sub> intermediate was recovered from cells treated for 6 h, similar to US11<sub>WT</sub> (Fig. 5B, lane 8). These results indicate that US11<sub>K200R</sub> undergoes proteasomal degradation in a manner similar to wild-type US11. More importantly, the data reveal that the possible ubiquitination of this substrate on lysine 200 was not required to initiate US11 degradation.

The proteasomal destruction of US11<sub>K200R</sub> does not exclude the possibility that ubiquitination of class I heavy chain complexes with US11 is the catalyst needed to target US11<sub>K200R</sub> for destruction. To examine whether US11<sub>K200R</sub> degradation is independent of class I heavy chain degradation, cells that express a US11 double mutant (US11<sub>Q192L,K200R</sub>) that is incapable of inducing class I degradation (data not shown) were generated. U373, US11<sub>WT</sub>, and US11<sub>Q192L,K200R</sub> cells were treated with ZL<sub>3VS</sub> (5 μM, 14 h) followed by lysis in 1% SDS. Whole cell lysates from equal number of cells were resolved by SDS-PAGE followed by immunoblot analysis with anti-US11 serum (Fig. 5C). As expected, US11 was not observed in U373 cell lysates (Fig. 5C, lanes 1 and 2), and increased amounts of glycosylated as well as deglycosylated US11 molecules accumulated after proteasome inhibition (Fig. 5C, lane 4). Similarly, an increased amount of glycosylated US11<sub>Q192L,K200R</sub> as well as deglycosylated species were also observed in lysates from US11<sub>Q192L,K200R</sub> cells treated with proteasome inhibitor (Fig. 5C, lane 6). These results indicate that US11<sub>Q192L,K200R</sub> was targeted for proteasomal degradation and further confirms the initiation of US11 degradation was likely independent of ubiquitin modification of lysine 200 or class I degradation.

**Proteasomal Inhibition Causes the Accumulation of Ubiquitinated, Glycosylated US11.**—High molecular weight US11-reactive species were recovered from ZL<sub>3VS</sub>-treated cells (Fig. 2). It is not clear whether these US11 molecules are derived from glycosylated or deglycosylated US11 species, as ubiquitination of lysine does not appear to participate in initiation of US11 dislocation (Fig. 5). To address this, US11 was recovered from cells treated with ZL<sub>3VS</sub> (2.5 μM, 14 h) using anti-US11 serum. The immunoprecipitates were subsequently untreated or treated with endoglycosidase H and subjected to immunoblot analysis using anti-US11 serum (Fig. 6A). As expected, ubiquitinated US11 species were not recovered from untreated cells (Fig. 6A, lanes 1 and 2), whereas high molecular weight ubiquitin-modified US11 polypeptides were recovered from ZL<sub>3VS</sub>-treated cells (Fig. 6A, lane 3). These US11 species migrated at 152, 130, 98, 58, and 42 kDa and were sensitive to endoglycosidase H, as evidenced by their faster migration pattern after treatment (Fig. 6A, lane 4). The molecular weight of a single ubiquitin molecule is ~8 kDa (40), whereas that of glycosylated US11 is ~27 kDa (10). Therefore, judging by the mobility of the slower migrating glycosylated US11 molecules, US11 proteins were modified with multiple ubiquitins (Fig. 6A, lane 3). These data demonstrate that a population of glycosylated US11 was modified with ubiquitin while still integrated in the ER membrane.

Lysine 200 is not required to initiate US11<sub>WT</sub> dislocation (Fig. 5, A and B). Does the ubiquitination of glycosylated US11<sub>WT</sub> (Fig. 6A) occur on luminal lysine residues that have become exposed to the cytosol during dislocation? To address this question, US11<sub>K200R</sub> and US11<sub>Q192L,K200R</sub> molecules were recovered from ZL<sub>3VS</sub>-treated cells (2.5 μM, 14 h), digested with endoglycosidase H, and subjected to immunoblot analysis using anti-US11 serum (Fig. 6B). High molecular weight forms of US11<sub>K200R</sub> (Fig. 6B, lanes 3 and 4) and US11<sub>Q192L,K200R</sub> (Fig. 6B, lanes 7 and 8) accumulated in the presence of ZL<sub>3VS</sub>. These high molecular weight US11<sub>K200R</sub> and US11<sub>Q192L,K200R</sub> species

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**FIGURE 6. Glycosylated/ubiquitinated US11 polypeptides accumulate upon proteasome inhibition.** A. US11 was recovered from SDS lysates of US11 cells treated with ZL<sub>3VS</sub> (2.5 μM, 14 h) using anti-US11 polyclonal serum. Immunoprecipitates were treated without (lanes 1–2) or with (lanes 3–4) endoglycosidase H (endo H) and subjected to SDS-PAGE followed by an anti-US11 immunoblot. High molecular weight polyubiquitinated (poly Ub), endoglycosidase H-sensitive US11 species are indicated by asterisks, and their conversion to a non-glycosylated form is indicated with arrows. B. US11<sub>K200R</sub> (lanes 1–4) and US11<sub>Q192L,K200R</sub> (lanes 5–8) cells were treated with ZL<sub>3VS</sub> as in A and subjected to endoglycosidase H digestion in an identical manner. Ubiquitin modifications and endoglycosidase H-sensitive species are indicated as in A.
Proteasome Degradation of an ER Membrane Protein

### A. US11 cells (-)ZL3VS

| Subcellular Fraction | Immunoblot: anti-p97 |
|----------------------|----------------------|
| 100 kDa              | 1 2 3 4 5            |
| 35 kDa               | TRAM1                |
| 25 kDa               | US11 (+CHO)          |

| Subcellular Fraction | Immunoblot: anti-US11 |
|----------------------|-----------------------|
| 100 kDa              | 1 2 3 4 5             |
| 35 kDa               | TRAM1                 |
| 25 kDa               | US11 (+CHO)           |

### B. US11 cells (+)ZL3VS

| Subcellular Fraction | Immunoblot: anti-p97 |
|----------------------|----------------------|
| 100 kDa              | 1 2 3 4 5            |
| 35 kDa               | TRAM1                |
| 25 kDa               | US11 (-CHO)          |

| Subcellular Fraction | Immunoblot: anti-US11 |
|----------------------|-----------------------|
| 100 kDa              | 1 2 3 4 5             |
| 35 kDa               | TRAM1                 |
| 25 kDa               | US11 (-CHO)           |

**FIGURE 7.** Deglycosylated US11 remains integrated within the ER membrane upon proteasome inhibition. Subcellular homogenates from US11 cells untreated (A) or treated (B) with ZL3VS were subjected to 15,000 × g centrifugation. The supernatant was treated without or with 4.5 M urea and centrifuged at 120,000 × g. Cell pellets from the 15,000 and 120,000 × g centrifugations and the 120,000 × g supernatant were resolved by SDS-PAGE and subjected to immunoblot analysis using anti-p97, anti-TRAM1 (lanes 6–10), and anti-US11 (lanes 11–15) serum. Glycosylated (+CHO) and deglycosylated (−CHO) US11 proteins are indicated.

**TABLE 1.** US11 and US11→K200R and US11→Q192L,K200R that lack lysines in the luminal region of US11 were sensitive to endoglycosidase H treatment (Fig. 6B, lanes 3 and 4 and lanes 7 and 8). It is possible that the presence of wild-type US11 that migrates at 58 kDa (Fig. 6A, lane 3) represents a US11 molecule that has been ubiquitinated on lysine 200. The accumulation of glycosylated and ubiqui-

**FIGURE 6.** US11 is released from the ER membrane upon proteasomal inhibition. (A) Subcellular homogenates from US11 cells untreated (lanes 1–5) and deglycosylated (lanes 6–10) were analyzed by immunoblot. US11 was recognized using p97 and anti-US11 antibodies. (B) US11 (lanes 1–5) and US11→K200R and US11→Q192L,K200R that lack lysines in the luminal region of US11 were sensitive to endoglycosidase H treatment (lanes 6–10). Notably, US11→K200R and US11→Q192L,K200R that migrate at about 58 kDa were absent from the immunoblot (lanes 6–10).

**FIGURE 5.** Proteasome Degradation of an ER Membrane Protein. Proteasome inhibition blocks a latter step of the dislocation reaction—The disposal of ER substrates is a multistep process that involves the dislocation of substrates across the ER membrane followed by their proteasomal degradation in the cytosol. Our findings that proteasome inhibition causes an accumulation of both glycosylated, ubiquitinated US11 polypeptides and deglycosylated degradation intermediates suggest that the dislocation and degradation of US11 are tightly coupled processes (Figs. 2A, 3B, and 6A). Therefore, proteasome inhibition allows for the accumulation of diverse dislocation intermediates. To further characterize the deglycosylated US11 intermediates, we examined the membrane topology of glycosylated and deglycosylated US11 proteins upon proteasome inhibition using urea treatment. Incubation of cell-derived microsomes with urea causes the release of non-integral peripheral proteins from the membrane bilayer (41). In contrast, a polypeptide imbedded within the bilayer is insensitive to urea treatment. Therefore, this protocol can be used to determine whether the US11 polypeptide is integrated within the membrane or has been fully extracted from the membrane bilayer.

**TABLE 2.** US11 and US11→K200R and US11→Q192L,K200R that lack lysines in the luminal region of US11 were sensitive to endoglycosidase H treatment (Fig. 6B, lanes 3 and 4 and lanes 7 and 8). It is possible that the presence of wild-type US11 that migrates at 58 kDa (Fig. 6A, lane 3) represents a US11 molecule that has been ubiquitinated on lysine 200. The accumulation of glycosylated and ubiquitinated US11→K200R and US11→Q192L,K200R that lack lysines in the luminal region of US11 was dissociated to the cytosol and modified with ubiquitin before its complete deglycosylation and extraction from the ER membrane. Together these data demonstrate that partially dislocated US11 molecules accumulate in the ER when proteasomal activity was impaired.

**TABLE 3.** US11 and US11→K200R and US11→Q192L,K200R that lack lysines in the luminal region of US11 were sensitive to endoglycosidase H treatment (Fig. 6B, lanes 3 and 4 and lanes 7 and 8). It is possible that the presence of wild-type US11 that migrates at 58 kDa (Fig. 6A, lane 3) represents a US11 molecule that has been ubiquitinated on lysine 200. The accumulation of glycosylated and ubiquitinated US11→K200R and US11→Q192L,K200R that lack lysines in the luminal region of US11 was dislocated to the cytosol and modified with ubiquitin before its complete deglycosylation and extraction from the ER membrane. Together these data demonstrate that partially dislocated US11 molecules accumulate in the ER when proteasomal activity was impaired.

**TABLE 4.** US11 and US11→K200R and US11→Q192L,K200R that lack lysines in the luminal region of US11 were sensitive to endoglycosidase H treatment (Fig. 6B, lanes 3 and 4 and lanes 7 and 8). It is possible that the presence of wild-type US11 that migrates at 58 kDa (Fig. 6A, lane 3) represents a US11 molecule that has been ubiquitinated on lysine 200. The accumulation of glycosylated and ubiquitinated US11→K200R and US11→Q192L,K200R that lack lysines in the luminal region of US11 was dislocated to the cytosol and modified with ubiquitin before its complete deglycosylation and extraction from the ER membrane. Together these data demonstrate that partially dislocated US11 molecules accumulate in the ER when proteasomal activity was impaired.

**TABLE 5.** US11 and US11→K200R and US11→Q192L,K200R that lack lysines in the luminal region of US11 were sensitive to endoglycosidase H treatment (Fig. 6B, lanes 3 and 4 and lanes 7 and 8). It is possible that the presence of wild-type US11 that migrates at 58 kDa (Fig. 6A, lane 3) represents a US11 molecule that has been ubiquitinated on lysine 200. The accumulation of glycosylated and ubiquitinated US11→K200R and US11→Q192L,K200R that lack lysines in the luminal region of US11 was dislocated to the cytosol and modified with ubiquitin before its complete deglycosylation and extraction from the ER membrane. Together these data demonstrate that partially dislocated US11 molecules accumulate in the ER when proteasomal activity was impaired.
amount of the deglycosylated US11 protein localized as a membrane protein (Fig. 7B, lanes 11, 12, and 14), whereas only a minute amount of deglycosylated US11 was observed in the cytosolic fraction (Fig. 7B, lane 13). Interestingly, treatment with urea caused only a small increase in the amount of deglycosylated US11 released as a cytosolic polypeptide (Fig. 7B, lane 15). The efficacy of urea treatment was confirmed by the exclusive localization of p97 in the 120,000 × g supernatant (Fig. 7B, lane 5). Together, these results indicate that in the absence of proteasome function, deglycosylated US11 polypeptides accumulate in the ER membrane and are unable to be fully released into the cytosol. These findings demonstrate that proteasome inhibition induces the accumulation of unique degradation intermediates and supports a model in which proteasome function is involved in the final steps of extracting US11 from the ER membrane. Finally, US11 is completely extracted from the ER membrane and degraded by the proteasome.

The US11 polypeptide is a 215-residue type I glycoprotein with a predicted luminal domain (amino acids 1–180), transmembrane domain (amino acids 181–199), and cytoplasmic tail (amino acids 200–215). The protein is modified with an N-linked glycan (amino acids 73) and contains four lysine residues at positions 109, 123, 179, and 200 (35). The initiation of US11 degradation proceeds with the partial dislocation of US11 across the ER bilayer; however, the ER proteins or complexes that mediate the partial extraction of US11 have not yet been defined. Molecular chaperones and transmembrane proteins such as Derlin-1 might mediate in part the dislocation of US11 across the membrane bilayer. Once partially dislocated, glycosylated US11 polypeptides are ubiquitinated (Figs. 6 and Fig. 8, step 2).

The lysine at position 200 is a likely candidate for ubiquitination. However, the US11 mutant US11K200R continues to be targeted for degradation and can be modified with ubiquitin by a cellular E3 ligase. Substrate cysteine (45), threonine, and serine residues (46) can be targets for viral E3 ligases MIR1 and mK3. US11 possesses a serine residue in the cytoplasmic tail at position. The possibility exists that this serine residue could accept ubiquitin moieties during dislocation; however, these non-traditional ubiquitination events have thus far only been observed for viral E3 ligases. We maintain that US11 is recognized as a degradation substrate and disposed of by cellular machinery in a manner similar to a misfolded protein. Therefore, we favor a model in which the US11 luminal domain is partially dislocated before ubiquitination and ubiquitinated on

**DISCUSSION**

Proteasomal degradation of HCMV US11 can be utilized as a new tool to define dislocation and degradation of ER type I glycoproteins. Inhibition of US11 degradation through interference of proteasome function resulted in the accumulation of both glycosylated, ubiquitinated and membrane-integrated deglycosylated US11 molecules. The formation of these distinct degradation intermediates further clarifies the mechanism of substrate extraction into the cytosol and allows for the proposal of an expanded model of ER dislocation (Fig. 8).
lysine residues by a cellular E3 ligase. Because the ubiquitinated US11 species maintain an N-linked glycan, the topology of glycosylated/ubiquitinated US11 molecules must consider that the region around the N-linked glycan is within the ER lumen.

There are three possibilities how ubiquitinated/glycosylated US11 could occur. 1) US11 could be extracted through the membrane in a C-terminal manner. This is most likely not the case because deglycosylated US11 remains integrated within the ER membrane after treatment with urea (Fig. 7B). This suggests that the transmembrane region of US11 is the last domain to be dislocated out of the ER. 2) The US11 luminal domain between amino acids 73 and the transmembrane region are dislocated across the membrane into the cytosol. This configuration is supported by the presence of multiple lysines that can be ubiquitinated. 3) The N terminus of US11 is dislocated across the membrane, and the N-terminal methionine residue is modified with ubiquitin (47). Even though it is not possible to state with certainty the exact topology of the glycosylated/ubiquitinated US11 molecules, the results support the hypothesis that the luminal domain is initially dislocated across the ER membrane in a ubiquitin-independent manner. The model of partial dislocation and subsequent ubiquitination of US11 is supported by the accumulation of membrane-bound ubiquitinated CD4 in the context of HIV-1 Vpu in yeast (48).

The role of substrate ubiquitination in dislocation is poorly understood. Ubiquitin conjugation does not appear to be critical for initiation of the process, but the data presented here support a model in which ubiquitination of the substrate is important for the latter steps of substrate extraction before deglycosylation and proteasome degradation. Attachment of ubiquitin may participate in the recruitment of dislocation machinery, including p97 or perhaps the proteasome itself.

Based upon what is known about US11-mediated degradation of MHC class I heavy chains, we predict that ubiquitinated US11 will most likely then be targeted for dislocation via the p97-Ufd1-Npl4 complex. This prediction remains to be tested, however. The luminal domain of the glycosylated/ubiquitinated US11 molecule would most likely then be completely extracted through the bilayer. Upon exposure of the N-linked glycan to the cytoplasm, peptide N-glycanase removes the glycan, generating a deglycosylated and ubiquitinated US11 species (Fig. 8, step 3). Once engaged with the dislocation machinery, ubiquitins may be removed by de-ubiquitin hydrolases in preparation for proteasome degradation (49). The accumulation of membrane-integrated deglycosylated US11 intermediates upon proteasome inhibition suggests that the transmembrane domain is dislocated in one of the final steps of dislocation and its dislocation may indirectly require proteolytic activity of the proteasome.

The 19 S proteasome cap may be important in substrate extraction from the ER (50). An interesting question is whether the 19 S complex is involved in recognition of ubiquitinated dislocation substrates as they exit the ER, a function that could be separate and upstream from subsequent proteolytic activity. The 19 S cap of the proteasome is subdivided into a base that is composed of at least 6 AAA ATPase subunits (Rpt1–6) and a lid comprised of non-ATPase subunits (for review, see Ref. 51). The 19 S base also contains two non-ATPase subunits, Rpn1 and Rpn2, that bind ubiquitin-like protein domains (52, 53). Ubiquitin binding capabilities have also been demonstrated for the non-ATPase subunit Rpn10 (54, 55) and the ATPase base member Rpt5 (56). De-ubiquitination of substrates must occur before proteolysis, and this activity has been described for the non-ATPase lid subunit Rpn11 (57) and the p97-associated non-proteasomal protein ataxin-3 (58, 59).

The non-proteasomal protein Rad23 has been proposed to play a role in delivering ubiquitinated substrates to the proteasome, as it possesses both ubiquitin binding and ubiquitin-like domains (60, 61). Similarly, the ER transmembrane protein Herp could potentially interact with and recruit the proteasome to the ER via its N-terminal ubiquitin-like domain (62–64). p97 binding to ubiquitinated proteins may play a role in extracting substrates from the ER, but the dynamics between p97 and the proteasome are not clear. These proteins and complexes may be important for US11 degradation, but the role of these proteins in US11 destruction remains to be determined.

The data presented here suggests that the proteasome may potentially be involved in the latter steps of extraction of ER degradation substrates. Cellular proteasomes are ubiquitous and can bind to the Sec61 translocation channel of the ER (65). Proteasome function has been implicated in the removal of T cell receptor subunits TCRα and CD3-δ (66) and unassembled μ chains of IgM molecules (67), indicating that the proteasome participates in the dislocation of a wide range of cellular substrates. The data presented here provide more insight into the mechanism of type I glycoprotein dislocation through the identification of novel degradation intermediates. The exact nature of the activity of the proteasome in this process remains to be further investigated.

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