Dislocation of a Type I Membrane Protein Requires Interactions between Membrane-spanning Segments within the Lipid Bilayer

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The human cytomegalovirus gene product US11 causes rapid degradation of class I major histocompatibility complex (MHC-I) heavy chains by inducing their dislocation from the endoplasmic reticulum (ER) and subsequent degradation by the proteasome. This set of reactions resembles the endogenous cellular quality control pathway that removes misfolded or unassembled proteins from the ER. We show that the transmembrane domain (TMD) of US11 is essential for MHC-I heavy chain dislocation, but dispensable for MHC-I binding. Cells that express US11 TMD mutants allow formation of MHC-I-β2m complexes, but their rate of egress from the ER is significantly impaired. Further mutagenesis data are consistent with the presence of an alpha-helical structure in the US11 TMD essential for MHC-I heavy chain dislocation. The failure of US11 TMD mutants to catalyze dislocation is a unique instance in which a polar residue in the TMD of a type I membrane protein is required for that protein’s function. Targeting of MHC-I heavy chains for dislocation by US11 thus requires the formation of interhelical hydrogen bonds within the ER membrane.

INTRODUCTION

Many viruses target components of the MHC-I antigen presentation pathway to prevent recognition of infected cells by cytotoxic T lymphocytes (Tortorella et al., 2000). The human cytomegalovirus (HCMV) encodes two ER-resident type I membrane glycoproteins, US2 and US11, both of which specifically target MHC-I heavy chains for dislocation from the ER membrane to the cytosol, where they are processed by an N-glycanase and degraded by the proteasome (Wiertz et al., 1996a, 1996b). Dislocation is rapid, occurring soon after insertion and glycosylation of the MHC-I heavy chain in the ER. Many similarities exist between the sequence of events catalyzed by US2 and US11 and the means by which cells dispose of misfolded or unassembled proteins that accumulate in the ER, suggesting that the viral proteins have co-opted the endogenous cellular pathway to bring about the specific degradation of MHC-I heavy chains.

Disposal of MHC-I heavy chains and cellular proteins involves the coordinated action of many protein complexes that recognize the substrate within the ER lumen and subsequently move it across the ER membrane into the cytosol. It is unclear how proteins that fail to fold properly or that do not assemble into their correct oligomeric state are recognized and selectively targeted for removal from the ER, but several mechanisms have been proposed (Cabral et al., 2001). After recognition, substrates for dislocation must be brought into contact with the as yet unidentified protein machinery that initiates their removal from the ER (Tsai et al., 2002).

Genetic studies in yeast and biochemical studies in mammalian cells have suggested that the Sec61 channel, involved in protein import into the ER, may also be an exit route for substrates (Tsai et al., 2002). Ubiquitin conjugation of cytosolic proteins is not only important for their degradation by the proteasome, but is also essential for ER-to-cytosol dislocation. When ubiquitin conjugation is blocked, using either genetic ablation in yeast, semi-intact mammalian cells depleted of ubiquitin or mammalian cells expressing a temperature-sensitive E1 ubiquitin activating enzyme, dislocation substrates remain within the ER (Kikkert et al., 2001; Shamu et al., 2001; Jarosch et al., 2002). The Cdc48(p97)/Npl4/Ufd1 complex acts in an ATP-dependent manner at the cytosolic face of the ER to promote removal of ubiquitinated disloca-

Abbreviations used: β2m, β2-microglobulin; EndoH, endoglycosidase H; ER, endoplasmic reticulum; MHC-I, class I major histocompatibility complex; TMD, transmembrane domain; Ub-bio, biotinylated ubiquitin.
tion substrates from the ER membrane (Ye et al., 2001). Proteolysis is then carried out by the 26S proteasome (Wiertz et al., 1996a). For glycoproteins, the N-linked glycan is removed before proteolysis by a cytosolic N-glycanase (PNG1), which has a preference for unfolded proteins (Hirsch et al., 2003).

US2- and US11-mediated dislocation of MHC heavy chains resembles dislocation of misfolded cellular proteins. However, MHC heavy chains in US2- or US11-expressing cells have a half-life of only minutes, whereas misfolded cellular proteins have a half-life of between 30 min and several hours (Wiertz et al., 1996a, 1996b; Hughes et al., 1997; Huppa and Ploegh, 1997). US2 and US11 must somehow cause MHC heavy chains to bypass normal quality control, which grants cellular proteins a chance to assume their proper conformation. How US2 and US11 accomplish this task remains unknown, but analysis of both MHC heavy chain mutants and the US2 protein have revealed some of the requirements for dislocation. The cytosolic portion of the MHC heavy chain is required for dislocation by both US2 and US11, but Lys residues in this region are dispensable, suggesting that ubiquitination of the cytosolic region of the MHC heavy chain is not required (Shamu et al., 1999; Shamu et al., 1999). The structure of a US2 fragment bound to a complex of MHC heavy chain, β2m, and peptide suggests that an interaction between their luminal domains is how US2 selectively targets ER-resident MHC heavy chains for dislocation (Gewurz et al., 2001). However, mere interaction of US2 with MHC heavy chains is not sufficient: US2 mutants that lack the cytosolic tail but remain membrane anchored continue to interact strongly with MHC heavy chains, yet do not catalyze dislocation (Furman et al., 2002). US11, in all likelihood, causes dislocation in a manner distinct from US2 (Furman et al., 2002).

Extraction of the MHC heavy chain membrane anchor represents a critical step in dislocation, but events that occur within the lipid bilayer have yet to be examined. Here, we explore interactions within the lipid bilayer by examining the role of the US11 transmembrane domain (TMD). MHC heavy chain dislocation is blocked by mutation of the single Gln residue within the US11 TMD, suggesting that interhelical hydrogen bonds formed by the US11 TMD are essential for US11’s function. Our results suggest that US11 uses interactions within the ER lipid bilayer to manipulate the cellular quality control pathway to bring about MHC heavy chain degradation.

**MATERIALS AND METHODS**

**Cell Lines, Antibodies, and Chemicals**

U373-MG astrocytoma cells transfected with US11 and US11–180 have been described (Jones et al., 1995; Rehm et al., 2001). All astrocytoma cell lines were cultured in DMEM as described (Tortorella et al., 1998), and the retroviral packaging cell line 293GPG was maintained as described (Ory et al., 1996). Astrocytoma cell lines that stably express the US11 variants generated in this study were initially selected and subsequently maintained in DMEM containing 0.5 mg/ml Geneticin (Life Technologies, Rockville, MD). Antibodies used in this study have been described (Parham et al., 1979; van de Rijn et al., 1983; Stam et al., 1986; Hochstenbach et al., 1992; Tortorella et al., 1998; Rehm et al., 2001). 12CA5 (anti-HA) was coupled to CNBr-activated Sepharose 4 Fast Flow (Amersham-Phar- macia Biotech, Piscataway, NJ) according to the manufacturer’s specifications. Reagents used for immunofluorescence analysis were: biotinylated 12CA5 (Roche, Indianapolis, IN), Alexa-Fluor 488–conjugated goat anti-mouse (Molecular Probes, Eugene, OR) and Cy3-conjugated streptavidin (Rockland, Gilbertsville, PA). Horse radish peroxidase-conjugated goat anti-mouse and goat anti-rabbit antibodies (Jackson ImmunoResearch, West Grove, PA) as well as HRP-conjugated streptavidin (Roche) were used according to the manufacturer’s specifications. Digitonin was purchased from Merck (Whitehouse Station, NJ) and was purified as described (Gorlich and Rapoport, 1993).

**Plasmid Constructs and Retroviral Infections**

A pcDNA3.1+ (Invitrogen, Carlsbad, CA) construct containing the US11 gene from the AD169 strain of human cytomegalovirus (Rehm et al., 2001) was used as a template for the introduction of all missense mutations which were generated using the Quick Change method (Stratagene, La Jolla, CA). The 11-C-11 construct is a chi-meric molecule in which the TMD of US11 (amino acids 179–199) was replaced with that of the human CD4 protein (residues 395–418). In the constructs specified, the signal sequence of US11 (amino acids 1–17) was replaced with the signal sequence of the H-2Kb molecule. HA epitope-tagged versions of US11 were generated by inserting US11 sequence (amino acids 18–215) downstream of a sequence containing (from 5’ to 3’) the H-2Kb signal sequence and the HA epitope. All US11-related constructs were verified by sequencing and were subcloned into a modified pLNCX-based (Clontech, Palo Alto, CA) retroviral expression vector to be described elsewhere. 293GPG retroviral packaging cells were transfected with the retroviral plasmids using FuGene6 transfection reagent (Roche) according to the manufacturer’s specifications. Cell supernatants containing retrovirus were used to infect U373 astrocytoma cells.

**Metabolic Labeling, Pulse-chase Analysis, and Immunoprecipitation**

Metabolic labeling, pulse-chase analysis, detergent solubilization, and immunoprecipitation were performed as described (Tortorella et al., 1998; Rehm et al., 2001). Immune complexes were recovered from digitonin lysates (1% digitonin, 25 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl2 with 1 mM PMSF, 1 μM leupeptin, and 1.5 μg/ml aprotinin) using Protein A-agarose (RepliGen, Needham, MA) and were washed in 0.2% digitonin in 10 mM PMSF, 1 mM leupeptin, and 1.5 μg/ml apro tinin containing 0.5 mg/ml aprotinin (Life Technologies, Rockville, MD). The 11-C-11 construct is a chim-eric molecule in which the TMD of US11 (amino acids 179–199) was replaced with that of the human CD4 protein (residues 395–418). For glycoproteins, the N-linked glycan is re- moved as described (Gorlich and Rapoport, 1993).

**Gel Electrophoresis, Immunoblotting, and Immunofluorescence**

Immune complexes were analyzed using SDS-PAGE and fluorography (Ploegh, 1995). Quantitation of radiolabeled MHC heavy chains was performed using a STORM PhosphorImager and Imagequant software (Molecular Dynamics, Sunnyvale, CA). In immunoblotting experiments, proteins were transferred electrophoretically on to PVDF membranes (NEN, Boston, MA), and were probed with the specified antibodies. The analysis of ubiquitinated MHC heavy chains was performed essentially as described (Shamu et al., 1999) except 10 μM biotinylated ubiquitin (Ub-bio; Mitsui and Sharp, 1999) was substituted for iodinated ubiquitin and immune complexes were recovered with Protein A-agarose and were analyzed using SDS-PAGE and immunoblotting. For immunofluorescence analysis, cells were seeded onto glass coverslips 18 h before fixation with 3.7% paraformaldehyde/initiator (50 mM Hepes, pH 7.4 at room temperature). Immunohistochemistry and epifluorescence microscopy were performed as described (Tirabassi and Ploegh, 2002).
RESULTS

Features of the US11 TMD Are Essential for Causing Dislocation of MHCI Heavy Chains

To investigate how the US11 protein causes dislocation of MHCI molecules, we examined the minimal sequence requirements necessary for this function. A US11 molecule that lacks the cytosolic portion of the protein still catalyzes the dislocation of MHCI heavy chains (Furman et al., 2002). We produced a C-terminally truncated US11 molecule from which both the cytosolic and transmembrane domains of US11 were lacking (Rehm et al., 2001). We explored the importance of the identity of the TMD of US11 by replacing it with that of the human CD4 protein, an unrelated type I membrane protein used in other studies of TMD function (Cocquerel et al., 1998), resulting in a chimera designated 11-C-11. We then assessed the stability of MHCI heavy chains in cells expressing wild-type US11, US11–180, or 11-C-11. Lysates were prepared under fully denaturing conditions to ensure recovery of all MHCI heavy chains by immunoprecipitation with the αHC serum (Shamu et al., 1999).

MHCI heavy chains are completely degraded within 30 min of synthesis in cells that express wild-type US11, but are stable in cells expressing US11–180 or 11-C-11 (Figure 1B). Expression levels of US11–180 and 11-C-11 were comparable to that of US11. At the onset of the chase, we observe both the signal peptide-containing and mature US11–180 and 11-C-11 proteins (Figure 1B, bottom panel, lanes 3–6), as reported for the US11–180 molecule (Rehm et al., 2001). Wild-type US11 binds to complexes of MHCI heavy chains and β2m, recognized by the conformation-specific antibody W6/32 (Story et al., 1999). Can US11–180 and 11-C-11 proteins bind to such MHCI complexes? Immunoprecipitation using W6/32 showed coprecipitation of US11–180 and 11-C-11 with MHCI complexes in nondenaturing lysis buffers (NP-40 or digitonin, our unpublished results). Therefore, although US11 mutants lacking a TMD or containing a heterologous TMD retain the ability to bind to MHCI heavy chains, specific features of the US11 TMD itself are required for dislocation.

The US11 sequence contains a Gln residue at position 192, predicted to lie in the center of the US11 TMD (Figure 1C). The TMDs of bitopic proteins usually contain few, if any, polar or charged residues (Landolt-Marticorena et al., 1993). Hydrogen bonding between polar amino acids can mediate strong interactions between alpha-helices within the lipid bilayer (Gratkowski et al., 2001; Zhou et al., 2001). Polar or charged amino acid side chains in the single TMDs of type I and type II membrane proteins likely form favorable contacts with residues of other proteins within the cellular lipid bilayer and facilitate assembly of protein complexes (Popot and Engelman, 2000). Replacement of Gln192 with Leu, a hydrophobic residue with a size roughly similar to that of Gln yielded US11 Q192L. MHCI heavy chains were rapidly degraded in cells expressing wild-type US11, yet they were stable in cells expressing US11 Q192L (Figure 1D, top panel). Analysis of the US11 Q192L protein shows an additional, more slowly migrating form of US11 Q192L present at the onset of the chase, which corresponds to the US11 Q192L molecule that has retained its signal peptide post-translationally (spUS11 Q192L), but is processed during the chase into the mature form (mUS11 Q192L; Figure 1D, lower panel). This behavior resembles that of US11–180 (Rehm et al., 2001) and 11-C-11 (Figure 1B, bottom panel). At present, we do not understand the reason for the delayed signal sequence cleavage phenotype observed for US11 TMD mutants, but this phenomenon will be addressed in detail elsewhere. Delayed signal peptide cleavage of US11 Q192L did not contribute to its inability to cause MHCI heavy chain dislocation. Replacement of the signal sequence of the US11 Q192L molecule with that of H-2Kβ resulted in cotranslational signal sequence cleavage (Rehm et al., 2001), but failed to rescue US11 Q192L’s activity (our unpublished results).
During US11-mediated dislocation, ubiquitin conjugation is required to move the MHCI heavy chain from the ER membrane into the cytosol, and MHCI heavy chains are themselves ubiquitinated before degradation by the proteasome (Shamu et al., 1999, 2001; Kikkert et al., 2001). A small fraction of these ubiquitinated MHCI heavy chains is associated with the membrane, indicating that they may be exposed to the cytosol, yet still inserted in the membrane. Poly-ubiquitination may occur on the lumenal domain of the MHCI heavy chain upon exposure to the cytosol and may serve to prevent MHCI heavy chains from moving back into the ER lumen (Shamu et al., 2001). We therefore examined whether MHCI heavy chains in cells expressing US11 Q192L were ubiquitinated by using a permeabilized cell system (Shamu et al., 1999).

Only when cells were permeabilized with a low concentration of digitonin in the presence of biotinylated ubiquitin (Ub-bio) and an ATP-regenerating system did Ub-bio conjugation to cellular proteins occur (Figure 2, left panel). Control U373 cells, US11-expressing cells and US11 Q192L-expressing cells were treated with proteasome inhibitor and permeabilized in the presence of Ub-bio, and immunoprecipitations for MHCI heavy chains from denaturing lysates were performed and analyzed by immunoblotting for Ub-bio and MHCI heavy chains (Figure 2, top and bottom panels, respectively). In US11-expressing cells, the characteristic deglycosylated MHCI heavy chain species was recovered, indicating dislocation to the cytosol (Figure 2, bottom panel, lanes 7–9; Wiertz et al., 1996a). A fraction of the recovered heavy chains was poly-ubiquitinated (Figure 2, top panel, lane 9). No deglycosylated or poly-ubiquitinated species were recovered from control U373 or US11 Q192L cells (Figure 2, top panel, lanes 6 and 12). MHCI heavy chains in US11 Q192L cells are apparently not exposed to the cytosol, where ubiquitination and deglycosylation occur. Gln192 in the US11 TMD is thus essential for dislocation at a stage that precedes exposure of MHCI heavy chains to the cytosol.

**US11 Q192L Is an ER-resident Protein and Causes Retention of MHCI Complexes in the ER**

US11 resides in the ER where it acts on MHCI heavy chains (Wiertz et al., 1996a). Polar residues within TMDs of single spanning proteins can affect localization and cause retention of proteins within the ER (Bonifacino et al., 1991). Does replacement of Gln192 alter the subcellular localization of US11? Because the mAb HC-10 serum used for immunoprecipitations was not suitable for immunohistochemistry, we analyzed cells expressing HA epitope-tagged versions of US11 and US11 Q192L (termed HA-11 and HA-11 Q192L, respectively). These tagged versions are as effective at MHCI heavy chain dislocation as their untagged counterparts (our unpublished results). We observed complete colocalization of HA-11 and HA-11 Q192L with the ER-resident protein calnexin (Figure 3A). The mechanism by which US11 is retained in the ER is unclear, because US11 lacks a known ER retention motif, but the luminal domain of US11 appears to be insufficient to confer ER localization to the protein (B.L., unpublished results).

The US11 Q192L protein is present at the correct site to mediate dislocation, but is there a role for Gln192 in binding to MHCI heavy chains? We examined the kinetics of association of MHCI heavy chains with HA-11 and HA-11 Q192L in a pulse-chase experiment. MHCI heavy chains were recovered in a complex with HA-11 after the onset of the chase, and the binding of MHCI heavy chains to HA-11 was rapidly lost, with kinetics that resembled that of MHCI heavy chain dislocation (Figure 3B, lanes 1–3 and our unpublished results). At the onset of the chase, we recovered similar levels of MHCI heavy chains in HA-11 Q192L cells as seen for HA-11. However, the complex of MHCI heavy chains and HA-11 Q192L was stable and persisted throughout the chase (Figure 3B, lanes 4–6). Therefore, Gln192 is not required for the interaction of US11 with its substrate, and in fact, mutation of Gln192 to Leu results in a persistent interaction with MHCI heavy chains.

We next examined the effect, if any, of US11 Q192L expression on MHCI complex assembly and stability. Early after insertion into the ER and chaperone-assisted folding, MHCI heavy chains associate with β2m to form a heterodimeric MHCI complex recognized by the mAb W6/32 (Parham et al., 1979). In US11-expressing cells, this complex can be recovered only transiently before dislocation and degradation, consistent with its disassembly during dislocation (Wiertz et al., 1996a; Tortorella et al., 1998). Disassembly most likely occurs before exposure of the MHCI complex to the cytosol (Tortorella et al., 1998). In control U373 cells, MHCI complexes were stable throughout a 2-h chase period.
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out with NP-40 lysis mix, a second immunoprecipitation was carried by boiling in 1% [wt/vol] SDS with 5 mM DTT. After dilution HA). Bound material was eluted from the beads and denatured of calnexin and US11. (B) HA-US11 cells (lanes 1 – of the left and middle panels (right panels) shows colocalization the ER chaperone calnexin (left panels, green), biotinylated an-

Figure 3. (A and B) Mutation of Gln192 does not affect subcel-

ular localization or MHCI heavy chain binding. (A) HA-US11 cells (top panels) or HA-US11 Q192L cells (bottom panels) were stained with the AF8 mAb (Hochstenbach et al., 1992) recognizing the ER chaperone calnexin (left panels, green), biotinylated anti-HA (middle panels, red), and DAPI (blue, all panels). A merge of the left and middle panels (right panels) shows colocalization of calnexin and US11. (B) HA-US11 cells (lanes 1–3) or HA-US11 Q192L cells (lanes 4–6) were labeled for 10 min and chased for 0, 15, or 30 min. A first immunoprecipitation was performed from lysates (1% [wt/vol] digitonin in 25 mM Tris-HC, pH 7.4, 150 mM NaCl, 5 mM MgCl2) with 12CA5-coupled Sepharose (anti-

HA). Bound material was eluted from the beads and denatured by boiling in 1% [wt/vol] SDS with 5 mM DTT. After dilution with NP-40 lysis mix, a second immunoprecipitation was carried out with αHC (top panel) and αUS11 (bottom panel). (C) MHCI-βm complexes are stable in cells expressing US11 Q192L, but are retained in the ER. Control U373 cells (lanes 1–6) and US11 Q192L cells (lanes 7–12) were pulse-labeled for 15 min and were chased for 0, 1, or 2 h. Immunoprecipitations were performed with antitransferrin receptor (van de Rijn et al., 1983; top panels) or W6/32 (Parham et al., 1979; bottom panels). Immune complexes were treated with EndoH where indicated. The positions of the EndoH-resistant (EndoH+) and EndoH-sensitive (EndoH–) MHCI heavy chains, US11 Q192L, and βm are indicated.

and >90% of the complexes acquired EndoH resistance (Figure 3C, bottom left panel), indicating passage through the Golgi. In cells expressing US11 Q192L, MHCI complexes retained W6/32 reactivity and were stable during the 2-h chase (Figure 3C, lower right panel). Therefore, consistent with its role in MHCI heavy-chain dislocation and ubiquitini-

nation, the Gln residue at position 192 is also essential for destabilizing the MHCI heavy chain-βm complex.

Although MHCI complexes were stable, their trafficking through the secretory pathway was dramatically slowed in US11 Q192L cells, as only ~50% of the MHCI complexes acquired EndoH resistance, compared with full resistance seen in U373 cells (Figure 3, compare lanes 5 and 6 with 11 and 12). The expression of US11 Q192L did not affect traffi-

cicking of the transferrin receptor, which acquired resistance to EndoH at equivalent rates in control cells and US11 Q192L cells (Figure 3C, top panels). Also, the US11 Q192L protein itself was coinmunoprecipitated with MHCI complexes at all time points tested and remained fully EndoH sensitive, consistent with its ER localization. We also ob-

served retention of MHCI complexes in the ER in cells expressing US11-180 and 11-C-11 as well as coinmunopre-

cipitation of the US11 TMD mutants with MHCI complexes (our unpublished results). Therefore, the ER lumenal do-

main of US11 must be responsible for both MHCI heavy chain binding and retention in the ER.

Additional Polar Residues Can Substitute for Gln192 in Mediating MHCI Heavy-

chain Degradation

The Gln side chain contains a carboxamide capable of acting as both a hydrogen bond donor and acceptor. Mutant forms of US11 in which Gln192 was substituted with Asn, Glu, Cys, Ala, and Ser were stably expressed in U373 cells and their ability to cause MHCI heavy chain dislocation was examined. When we mutated Gln192 to Leu, Ser, Cys, and Ala, the activity of US11 was largely eliminated, because little or no MHCI heavy chain degradation occurred in cells expressing these mutants (Figure 4). Changing Gln192 to Asn had a modest effect on US11 activity. Mutation of Gln192 to Glu did not significantly alter the rate of MHCI heavy chain degradation. Therefore, a residue containing either a carboxamide or carboxylate, both of which can promote strong interhelical interactions (Zhou et al., 2001), is required at position 192 to mediate rapid MHCI heavy chain dislocation.

The US11 TMD Contains a Structural Feature That Is Essential for MHCI Heavy chain Dislocation

Although polar amino acids can mediate strong interactions between TM helices in model systems, additional structural elements of TM helices also play an important role (Popot and Engelman, 2000). Based on a helical wheel projection of the US11 TMD, Gly196 is predicted to be on the same face of the helix as Gln192 (Figure 5A). Gly residues, frequently found in membrane-spanning helices, allow for close pack-

ing and favorable Van der Waals contacts between helices that have a complementary interface (Russ and Engelman, 2000). We thus examined the role of Gly196 in MHCI heavy chain dislocation. In cells expressing a US11 mutant with Leu substituted for Gly196 (US11 G196L), only minimal
Figure 4. Other polar residues can substitute for Gln at position 192. (A) Pulse-chase immunoprecipitation analysis of cell lines expressing wild-type US11 (WT, lanes 1 and 2), or with Gln192 mutated to Glu (lanes 3 and 4), Asn (lanes 5 and 6), Ser (lanes 7 and 8), Leu (lanes 9 and 10), Ala (lanes 11 and 12), or Cys (lanes 13 and 14) was performed as in Figure 1. The aHC and αUS11 immunoprecipitations were run on separate SDS-PAGE gels, and the data shown are from nonconsecutive lanes of the individual gels. The positions of the signal peptide-containing form (spUS11) and the mature form (mUS11) are indicated. Differences in the mobilities of the US11 mutants were consistently observed in SDS-PAGE gels, attributable to the introduction of the various residues at position 192. (B) Quantitation of the MHCI heavy chain amounts recovered in the experiment shown in A was performed using PhosphorImager analysis for each individual cell line. The values shown represent the amount of MHCI heavy chain recovered at 30 min as a percentage of that recovered at the 0-min time point.

DISCUSSION

The study of US11- and US2-mediated dislocation of MHCI heavy chains has thus far focused on either the interactions between the luminal domain of MHCI heavy chains and the viral proteins or on their cytosolic portions. The luminal domains of both US11 and US2 mediate a recognition event that most likely allows them to target MHCI heavy chains selectively for dislocation (Gewurz et al., 2001). The luminal domains of US2 and US11 may have other, as yet undefined, roles.

However, events that occur within the ER lipid bilayer have not been examined in any detail. During dislocation, the MHCI heavy chain, a type I membrane protein, is removed from the membrane and can be retrieved from the cytosol when the proteasome is inhibited (Wiertz et al., 1996a, 1996b). Other membrane proteins known to be dislocated due to their misfolding or improper assembly have also been identified as soluble species in the cytosol (Hughes et al., 1997; Huppa and Ploegh, 1997; Johnston et al., 1998). Therefore, removal of the stably integrated TMDs from the lipid bilayer likely represents a crucial, though energetically unfavorable, step in the disposal of membrane proteins. How the cell accomplishes this is unclear, but may involve partitioning of the TMD from the lipid environment into an aqueous, protease-competent channel. This would represent the reverse of what happens during TMD insertion into the lipid bilayer, where the Sec61 channel opens laterally to allow hydrophobic sequences to insert (Heinrich et al., 2000). The energetic cost of such an event, regardless of the precise mechanism, would be high because of the removal of a hydrophobic sequence from the lipid phase into an aqueous phase. Accordingly, dislocation is ATP-dependent (Wiertz et al., 1996b).

Despite the importance of events that must occur within the ER lipid bilayer, the roles of the US2 and US11 TMDs in dislocation had not been examined. Surprisingly, when the US11 TMD was replaced with that of the CD4 protein, a typical, nonpolar membrane anchor, dislocation was abrogated (Figure 1B). Thus, the identity of the US11 TMD is essential.

TMDs of bitopic membrane proteins function as more than simple membrane anchors, by promoting protein assembly and folding and regulating subcellular localization (Cocquerel et al., 2000; Call et al., 2002). There are many documented cases where TMDs form homo- or hetero-oligomers, essential for the function of the respective proteins (MacKenzie et al., 1997; Cocquerel et al., 2000; Constantinescu et al., 2001; Call et al., 2002). In the striking case of the T cell receptor complex, lateral associations formed by the TMDs of the individual subunits contribute to the formation of a macromolecular signaling complex (Call et al., 2002). Many of the known instances of TMD interactions involve Gly motifs or charge pair interactions (Cosson et al., 1991; Russ and Engelman, 2000), but interhelical hydrogen bonding by polar residues can also provide a major force for TMD association, as judged from the behavior of model TMDs in vitro (Gratkowski et al., 2001; Zhou et al., 2001). Although the functional relevance of such interactions has been shown for a small number of proteins only, there are two notable examples. The TMD of the bovine papillomavirus E5 protein contains a Gln residue that contributes to the formation of an E5 homodimer. The E5 dimer then forms a complex with the platelet-derived growth factor β receptor, inducing ligand-independent autophosphorylation and cellular transformation (Klein et al., 1998). A Gln residue in the TMD of the
invariant chain (II) contributes to the assembly of II trimers, the formation of which is required to mediate II-class II MHC association in the ER (Ashman and Miller, 1999). In both of these instances, Gln residues in the TMDs are required for homo-oligomerization, a prerequisite for subsequent assembly into a hetero-oligomeric complex.

Here, we show an absolute requirement for a helical structure encoded by the US11 TMD. Without a polar residue capable of forming hydrogen bonds at position 192 of the US11 TMD, MHCI heavy chains, complexed with US11, are not exposed to the cytosol. Instead, they persist as folded complexes that are retained in the ER. The importance of the Gly residue at position 196, on the same face of the US11 TM helix as Gln192, suggests that the US11 TMD forms multiple contacts, each of them required for MHCI heavy chain dislocation. Gln192 in the US11 TMD may act in a manner similar to the Gln residues in the E5 and II TMDs by promoting the association of US11 with other ER membrane proteins involved in dislocation. However, the binding of US11 to its one known ligand, the MHCI heavy chain, is not dependent on Gln192. We are currently investigating the nature of the interactions mediated by the US11 TMD and potential cellular proteins that associate with US11 in a Gln192-dependent manner.

What sort of interaction might the US11 TMD form within the ER membrane? Because of the rapid kinetics of US11-induced degradation, there may be a direct interaction between US11 and a component of the dislocation machinery. Candidates include the Sec61 complex or its associated components. Such an interaction would place US11 in an ideal location for catalyzing dislocation through the Sec61 channel. The US11 TMD could influence the lateral gating of a proteinaceous channel such as Sec61, facilitating extraction of the MHCI heavy chain membrane anchor. Of note, when ubiquitin conjugation is blocked, MHCI heavy chains are not dislocated, but form complexes with US11 that remain in the ER (Kikkert et al., 2001). We observe similar stable complexes that form between US11 Q192L and MHCI heavy chains. This similarity indicates that the US11 TMD positions the MHCI heavy chain for ubiquitin conjugation, possibly by forming interactions with the ER-associated components of the ubiquitin conjugation machinery.

US11 is not itself a substrate for the ER-to-cytosol dislocation pathway, suggesting that US11 directs dislocation in trans via interactions with cellular components. We observe US11-MHCI heavy chain complexes that form after MHCI synthesis, but are rapidly lost with kinetics that resemble those of dislocation to the cytosol (Figure 3B). Such a US11-MHCI heavy chain complex may be a dislocation intermediate that forms within the ER before exposure to the cytosol. We hypothesize that the interactions mediated by the US11 TMD are required for bringing the MHCI heavy chain into contact with the machinery that physically removes the MHCI heavy chain from the ER (Figure 6). When the inter-
actions formed by the US11 TMD are disrupted, MHCI heavy chains can no longer access this machinery. Consistent with this hypothesis, we observe stable association of MHCI heavy chains with US11 Q192L, whereas MHCI heavy chains associate only transiently with wild-type US11 (Figure 3B).

We propose that US11 and its functional analog, US2, act as molecular links between MHCI heavy chains and the proteins that remove misfolded cellular proteins from the ER. The normal recognition and targeting machinery that acts on misfolded cellular substrates may thus be bypassed. Although both US2 and US11 bind to their MHCI substrate through their luminal domains and trigger a similar series of biochemical events, the elements of the MHCI heavy chain that are recognized by US2 are different from US11. US11 preferentially recognizes incompletely folded MHCI heavy chains (J. Loureiro and B.L., unpublished observations) in contrast to US2, which recognizes a folded MHCI-β₂m complex (Gewurz et al., 2001). Unlike US11, US2 is itself a substrate of the ER-to-cytosol dislocation pathway and US2 may accompany the MHCI heavy chain on its way out of the ER (Wiertz et al., 1996b). The differences in the details of US11- and US2-mediated dislocation indicate that these viral proteins may use distinct mechanisms to cause MHCI heavy chain degradation. The definition of US11 and US2 mutants that are defective in dislocation will aid in the identification of the cellular proteins involved in this remarkable reaction.

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