The Degradation of Membrane Proteins from 
The Mammalian Endoplasmic Reticulum

by

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B.S. Animal Systems
B.S. Microbiology
Louisiana State University, 1995

Submitted to the Department of Biology in partial fulfillment
of the requirements for the degree of

Doctor of Philosophy in Biology
at the
Massachusetts Institute of Technology

May 2003

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Abstract

Membrane glycoproteins of the secretory pathway that cannot adopt their native
conformation are targeted for dislocation from the endoplasmic reticulum (ER)
membrane for subsequent degradation by the cytosolic proteasome. This thesis
investigates factors influencing the catalyzed destruction of MHC class I molecules by
the HCMV glycoproteins US2 and US11 and the degradation of the model substrate
TCRα.

The ER chaperone calnexin, implicated in glycoprotein folding, and the ER
chaperones protein disulfide isomerase and Ero1α, implicated in substrate disulfide bond
formation, were examined for their roles in protein dislocation. By targeting these ER
chaperones with siRNA constructs, the cellular levels of these ER chaperones were
significantly reduced. Nevertheless, the rates of degradation of TCRα and the US2- and
US11-catalyzed destruction of MHC class I molecules were similar to wild-type cells.

The Unfolded Protein Response (UPR) transcriptionally regulates ER chaperones
and is essential in S. cerevisiae for efficient degradation of model ER substrates. In
mammalian cells, neither the ATF6-dependent response nor the IRE1α-XBP-1-dependent
response of the UPR was found to be essential for efficient degradation of TCRα, US2,-
or US11-catalyzed destruction of MHC class I molecules. Interestingly, the ATF6
response, but not the IRE1α-XBP-1 response, is essential for cellular viability.

To better define the substrate requirements of US2 and US11, the 30 residue
cytoplasmic tail of MHC class I molecules was mutated. US2 can degrade MHC class I
colourless with a cytoplasmic tail shortened to 10 residues or lengthened, by the fusion of
GFP to the C-terminus, to several hundred residues. In contrast, US11 only degrades MHC class I molecules possessing a cytoplasmic tail of 30 amino acids. These data support a model that US2 and US11 act through distinct degradative mechanisms.

To define modular functional domains of US2 and US11, lumenal or cytoplasmic domains were reciprocally exchanged between the viral molecules and between each viral molecule and their degradation substrate, the HLA-A2 heavy chain. Most chimeric molecules were not targeted for degradation when expressed alone or with their complementary construct. However, the US11 molecule in which the cytoplasmic tail was exchanged for that of HLA-A2 was rapidly dislocated from the ER to the cytosol. In addition, lumenal GFP possessing the transmembrane domain of US11 and the cytoplasmic tail of HLA-A2 was rapidly dislocated from the ER to the cytosol. Mutation of the critical glutamine residue in the transmembrane domain of US11 essential for destruction of MHC class I molecules resulted in a marked stabilization of the GFP construct.

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Acknowledgements

I would like to thank Hidde Ploegh for the invaluable education he has provided me over the past six years. His experimental insight and elegant turn-of-phrase are but two of his qualities I will spend a career attempting to surpass. Under his watchful eye, I have learned to swim. I am particularly grateful to Hidde for the wonderful people he has brought to the lab, and for the many experiences I have been afforded by this diverse array of unique individuals.

I owe a great debt to Domenico Tortorella, who was always there to guide, cajole, harass, and encourage me. Dom, you will be a great advisor. I would like to thank Luk Van Parijs, for showing me the light at the end of the tunnel. I thank Dennis Ingram and Ronald Siebeling, for starting me on the path that ends (and begins) here, and Herb Oshiva, for alternate points of view.

For the work and the play, I thank the Ploegh lab members who have made such a difference: Karen Flynn, for three extra years of grad school; Rocco Casagrande, for years of obscure conversations and lunch; Dina Gould, for keeping our bay moving; Madelon Maurice, for sharing brains; Margo and Mayra, for keeping the reputation strong; Daniël, Jay, Joana, and Shahram, the New Kids, for making my last days as fun as the first.

I would like to thank my mom and dad, for always encouraging and supporting me. I dedicate this thesis to my father, Joseph Stern, and to my mother, Cynthia. In particular, I would like to thank Lakshmi Balachandra, my wife, for her strength, focus, and insightful edits. Lak, you have been my inspiration.
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Chapter 1

The Degradation of Proteins from the Endoplasmic Reticulum
Introduction

Protein synthesis is an imperfect process. About forty percent of all proteins are destroyed co-translationally or within minutes of their synthesis by the degradation machinery present in the cytoplasm (Schubert, et al., 2000). Approximately one-fifth of the proteins encoded by the human genome are predicted to be secretory proteins, which are segregated either partially or completely from the cytoplasm behind a membrane-limited compartment (Lander, et al., 2001). The efficient elimination of misfolded polypeptides from the secretory pathway requires their mobilization across a lipid membrane for disposal in the cytoplasm (Brodsky, et al., 1999b). The process of substrate selection and removal presents an intriguing topological conundrum.

Proteins of the secretory pathway may be degraded for a number of reasons. A protein may be unable to adopt a proper tertiary or quaternary structure due to genetic lesions or to the absence of essential cofactors. These defects render the polypeptides unable to pass quality control in the endoplasmic reticulum (ER) and subsequent targets for degradation (Ellgaard, et al., 1999). Alternatively, a key protein in a biosynthetic pathway may be subject to regulated degradation as a means of maintaining cellular homeostasis. The aim of this thesis is to contribute to a better understanding of the mechanisms that target membrane proteins in the ER for destruction.

Protein folding and quality control in the ER

Most secretory proteins enter the secretory pathway by virtue of their N-terminal cleavable signal sequence that targets the nascent polypeptide to the ER. As the N-
terminus of the polypeptide emerges from the translating ribosome, the signal recognition particle (SRP) binds the signal sequence, arrests translation, and directs the complex to the signal recognition particle receptor at the ER membrane. The ribosome then associates with a proteinaceous pore in the membrane, the Sec61 complex, and the polypeptide is co-translationally translocated into the ER (Matlack, et al., 1997). The nascent protein encounters a number of chaperone complexes that facilitate import and mediate post-translational modifications that assist the immature polypeptide in adopting its properly folded conformation (Fewell, et al., 2001).

Proteins of the secretory pathway are subject to quality control processes in the ER. The processes assess each protein’s folding status and thereby determine its fate. The formation of proper disulfide bonds is essential for a protein to pass ER quality control. The ER provides an oxidizing environment by maintaining the requisite balance between oxidized and reduced glutathione (Hwang, et al., 1992). Although glutathione is the primary component sustaining the oxidizing redox-potential of the ER lumen, glutathione is dispensable for disulfide bond formation. Instead, the ER contains several resident protein catalysts, generally termed thiol-oxidoreductases, containing the thioredoxin CXXC motif that catalyze disulfide bond formation and disulfide bond reshuffling of protein substrates. Protein disulfide isomerase (PDI) has been shown to be essential for oxidative protein folding in yeast (LaMantia and Lennarz, 1993). PDI, in its oxidized state, can catalyze formation of disulfides within a protein (Lyles and Gilbert, 1991). In its reduced state, PDI can catalyze disulfide rearrangement in a polypeptide that contains both oxidized and reduced thiols (Laboissiere, et al., 1995). Reduced PDI is regenerated to an oxidized state by the transfer of oxidizing equivalents from the ER-
resident protein ERO1 (ER oxidation 1) (Frand and Kaiser, 1998), (Frand and Kaiser, 1999), (Pollard, et al., 1998). How the oxidation state of ERO1 is regenerated has yet to be fully elucidated, but the chaperone may utilize FAD-catalyzed transfer of electrons to molecular oxygen as a terminal electron acceptor (Tu and Weissman, 2002). *S. cerevisiae* possesses a single ERO1 gene product and mammals possess two isoforms, Ero1α (Cabibbo, et al., 2000) and Ero1β (Pagani, et al., 2000). Ero1α is constitutively expressed, whereas Ero1β expression is induced by stress in the ER, such as the accumulation of misfolded proteins.

Many nascent secretory proteins are covalently modified by the addition of a branched core oligosaccharide to asparagine residues (N-linked glycosylation) within the consensus sequence Asn-X-Ser/Thr (where X cannot be Pro). Oligosaccharide assembly begins in the cytosol, where Man₃GlcNAc₂-dolicholpyrophosphate is synthesized and translocated to the lumenal side of the ER by the flippase Rft1 (Helenius, et al., 2002). In the ER, the carbohydrate is elaborated by glycosyltransferases to generate Glc₃Man₉GlcNAc₂ that is transferred *in toto* to the acceptor site of the polypeptide. The carbohydrate moiety serves various functions depending on the cellular localization of the polypeptide. At the cell surface, carbohydrates may modulate protein-protein interactions and protect polypeptides from proteases (Moody, et al., 2001). In the ER, N-linked glycosylation facilitates protein folding by serving as a signal for chaperone association with the immature polypeptide.

After transfer to the nascent protein, the Glc₃Man₉GlcNAc₂ oligosaccharide is exposed to the successive actions of the ER-resident enzymes glucosidase I and II. These enzymes remove the terminal first and second glucose residues, respectively, to yield a
monoglucosylated oligosaccharide. The monoglucosylated moiety is a target for the
lectin-like chaperones calnexin (CNX) and calreticulin (CRT) (Hammond, et al., 1994).
Membrane bound CNX and soluble CRT have overlapping substrate specificities dictated
in part by their differing cellular localizations. The initial interaction of lectin with the
monoglucosylated glycan leads to protein-protein interactions between the chaperone and
the immature glycoprotein that facilitates proper folding of the polypeptide. This
interaction of lectin and glycoprotein is terminated by the action of ER-glucosidase II as
it removes the single terminal glucose. If the released glycoprotein has adopted the
proper conformation it can progress through the secretory pathway. If the glycoprotein
has not folded properly, UDP-glucose:glycoprotein glycosyltransferase (UGGT) will act
on it by catalyzing the addition of a single glucose residue to the N-linked glycan of
misfolded polypeptides, making the glycoprotein a substrate for CNX/CRT interaction
(Figure 1)(Trombetta and Helenius, 2000), (Parodi, 2000). The glucosylation cycle
retains incompletely folded glycoproteins in the ER until the substrate has either properly
folded or is targeted for degradation (Figure 1).

Although integral to glycoprotein folding, the lectin-like chaperones do not
appear to be essential. Cell lines that lack CNX or CRT are viable under standard tissue
culture conditions. Transgenic mice with genetic deletions of CNX are viable, but
develop debilitating tremors by 12 weeks of age (Denzel, et al., 2002). CRT knockout
mice die during embryonic development due to a failure of cardiac development
(Mesaeli, et al., 1999). Different protein folding pathways are physically interconnected.
Interaction with CRT and CNX exposes the immature glycoprotein to the action of the
associated chaperone Erp57, a thiol-oxidoreductase of the PDI family (Figure 1)(Oliver, et al., 1997).

Other ER resident molecules assist protein folding and assembly. The ER chaperone BiP (immunoglobulin binding protein), a member of the hsp70 family, binds exposed hydrophobic regions of the nascent polypeptide as it is translocated into the lumen of the ER and thereby acts as a “molecular ratchet” to ensure mono-directional movement of the protein into the ER (Panzner, et al., 1995). Interaction of BiP with exposed hydrophobic regions also prevents intra- and inter-molecular aggregation of immature ER proteins (Getling, 1999). Proteins that have oligosaccharides within about 50 residues of their amino-terminus interact with the CNX/CRT system without prior binding to BiP (Molinari and Helenius, 2000). Glycoprotein 96 (GP96), a member of the hsp90 family, is essential for the proper multimeric assembly and export of some members of the integrin and toll-like receptor protein families (Randow and Seed, 2001). In the absence of GP96, the unassembled monomers are retained in the ER.

The Unfolded Protein Response and signaling from the ER

Under certain physiologic conditions, a significant increase in secretory load may exceed the protein-folding capacity of the ER, such as the differentiation of B-cells into antibody secreting plasma cells. The ER must respond to the stress of increased secretory load or risk organelle dysfunction. Certain cell types are particularly vulnerable and a failure to adequately respond to ER stress can result in pathologic conditions, such as stress-induced pancreatic β-cell apoptosis and diabetes (Oyadomari, et al., 2002). The cellular response to ER stress is termed the Unfolded Protein Response (UPR). The UPR
Figure 1.

The calnexin-calreticulin cycle. When two of the glucose residues in the N-linked core glycans have been trimmed away by glucosidases I and II, the nascent or newly synthesized glycoproteins bind to CNX and/or CRT. The protein is thereby exposed to another folding factor, Erp57, a thiol oxidoreductase that binds both CNX and CRT. When the remaining third glucose is trimmed by glucosidase II, the complexes dissociate. If the glycoprotein is not folded at this time, the oligosaccharides are reglucosylated by an ER glucosyltransferase (UGGT), and the protein reassociates with the lectins. The cycle is repeated until the protein is either folded or degraded. Once correctly folded, a glycoprotein is no longer recognized by the glucosyltransferase, and because it is no longer glucosylated, it will not bind back to CNX and/or CRT. It can now exit the ER. Exit of certain glycoproteins from the ER to the Golgi is facilitated by the lectin ERGIC-53, which binds mannose residues. The CNX/CRT cycle promotes folding, inhibits aggregation of folding intermediates, blocks premature oligomerization, regulates ER degradation, and provides quality control by preventing incompletely folded glycoproteins from exiting to the Golgi complex.
was initially defined in *S. cerevisiae* as an increased concentration of ER chaperones in response to agents that result in protein misfolding, such as tunicamycin that inhibits oligosaccharide synthesis or the reducing agent dithiothreitol that disrupts the ER redox environment (Mori, et al., 1996), (Chapman, et al., 1998). Subsequent experiments in yeast revealed UPR signaling to be essential for efficient degradation of proteins from the ER and for stress-induced remodeling of the secretory pathway, as part of a global transcriptional response (Casagrande, et al., 2000), (Travers, et al., 2000).

In yeast, UPR signaling is initiated by a transmembrane ER-resident kinase Ire1p (Inositol REquiring), which was initially identified as a gene required for inositol auxotrophy (Cox, et al., 1993). This kinase is maintained in a monomeric state by association of the ER chaperone Kar2p (Karyogamy 2, the yeast BiP homolog) with the lumenal portion of Ire1. As unfolded proteins accumulate in the ER and sequester ER chaperones, BiP is titrated away from Ire1p. The lumenal domains of free Ire1p homodimerize, which induces trans-autophosphorylation by the cytoplasmic kinase domain (Shamu and Walter, 1996). The phosphorylation event unmasks a cytoplasmic endoribonuclease activity that removes a 26-base pair intron from the mRNA encoding the transcription factor Hac1p (Kawahara, et al., 1998). The cleaved mRNA is then ligated by tRNA synthase (Sidrauski, et al., 1996).

The protein product of the unspliced Hac1 mRNA is rapidly degraded. Removal of the intron from Hac1 generates a frame shift in the coding sequence that results in a significant stabilization of the protein and greatly increased transcriptional transactivation activity. Hac1p is solely responsible for the increase of ER chaperones and other components that constitute the UPR (Shamu, 1997).
Mammals possess two signaling pathways for responding to increased secretory load. The initial response to increased ER stress is initiated by ATF6 (Yoshida, et al., 2003). ATF6 is a type-II 90 kDa protein whose ER-localization is maintained by the interaction of BiP with the luminal portion of ATF6. Upon accumulation of unfolded proteins in the ER and subsequent titration of BiP from ATF6 to unfolded client proteins, ATF6 transits to the Golgi (Shen, et al., 2002). In the Golgi, S1P and S2P, the two proteases that also mediate the regulated intramembrane proteolysis (RIP) of the transcription factor SREBP (Sterol Regulatory Element Binding Protein) cleave the transmembrane region of ATF6 (Ye, et al., 2000). Intramembrane cleavage results in release of the 50 kDa cytoplasmic domain that enters the nucleus and activates transcription of ER chaperones (Haze, et al., 1999). Cleaved and uncleaved forms of ATF6 are present during steady-state conditions, indicating the ATF6 signaling pathway is constitutively active and helps maintain the steady-state concentrations of ER chaperones, as well as responding to increased ER stress.

In addition to increasing transcription of ER chaperones, ATF6 up-regulates XBP-1 (X-box Binding Protein-1)(Yoshida, et al., 2001) (Lee, et al., 2002). XBP-1 is the mammalian homolog to yeast Hac1p and encodes a highly unstable protein (Figure 2). Upon increased ER stress and the accumulation of misfolded proteins, the ubiquitously expressed mammalian homolog to yeast IRE1p, IRE1α, is activated in an identical fashion to IRE1p by the sequestration of BiP to ER client proteins and subsequent homodimerization (Bertolotti, et al., 2000). IRE1α splices an intron from XBP-1 mRNA to generate a transcript that encodes a relatively stable, potent transcription factor.

Spliced XBP-1 up-regulates transcription of ER chaperones, as well as genes distinct
Figure 2.

Both ATF6 and XBP-1 contribute significantly to the UPR. Transcriptionally active ATF6 is generated by cleavage of the membrane-bound precursor. ATF6 initiates induction of ER chaperone and XBP-1 genes via direct binding to the ERSE in response to ER stress. The spliced form of XBP-1(S) produced from induced XBP-1 mRNA by the action of activated IRE1α enhances transcription of ER chaperone genes further via heterodimerization with ATF6 and direct binding to the ERSE. In addition, spliced XBP-1 activates gene transcription via direct binding of the UPRE. Activation of ATF6 proteolysis is absolutely required for initiation of the UPR.
from those targeted by ATF6. The splicing reaction of XBP-1 has been termed a splice shift, as the excised intron results in a shift of the reading frame of the transcript. In addition to signaling through XBP-1, IRE1α can signal through binding of TRAF2 to the phosphorylated cytoplasmic domain of IRE1α, resulting in stress kinase (JNK) activation and apoptosis (Urano, et al., 2000).

Mammals also possess a second isoform of IRE1, IRE1β, whose expression is restricted to epithelial cells of the gut. Overexpression of IRE1β induces apoptosis through activation of its ribonuclease activity, which results in cleavage of 28S ribosomal RNA that leads to translation attenuation (Yoneda, et al., 2001),(Iwawaki, et al., 2001).

The ATF6 dependent response can buffer activation of the XBP-1-IRE1α pathway. The chaperone BiP regulates activation of the effectors of the pathways and BiP up-regulation by ATF6 can prevent IRE1α dimerization, thus attenuating the IRE1α response. One model presents the ATF6 pathway as sufficient to respond to minor disturbances in ER homeostasis, while significant perturbations require a maximal response that includes the XBP-1-IRE1α pathway (Yoshida, et al., 2003).

In addition to increasing the carrying capacity in the secretory pathway, the cellular response to ER stress also reduces flux through the pathway by attenuating translation. Accumulation of misfolded proteins induces activation of the ER kinase PERK, which phosphorylates the alpha-subunit of initiation factor 2 (eIF2α), which then results in decreased translation initiation (Shi, et al., 1998), (Harding, et al., 2000). Cells
lacking a functional PERK response are hypersensitive to ER stress. Naturally occurring human mutations in PERK result in Wolcott-Rallison syndrome, an autosomal recessive disease characterized by severe diabetes in infants (Delepine, et al., 2000).

A distinct stress response from the UPR is the ER overload response provoked by overexpression of certain client proteins or pharmacologic agents that result in activation of the transcription factor NF-κB (Pahl and Baueerle, 1997). The mechanism by which the overload response occurs and the physiologic role of this response remain unknown.

**The ubiquitin-proteasome system**

Despite their sequestration from the cytosol in membrane-limited compartments, proteins of the secretory pathway are subject to proteasomal degradation (Brodsky and McCracken, 1999a). Proteasomal proteolysis of cytosolic proteins generally proceeds through covalent modification of the substrate with ubiquitin that results in degradation of the substrate (Ciechanover, et al., 1984). Efficient disposal of proteins from the secretory compartment must overcome the topological and energetic barriers imposed by a membrane that separates the substrates from the cytosol. Ubiquitin is an integral factor in this process.

The 76 amino acid ubiquitin polypeptide is one of the most evolutionary conserved proteins among eukaryotes, with only three amino acid changes from yeast to human. By covalent conjugation to primary amines, ε-amino groups of lysine residues or the amino terminal residue of most proteins, ubiquitin serves as a molecular mediator of protein-protein interactions and as a signal for protein degradation (Breitschopf, et al., 1998), (Weissman, 2001). The regulated addition of a single ubiquitin moiety can
mediate protein interactions that result in receptor endocytosis, gene regulation, DNA repair, vesicle transport, and numerous other regulated cellular activities (Hicke, 2001). Covalent attachment of multimers of ubiquitin, composed of at least four monomers, generally serves as a signal for protein degradation (Thrower, et al., 2000).

Addition of ubiquitin to protein substrates proceeds through a highly regulated process. Free ubiquitin is first conjugated to an ubiquitin-activating enzyme, E1, through a thio-ester bond between the active site cysteine of the E1 and the C-terminal glycine of ubiquitin. The E1-bound ubiquitin is subsequently transferred to the active site cysteine of an ubiquitin conjugating enzyme, E2. A ubiquitin ligase, E3, usually dictates substrate specificity and mediates covalent attachment of the ubiquitin to the substrate protein by formation of an isopeptide bond (Figure 3A).

Ubiquitin may be covalently attached to the E3 and subsequently transferred to the target substrate, as occurs for E3s that contain HECT domains (Huibregtse, et al., 1995). The HECT (homologous to E6-AP carboxyl terminus) domain is a highly conserved 350 amino acid region that includes a conserved cysteine that forms a covalent thio-ester bond with ubiquitin. HECT was first identified in the p53 interacting cellular protein E6-AP, which catalyzes the ubiquitination of the cell cycle regulator p53 in the presence of the human papillomavirus oncoprotein E6 (Figure 3B) (Scheffner, et al., 1993), (Shi, et al., 1998).

Alternatively, the E3 may coordinate transfer of ubiquitin from the E2 directly to the substrate, as is true for E3s containing RING (Really Interesting New Gene) finger motifs (Joazeiro, et al., 1999). Two inter-leaved metal coordinating sites identify RING
Figure 3.

- Free ubiquitin (Ub) is activated in an ATP-dependent manner with the formation of a thiol-ester linkage between E1 and the carboxyl terminus of ubiquitin. Ubiquitin is transferred to one of a number of different E2s. E2s associate with E3s, which might or might not have substrate already bound. For HECT domain E3s, ubiquitin is next transferred to the active-site cysteine of the HECT domain followed by transfer to substrate (S) (as shown) or to a substrate-bound multi-ubiquitin chain. For RING-E3s, current evidence indicates that ubiquitin is transferred directly from the E2 to the substrate.

- Ternary complex of p53 with human papillomavirus E6 and E6-AP. Ubiquitin is transferred from the E2 to E6-AP, then to p53.

Complex of Mdm2 and p53.
Ubiquitin is transferred from the E2 directly to p53.

SCF^{Rncp} as a prototypical cullin-containing E3. The F-box recruits phosphorylated IκB for subsequent ubiquitination and degradation.
finger motifs and, although many have been described, few have been to shown to mediate ubiquitination of substrates other than of themselves (Freemont, 2000). Mdm2 is an E3 that binds to and coordinates the ubiquitination of p53 (Figure 3B) (Fang, et al., 2000). These activities of Mdm2 are highly regulated by modifications of the substrate, E3, and various interacting polypeptides (Shieh, et al., 2000), (Lohrum, et al., 2000), (Honda and Yasuda, 1999).

Multimeric E3 ubiquitin ligases distribute the activities of substrate selection and E2 recruitment among several subunits. These SCF complexes are composed of subunits Skp1, Cullin1, Rbx1, and an E-box (Lyapina, et al., 1998), (Page and Hieter, 1999). The cullin family proteins, acting as scaffolds, interact with linker proteins (Skp1) that recruit the substrate recognition components (F-box) (Bai, et al., 1996). The RING finger protein Rbx1 binds the cullin subunit and recruits E2 activity (Kamura, et al., 1999). The tetrameric complex may have multiple substrate specificities, and exchanging any one of the subunits may alter the substrate specificity of the complex (Figure 3B).

Fbx2, an F-box protein that recognizes N-linked high-mannose oligosaccharides and mediates substrate ubiquitination has recently been identified (Yoshida, et al., 2002). Overexpression of dominant-negative mutant versions can reduce the degradation rate of model substrates T-cell-receptor alpha (TCRα) and mutant cystic fibrosis transmembrane conductance regulator (CFTR). Interestingly, Fbx2 is expressed predominantly in neuronal cells in the adult brain. Overexpression in tissue culture cells has been shown to induce growth arrest.

Once a protein has been poly-ubiquitinated by the action of an E3 ligase, the substrate is degraded in a processive fashion by the proteasome. The proteasome is a
barrel-shaped multi-catalytic protease complex composed of four heptameric rings (the 20S particle) and a multi-subunit “cap” (the 19S cap) at each end (Kloetzel, 2001). The catalytic residues are contained in the two internal rings of β-subunits that are flanked by two external rings of α-subunits (Groll, et al., 1997). Unfoldases and ATPases present in the cap subunits unfold substrates and the extended polypeptides are fed through the central chamber where they are cleaved by the catalytic residues (Braun, et al., 1999). Each ring of β-subunits possesses three distinct catalytic activities that cleave the polypeptide after acidic, basic, or hydrophobic residues. As a polypeptide is unfolded by the proteasome, covalently attached ubiquitin moieties are released to the cytosol to be recycled (Borodovsky, et al., 2001).

There is some evidence that E3 ubiquitin ligases can be found in association with the proteasome, indicating coordination between substrate recognition and degradation. Similarly, a population of proteasomes is tethered to the ER membrane to facilitate degradation of proteins that fail to pass through ER quality control (Enenkel, et al., 1998).

**Dislocation of membrane proteins – a multi-step process**

To efficiently degrade a misfolded protein from the ER while sparing by-stander polypeptides, there must exist a selection process that marks the substrate for destruction. This degradation signal must occur by either direct post-translational modification of the substrate or intimate association with a co-factor that serves as a marker for destruction. The substrate, once marked for destruction, is recruited to sites in the ER where it may be extracted across the limiting membrane and discharged into the cytosol for degradation by the proteasome. The bulk of published data support a model that ubiquitination is
essential for dislocation of a substrate from the ER membrane (Jarosch, et al., 2002).

Given this ubiquitination requirement, the recruitment-extraction process likely involves at least two discrete steps.

First, either the polypeptide or the adapter with which it is complexed would be ubiquitinated on the cytoplasmic face of the membrane by the action of ubiquitin ligase complexes integral or peripheral to the ER membrane. Secondly, the ubiquitinated substrate would be recognized by the proteasome and/or associated factors in the cytosol and extracted from the membrane.

There are several examples of post-translational modification of protein substrates or adapters in mammals and yeast that act as signals for protein degradation. Modification of the N-linked glycan is an example of substrate modification serving as a signal for degradation. As the glycans of incompletely folded proteins in the ER are exposed to cycles of deglucosylation and glucosylation, the glycans are also exposed to the action of α-mannosidase. This ER-resident enzyme removes the terminal mannose of the Man₉GlcNAc₂ (Man9) oligosaccharide on misfolded proteins resulting in a Man₈ oligosaccharide (Liu, et al., 1999). This Man₈ structure is recognized by an ER-resident α-mannosidase-like protein that targets the protein for degradation (Jakob, et al., 1998). This protein, termed EDEM (ER degradation enhancing α-mannosidase-like protein) in the mouse and Mnl1p (mannosidase-like protein) in yeast, resembles mannosidases by sequence comparisons, but is catalytically inactive (Hosokawa, et al., 2001), (Nakatsukasa, et al., 2001). Presumably, EDEM/Mnl1 recruits the Man8 labeled polypeptide to the ER-associated ubiquitination machinery. EDEM and Mnl1p have been implicated in facilitating the destruction of glycoproteins while having no effect on the
turnover of non-glycosylated ER proteins (Jakob, et al., 2001). How EDEM/Mnl1 bridge glycoproteins to the ubiquitination machinery remains to be elucidated.

The degradation of the glycoprotein CD4 from the ER by HIV proceeds through a post-translational modification not of the substrate itself, but of the associated factor Vpu. CD4 is a cell-surface glycoprotein important for generating immune responses. Bound to the cytoplasmic domain of CD4, Vpu recruits an SCF ubiquitin ligase complex containing the F-box β-human transducin containing protein (βTrCP) (Figure 3B) (Margottin, et al., 1998). Substrate recognition by βTrCP is dependent on phosphorylated serine residues 52 and 56 in the motif DS(P)GXXS(P) present in Vpu, which results in ubiquitination of the cytoplasmic tail of CD4 and its subsequent dislocation from the ER and proteasomal destruction (Deshaises, 1999). Vpu recruits βTrCP by mimicking the physiologic substrates of βTrCP, β-catenin and IkBα, that are targeted for degradation. This is a prime example of a viral protein usurping a cellular pathway to facilitate viral pathogenesis.

Generally, the identities of the adapters or the natures of the signals of ER substrates recognized by the membrane-proximal ubiquitination machinery remain a mystery. Components of multiple ER-associated ubiquitin ligase activities responsible for substrate ubiquitination have been identified (Bays, et al., 2001), (Gardner, et al., 2001). The gene products identified participate in regulated degradation of biosynthetic enzymes to control cellular homeostasis as well as degradation of proteins that fail to pass ER quality control.

Yeast control sterol synthesis by the regulated degradation of the ER protein HMG-CoA reductase (HMGR), the rate-limiting enzyme in the mevalonate synthesis
pathway. Protein half-life is regulated by the cell's sterol requirements and can vary from less than 20 minutes to greater than 10 hours (Hampton and Rine, 1994). Several proteins essential for the turnover of HMG (HRD - HMG-CoA reductase degradation proteins) have been identified. The genes identified in these screens have been implicated in the destruction of misfolded polypeptides from the ER as well (Figure 4).

Hrd1p and Hrd3p are ER-anchored E3 ubiquitin ligases of the RING-finger motif family (Bays, et al., 2001). These two ubiquitin ligases form a complex that facilitates substrate ubiquitination by the associated E2 (Gardner, et al., 2001). The primary E2 is Ubc7p, and to a lesser extent Ubc1p (Hiller, et al., 1996). Ubc7p is a cytosolic ubiquitin-conjugating enzyme anchored to the ER membrane by the adapter Cue1p (Biederer, et al., 1997). In addition to regulated degradation, Hrd1 has been implicated in the degradation of the constitutively misfolded variant of the luminal protein carboxy-peptidase Y (CPY*) (Bordallo, et al., 1998). The degradation of CPY* requires the same components as the HMG-CoA degradation pathway, as well as several additional proteins required for degradation of luminal proteins. The additional components required for disposal of luminal proteins support a model in which multiple substrate recognition pathways converge on common membrane-ubiquitination components (Wilhovsky, et al., 2000).

Yeast Doa10p is another ER-anchored E3 ubiquitin ligase of the RING-finger motif family. It utilizes the E2s Ubc6p and Ubc7p and is essential for efficient degradation of a number of Hrd1p-independent ER substrates (Swanson, et al., 2001). Both Hrd1p and Doa10p degrade multiple substrates with no sequence similarity. The degradation of the ER-bound transcription factor Ole1p is not dependent on Hrd1p or
Figure 4. The dislocation of a membrane glycoprotein.

The N-linked glycan (CHO) of the misfolded polypeptide is trimmed by α-mannisodase to generate the Man8 form that is recognized by EDEM/Mnl1p. Mnl1p recruits the complex to the RING-finger E3s Der3p/Hrd1p and/or Hrd1p. The substrate is recruited to the Sec61p translocon for exit. The substrate is ubiquitinated by the E2s Ubc6 or Ubc7. BiP and other ER factors (such as thiol-reductases) assist the polypeptide in its egress to the cytosol. Cdc48p/ p97 present in the cytosol may actively extract the substrate from the ER (not pictured). After dislocation, the N-linked glycan is removed by peptide-N-glycanase and the ubiquitinated substrate is degraded by the proteasome. The sequence of events remains to be elucidated.)
adapted from Curr Opin Cell Biol 14 476 (2002)
Doa10, indicating other E3 ligase activities must be present in yeast (Braun, et al., 2002). The number of ER-localized ubiquitin ligases presents a model of multiple pathways to recognize and ubiquitinate the host of proteins present in the secretory pathway. Substrate ubiquitination then recruits protein complexes that mobilize substrates out of the membrane for degradation in the cytosol.

Yeast Npl4p is essential for efficient ER-associated degradation to proceed normally. Interestingly, Npl4p appears to act between the steps of substrate ubiquitination at the membrane and proteasomal degradation. In Npl4p mutants, HMG-CoA is stabilized, but ubiquitination of HMG-CoA reductase as well as other degradation substrates proceed normally (Bays, et al., 2001). Degradation of cytosolic proteins is not compromised, indicating an intact ubiquitin-proteasome pathway.

Mutants of the AAA ATPase cdc48 (p97 in mammals) also demonstrate a defect in ER-associated degradation similar to that observed for Npl4p. Protein ubiquitination proceeds normally and proteasome activity is unaffected, yet certain membrane-associated ubiquitinated substrates are not efficiently degraded (Rabinovich, et al., 2002). A mammalian model-degradation system utilizing US11-mediated dislocation of MHC class I molecules in a permeabilized cell system demonstrated that the addition of a dominant-negative version of p97 resulted in accumulation of ubiquitinated MHC class I heavy chains in the microsomal fraction, but not in the cytosolic fraction (Ye, et al., 2001). These data indicate that p97 has a role in extracting ubiquitinated proteins from the ER membrane.

In addition to membrane extraction, cdc48 may have a role in directly presenting ubiquitinated substrates to the proteasome. Cdc48 was shown to associate with the
proteasome in an ATP-dependent manner (Verma, et al., 2000). In addition, Cdc48 has been reported to physically associate with tetra-ubiquitin chains, but not with mono-, di-, or tri-ubiquitin (Dai and Li, 2001). Ubiquitinated substrates at the ER membrane may recruit Cdc48, which facilitates substrate extraction and localizes proteasomes near the site of export.

Ufd1 (ubiquitin fusion degradation) mutants also demonstrate a post-ubiquitination, pre-proteasomal defect in ER protein degradation. In the degradation of the luminal protein CPY*, membrane-associated ubiquitinated intermediates were recovered, but no cytosolic ubiquitinated intermediates could be visualized (Jarosch, et al., 2002). Ufd1p, cdc48p, and Npl4p have been isolated in a complex, indicating that these three proteins may form a functional complex that mobilizes ubiquitinated membrane-bound substrates into the cytosol (Meyer, et al., 2000).

One intriguing question of the dislocation process is the conduit through which the substrate traverses from lumen to cytosol. The most likely candidate, in fact the only candidate, is the Sec61 translocon. As proteins are translated into the ER, accessory proteins of the Sec61 pore complex assist the polypeptide in translocation and membrane integration. Transport of ER proteins out of the ER (also referred to as dislocation) may be akin to this process, in reverse.

The role for Sec61 in protein dislocation was first demonstrated in the US11-catalyzed dislocation of MHC class I molecules (Wiertz, et al., 1996b). The dislocated substrates were found in association with the Sec61 complex (Wiertz, et al., 1996a). In yeast, the dislocation of misfolded soluble and membrane proteins is sensitive to mutations in Sec61p (Plemper, et al., 1997), (Plemper, et al., 1999). Two regions of
Sec61p have been identified that are important for dislocation (Zhou and Schekman, 1999), (Wilkinson, et al., 2000). However, no one has identified a dislocation substrate receptor that would recruit substrates to the Sec61 complex.

Purified complexes indicate the pore size of the inactive complex to be about 20 angstroms, while the actively-translocating ribosome-bound pore complex is estimated to dilate to 40-60 angstroms (Hanein, et al., 1996), (Hamman, et al., 1997). Whether this increased pore size is due to conformational changes induced in the 3 - 4 molecule complex or if other subunits are recruited remains to be elucidated. One question for Sec61-mediated dislocation of polypeptides is the physical size of the polypeptide as it enters the lumen versus as it exits. Nascent proteins are translocated into the ER as extended polypeptides that have not yet achieved significant secondary structure. In the ER, domains fold into conformations whose volumes are considerably larger than that of an extended polypeptide. Intact folded polypeptides can exit the ER, raising the possibility that the translocon adopts a dilated conformation to facilitate protein egress (Fiebiger, et al., 2002).

During dislocation, thiol oxido-reductases may reduce substrate disulfides to relax the polypeptide into a more extended conformation. The role of oxido-reductases in catalyzing disulfide bond formation during protein folding is well established. Their role in reducing disulfide bonds to facilitate protein egress has only been demonstrated in a process arguably akin to the dislocation of ER substrates. Several bacterial and plant toxins are transported in retrograde fashion from the cell surface through the secretory pathway to ultimately reside in the ER, where the active toxins are dislocated from the ER to the cytosol (Tsai, et al., 2002). Once the toxin subunit is dislocated, its catalytic
activity can intoxicate the infected cell. This dislocation process from the ER has been likened to the route misfolded polypeptides follow. However, the toxin is not degraded by cytosolic proteasomes, therefore, the toxins have a strategy for being dislocated while avoiding degradation. This strategy may be as simple as a paucity of lysines and very rapid refolding, or it may constitute a functionally distinct pathway for ER exit (Rodighiero, et al., 2002).

For cholera toxin, the dimeric holotoxin traffics from the cell surface to the ER. Dislocation of the catalytic A1 subunit into the cytosol mediates intoxication. The A1 chain is released from the holotoxin by the action of PDI, resulting in a mixed disulfide linkage between A1 and PDI (Tsai, et al., 2001). The covalent A1-PDI complex is then reduced by Ero1, which oxidizes PDI and subsequently releases the A1 chain (Tsai and Rapoport, 2002). The toxin subunit is then dislocated to the cytosol. This process identifies PDI in breaking disulfide bonds to prepare a protein substrate for exit from the ER, but this same role for PDI in dislocation of model degradation substrates has yet to be demonstrated.

MHC class I molecules and viral evasins — a model system for dislocation

The folding of MHC class I complexes is one of the best-studied examples of multimeric proteins of the secretory pathway in mammals. The complex consists of a single-pass transmembrane 43 kDa heavy chain, a soluble 12 kDa light chain β₂m (β-2-microglobulin), and an 8-10 residue peptide. The heavy chain encounters first calnexin, then the calreticulin/ERp57 complex and the light chain to form a dimeric complex. The chaperone tapasin then bridges the dimer to the Transporter Associated with antigen
Presentation (TAP) for assembly with the peptide. Peptides are generated in the cytosol by proteasomal destruction of cytosolic proteins and are actively transported into the ER by TAP. Once the dimer of heavy chain and light chain are assembled with the peptide, the trimeric complex is competent for export from the ER (Figure 5).

MHC class I complexes are expressed on the cell surface of all nucleated cells and provide a means for the immune system to survey the intracellular peptide repertoire and thereby detect infected cells that are producing foreign proteins. During infection, pathogen-derived peptides are presented on MHC class I molecules and are recognized by cytotoxic T cells which eliminate the infected cell.

Inevitably, intracellular pathogens have developed means to counteract MHC class I antigen presentation and thereby evade immune surveillance (Tortorella, et al., 2000). As the ER is the point of entry for many immune recognition molecules, pathogens have evolved to utilize this organelle for their own purposes. The human cytomegalovirus (HCMV) possesses at least four transmembrane glycoproteins that disrupt MHC class I antigen presentation (Ahn, et al., 1996). US3 binds and retains MHC molecules in the ER, preventing their egress to the cell surface (Jones, et al., 1996). US6 binds to TAP and inhibits peptide transport, disrupting presentation of cytosolic peptides (Ahn, et al., 1997). US2 and US11 bind MHC class I complexes and mediate their dislocation from the ER to the cytosol for destruction by the proteasome, eliminating MHC class I antigen presentation and thereby permitting HCMV infected cells to escape immune surveillance by cytotoxic T cells.
Figure 5. MHC class I assembly.

As it translocates into the ER, the MHC class I heavy associates with the chaperones BiP and CNX. The heavy chain is exposed to the action of the associated thiol-reductase Erp57. The heavy chain is assembled with the light chain (β_{2m}) and a peptide and is bound by CRT. The CRT/Erp57 complex escorts the class I trimer to the “peptide loading complex” that consists of the ATP-transporter TAP and the bridging chaperone Tapasin. TAP actively translocates peptides from the cytosol into the lumen of the ER. Lower-affinity peptides are displaced by high-affinity peptides in the MHC class I peptide binding groove. The peptides are derived from proteasomal proteolysis of ubiquitinated proteins in the cytosol. Once assembled, the MHC class I trimer is competent for egress from the ER.
adapted from Curr Opin Immunol15 75 (2003)
US2 or US11 can induce degradation of MHC class I molecules with rapid kinetics. The MHC class I heavy chains are degraded with a half-life of 10 minutes or less. This degradation rate is an order of magnitude faster than the degradation of other model substrates. For example, TCRα (T-cell receptor α) is degraded from the ER in the absence of its folding partner TCRβ with a half-life of approximately 90 minutes. US2 and US11 accomplish this rapid destruction in the absence of any other viral factors. Therefore, the viral proteins interact with some step of the ER quality control process to target the MHC class I molecules for dislocation.

The preponderance of data concerning US2 and US11 has focused on the similarities between the two, but data is emerging demonstrating distinct differences. Both molecules degrade the properly folded MHC class I trimer, but only US11 degrades the free heavy chain (J. Loureiro, submitted). This difference demonstrates that US2 and US11 recognize distinct epitopes on the MHC class I molecule. These two viral polypeptides provide a unique opportunity to study an ER dislocation reaction mediated by two distinct effectors, possibly utilizing two distinct disposal pathways. The adapter molecules and ubiquitination machinery utilized by these two viral molecules remain to be elucidated.

The degradation of proteins from the secretory pathway is a sequential multi-step process. How the ER disposal machinery recognizes misfolded polypeptides and recruits ubiquitination of these misfits is intriguing. Presumably adapter proteins bridge the “tagged” substrates to the ubiquitin ligase activity, but these molecules remain elusive. As the mammalian ER-associated ubiquitin ligases are being identified, insights from yeast pose a model of targeted substrate ubiquitination leading to membrane extraction
and degradation. How the substrate crosses the topological barrier from lumen to cytosol is puzzling. The aim of this thesis is to identify, in the US2/US11 system, the substrate, effector, and cellular requirements for efficient dislocation of MHC class I molecules from the endoplasmic reticulum.
References


Chapter 2

The role of ER chaperones in the degradation of membrane proteins
Abstract

The role of the ER chaperones calnexin (CNX), protein disulfide isomerase (PDI), and Ero1α in potentiating dislocation of ER substrates to the cytosol was addressed by a siRNA approach. Stable knockdown cell lines were generated by retroviral transduction. The HCMV glycoprotein US2 and US11, which catalyze the rapid destruction of MHC class I molecules, efficiently degraded their target substrate in each of the knockdown cell lines. Similarly, the degradation of TCRα was not significantly affected by lowered levels of these ER chaperones. PDI and Ero1α have been shown to be essential for the dislocation of cholera toxin from the ER. Possible differences between these two systems are discussed.
Introduction chaperone knockdowns

Nascent secretory proteins are inserted into the lumen of the endoplasmic reticulum (ER) where they must acquire the proper tertiary and quaternary structure to progress through the secretory pathway (Rajagopalan, et al., 1994). The environment of the ER possesses a host of resident chaperones that facilitate the nascent polypeptide in adopting its proper conformation (Braakman, et al., 1991). ER chaperones assist protein folding to overcome energetically unfavorable folding intermediates, as well as mediating post-translational modifications such as glycosylation and disulfide bond formation (Ellgaard and Helenius, 2003). Proteins that adopt their proper conformation egress from the ER. These folding misfits that are unable to fold properly are recognized by the ER quality control machinery and targeted for degradation. The misfolded polypeptide is discharged from the lumen of the ER to the cytosol for subsequent degradation by the proteasome (Brodsky and McCracken, 1999a).

Nascent proteins are translationally translocated into the lumen of the ER as an extended polypeptide through the Sec61 translocon, a proteinaceous conduit that facilitates ER entry and membrane insertion of translocating proteins (Matlack, et al., 1997). Experimental evidence implicates the Sec61 translocon as the conduit through which proteins targeted for degradation exit the lumen of the ER (Zhou and Schekman, 1999). As proteins entering the lumen of the ER are exposed to the actions of a number of chaperones to assist protein folding, it is reasonable to assume proteins exiting the ER are also exposed to the action of various chaperones to assist protein unfolding (Brodsky, et al., 1999b).
Many nascent transmembrane glycoproteins associate with the membrane-bound ER chaperone calnexin (Hammond, et al., 1994). Calnexin possesses a lectin-like activity and associates with mono-glucosylated protein substrates to assist these polypeptides in adopting their properly folded conformation (Hammond, et al., 1994). Protein substrates undergo a cycle of association and dissociation with calnexin through the action ER-enzymes glucosidase II and UDP-glucose:glycoprotein transferase (UGTR). The terminal glucose residue is cleaved by glucosidase II, releasing the substrate from calnexin (Trombetta and Helenius, 2000). If the substrate displays regions indicative of not having achieved a properly folded conformation, UGTR adds a single glucose residue to the N-linked glycan, which serves as a recognition signal for calnexin to bind to the substrate (Parodi, 2000).

Persistent association of a substrate with calnexin may target that substrate for proteasomal degradation, in a process referred to as “molecular capture” (Cabral, et al., 2000). Persistent association can be mediated by the action of the ER enzyme mannosidase I, which cleaves the terminal mannose residue from the Man9 glycan to generate the Man8 form (Hosokawa, et al., 2001). The Man8 glycan is a substrate for glucosylation by UGTR, but a poor substrate for deglucosylation by glucosidase II (Liu, et al., 1999). Thus, the Man8 form remains monoglucosylated and associates with CNX persistently. This association may recruit the substrate to ER export sites for dislocation from the ER, but the precise mechanism remains to be elucidated.

Protein disulfide isomerase (PDI) is an ER-resident chaperone that assists proteins in disulfide bond formation and reshuffling (Lyles and Gilbert, 1991), (Laboissiere, et al., 1995). PDI has been recovered in disulfide-bonded association with protein substrates in
the ER (Molinari and Helenius, 1999). Just as PDI assists protein folding upon substrate entry into the ER, the chaperone may assist protein unfolding upon protein exit during the dislocation reaction. Several bacterial and plant toxins utilize the ER-quality control and dislocation apparatus to dislocate the active subunit from the ER to the cytosol to mediate cellular intoxication (Tsai, et al., 2002). The dislocation of cholera toxin has identified PDI as an associated protein essential for mediating ER export (Tsai, et al., 2001). Thus, PDI may break disulfide bonds to facilitate export of a protein substrate into the cytosol for degradation.

Ero1α is an ER-resident oxidase essential for maintaining proper disulfide bond formation (Pollard, et al., 1998). The oxidized form of PDI transfers oxidizing equivalents to protein substrates to form disulfide bonds in the nascent polypeptide. In doing so, PDI is reduced. Ero1α oxidizes reduced PDI (Franda and Kaiser, 1999). The action of Ero1α may facilitate the pathogenicity of cholera toxin. Ero1-alpha has been reported to recruit the PDI-toxin complex to the ER membrane and to mediate dissociation of the PDI-toxin complex, which frees the toxin for dislocation to the cytosol (Tsai and Rapoport, 2002). Thus, Ero1α may be an essential component in the process of substrate disassembly preceding dislocation from the ER.

One method of ablating gene function is through small interfering RNAs. siRNAs provide an opportunity to study a gene product's contribution to a biological process (Elbashir, et al., 2001). First identified in C. elegans and later verified in higher eukaryotes, small 22-26 nucleotide RNA molecules complementary to a cellular transcript are able to efficiently target the cellular transcript for degradation, thereby eliminating gene function. Several methods have been devised for generating small
interfering RNA species in mammalian cells. Most utilize the well-defined transcription initiation and termination signals of RNA polymerase III to generate self-complementary hairpin RNAs. These RNAs are processed by undefined cellular machinery to give small single-stranded RNAs that can efficiently silence complementary cellular transcripts (Brummelkamp, et al., 2002). The RNA polIII constructs can be incorporated into retroviral vectors, allowing stable production of siRNAs in tissue culture cells (Brummelkamp, et al., 2002).

In this study, the role of calnexin, PDI, and Ero1α in facilitating the dislocation of ER proteins to the cytosol is addressed by targeting the chaperones with retroviral siRNA constructs. The effects of chaperone gene “knockdowns” on dislocation are explored utilizing TCRα and US2/US11-catalyzed dislocation of MHC class I heavy chains as model ER degradation substrates. TCRα is degraded from the ER in the absence of its folding partner TCRβ with a half-life of 90 minutes (Huppa and Ploegh, 1997). The human cytomegalovirus glycoproteins US2 and US11 catalyze the dislocation of MHC class I molecules from the ER with a half-life of 10 minutes (Tortorella, et al., 2000). This approach allows us to address differences between the slow degradation kinetics of TCRα and the rapid kinetics of US2/US11, as well as differences that may arise between the pathways utilized by US2 and US11 to destroy MHC class I molecules.
Materials & Methods

DNA Constructs

The multiple cloning site (mcs) of the retroviral vector pLNCX (Clontech, Palo Alto, CA) was expanded from three to eight unique sites to generate the vector LNm. The Woodchuck Post-transcriptional Response Element (WPRE) was inserted into the ClaI site located 3' to the expanded mcs in LNm to produce the vector LNW. The WPRE mediates nuclear export of unspliced transcripts driven by the LTR and CMV promoter in LNm, thus increasing protein expression.

The siRNA expressing retroviral vector pRETRO-SUPER (pRS) was a gift from T. Brummelkamp (Brummelkamp, et al., 2002). For each gene targeted by siRNA, three different regions of the coding sequence were tested and their efficacy determined empirically. The oligos corresponding to the targeted sequences in: calnexin: C1 – CCAGATACCACTGCTCCTC, C2 – CAAGCCCTTCTGTGTTGAC, C3 – TGGGAGGCTCCTCAGATTG; Ero1α: E1 – CCATGTCAATCTGATGAAG, E2 – TGTGAACAGCTGAAGC, E3 – GGAGAAGGCTCAAGAAGAGG; PDI: P1 – GCTGAAGGCAAGGGTTCC, P2 – GGGCAAGATCTGTTTCATC, P3 – GGTGCTTGGTTGGGAAGAAC.

Cell lines, reagents, and antibodies

U373-MG astrocytoma cells were transduced with LNm expressing TCRα or LNW expressing US2 or US11. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum and 5% calf serum and 0.5μg/ml geneticin (Gibco, Frederick, MD). U373 cells expressing the model substrate of interest
were subsequently transduced with pRS expressing the siRNA of interest. Transduced cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% fetal calf serum and 5% calf serum, 0.5mg/ml geneticin (Gibco, Frederick, MD), and 0.4μg/ml puromycin. The anti-heavy chain serum (Tortorella, et al., 1998), anti-TCRα serum (Huppa and Ploegh, 1997), W6/32 (Parham, 1979), the calnexin-specific monoclonal AF8 (Rajagopalan, et al., 1994), anti-US2 (Wiertz, et al., 1996a), anti-US11 (Wiertz, et al., 1996b), and anti-PDI (Fiebiger, et al., 2002) were used as described. Proteins consisting of the full-length of p97 and the lumenal domain of Ero1α were expressed in bacteria. Rabbits were immunized with the recombinant proteins and polyclonal antisera were obtained. Anti-β-actin monoclonal antibody and N-ethylmaleimide was purchased from Sigma Chemical Co (St. Louis, MO).

Metabolic labeling of cells and pulse-chase analysis

Cells were detached from their substrate by trypsin treatment, followed by starvation in methionine/-cysteine-free DMEM for 45 min at 37°C. Cells were metabolically labeled with 500 μCi/ml of [35S] methionine/ cysteine (1200 Ci/mM; NEN-Dupont, Boston, MA)/ml at 37°C for the times indicated. In pulse-chase experiments, cells were radiolabeled as above and were chased for the times indicated in DMEM containing non-radiolabeled methionine (2.5 mM) and cysteine (0.5 mM). Cells were then lysed in NP-40 lysis buffer (10 mM Tris pH 7.8, 150mM NaCl, 5 mM MgCl2, 0.5% NP-40) supplemented with 1.5 μg/ml aprotinin, 1μM leupeptin, 2mM phenylmethylsulfonyl fluoride (PMSF) followed by immunoprecipitation (see below). For cells lysed in 1%
SDS, the final concentration of SDS was adjusted to 0.067% with NP-40 lysis mix prior to immunoprecipitation.

**Immunoprecipitation**

Following cell lysis, debris was removed by centrifugation at 15,000g for 5 min. Non-specific binding proteins were removed from the cell lysates by the addition of 3µl/ml normal rabbit serum, 3 µl/ml normal mouse serum and formalin-fixed, heat-killed *Staphylococcus aureus* for 1 h at 4°C. Immunoprecipitation was performed by incubation with antiserum for 45 min at 4°C, followed by addition of *S.aureus* for 45 min at 4°C. The pelleted *S.aureus* were washed four times with washing buffer (0.5% NP-40 in 50 mM Tris pH 7.4, 150 mM NaCl and 5 mM EDTA). The pellet was resuspended in SDS sample buffer (4% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.025% bromophenol blue in 62.5 mM tris pH 6.8) and the released materials were subjected to 12.5% SDS-PAGE.

**Generation of cell lines by retroviral transduction**

To produce VSV-G pseudotyped retrovirus, HEK 293 cells were plated in HEK 293 media to yield 50% confluent monolayers the following day. Cells were transiently transfected with GagPol, VSV-G and retroviral vectors expressing the cDNA of interest at a ratio of 3:2:5 using Fugene 6 (Roche) following the manufacturer's guidelines. The media was changed 16-20 hours after transfection. The following day, supernatant was collected and used to infect target cells.
For infection, target cells were plated the day before infection in 6-well dishes at 2x10⁵/well. Retroviral supernatants were filtered through 0.8-μm syringe filters and diluted 1:2 with DMEM+10% FCS plus 4μg/ml (final conc) hexadimethrine bromide (Sigma Chemical Co.) to produce the retroviral cocktail. The U373 media was aspirated and 1ml of infectious cocktail added to each well of plated U373 cells. Plates were centrifuged in a Beckman Allegra 6KR centrifuge for 2 h at 1000 x g at 30°C. After 2 h, plates were moved to a humidified CO₂ incubator overnight. The following day, the cocktail was replaced with DMEM+10% FCS. Drug selection was imposed 2 days post infection.
Results

Generation of calnexin knockdown cell lines

The ER chaperone calnexin (CNX) interacts with nascent glycoproteins and assists these substrates in adopting their properly folded conformation. Pharmacologic inhibition of CNX-substrate interactions by inclusion of inhibitors of glycosyl transferases has indicated CNX may play a role in protecting misfolded substrates from degradation (Kearse, et al., 1994). Other evidence indicates that CNX may be important for targeting misfolded substrates for degradation (Liu, et al., 1999). To define the role of CNX in the degradation of TCRα and the US2- and US11-catalyzed destruction of MHC class I molecules, cell lines stably expressing siRNA constructs targeting CNX (CNX knockdowns) were constructed.

U373-MG cells were transduced with pRetro-Super (pRS; Brummelkamp.), a retroviral vector conferring puromycin resistance and expressing small, hairpin RNAs (siRNA) from a RNA polymerase III promoter. Three different siRNAs were tested for their efficacy in lowering expression of CNX (Figure 1A). The immunoblot for CNX of cells expressing either empty pRS or one of three siRNAs (C1, C2, C3) demonstrates the constructs effectively lowered cellular levels of CNX. Although all three siRNA constructs appeared to lower the steady-state levels of CNX compared to empty vector (pRS), C1 had the most robust activity and was utilized in subsequent experiments.

C1 efficiently silenced CNX expression in US2- and US11-expressing cells (Figure 1B). U373-MG cells stably expressing US2 or US11 were transduced with pRS-C1 and cells were selected for puromycin resistance for one week. Cells were metabolically labeled for 30 minutes, lysed, and subjected to immunoprecipitation and
Figure 1. Calnexin is efficiently knocked-down in US2- and US11-expressing cells.

A) An immunoblot for CNX on 40 μg of SDS lysate prepared from each knockdown cell line. MG-U373 cells were transduced with empty pRS or pRS expressing 1 of 3 CNX knockdown constructs. C1 most efficiently reduced CNX levels.

B) IP of CNX from SDS lysates of metabolically labeled cell lines. MG-U373 cells were transduced and selected with LNW-US2 or LNW-US11. Cells were subsequently transduced with empty pRS or pRS-C1 and metabolically labeled for 30 min. Cells were lysed and immunoprecipitated for CNX and p97. CNX levels are significantly reduced in pRS-C1 cell lines. The p97 IP serves as a protein loading control.
A) **Calnexin knockdowns**

pRS  C1  C2  C3

Blot: anti-calnexin

B) **US2 cells**

<table>
<thead>
<tr>
<th>US2 cells</th>
<th>pRS</th>
<th>pRS-C1</th>
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Pulse 30 min
IP: anti-CN

**US11 cells**

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<thead>
<tr>
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<td>3</td>
<td></td>
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<tr>
<td>4</td>
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</tbody>
</table>

Pulse 30 min
IP: anti-CN

IP: anti-p97

IP: anti-p97
SDS-PAGE analysis. Lanes 1 - 4 are immunoprecipitates of CNX from 50,000 TCA-precipitable DPM of lysates. C1 significantly lowered CNX expression in both cell lines tested.

The following experiments examined the role of CNX in US2- and US11-mediated dislocation of MHC class I molecules (Figure 2). Cell lines expressing the constructs of interest were pulsed for 12 minutes, chased for the indicated times, lysed, and subjected to immunoprecipitation and SDS-PAGE analysis. Lowered CNX expression did not significantly affect the rate of US2- or US11-catalyzed degradation of MHC class I molecules (compare lanes 1-3 with 4-6 and 7-9 with 10-12).

Knockdown of PDI and Ero1α

The ER chaperones PDI and Ero1α have been implicated in catalyzing disulfide bond formation in newly synthesized ER client proteins. Their role in dislocation of folding misfits from the ER to the cytosol has been inferred from their activity in facilitating dislocation of cholera toxin from the ER. However, the role of these two chaperones in the dislocation of model ER substrates has not been addressed directly. To this end, constructs expressing siRNAs to PDI and Ero1α were constructed and transduced into U373-MG cells. Puromycin resistant cells were selected for one week and assayed by immunoblot for efficient knockdown of PDI or Ero1α.

SiRNA constructs directed against PDI and Ero1α could efficiently silence these genes (Figure 3). An immunoblot for PDI in cells containing empty pRS or the siRNA constructs demonstrates PDI was efficiently knocked down by pRS-P1 and P2, but less so by pRS-P3. For P2 and P3, multiple clones were tested because the hairpins in these
Figure 2. Decreased CNX levels do not affect US2- or US11-mediated destruction of MHC class I heavy chains.

Degradation of MHC class I heavy chains was examined by pulse-chase analysis and IP from SDS lysates. MG-U373 cells were transduced and selected with LNW-US2 or LNW-US11 and subsequently transduced and selected with empty pRS or pRS-C1. Cells were metabolically labeled for 12 min and chased for 0, 20, or 40 min., lysed, and subjected to immunoprecipitation for MHC class I heavy chains. CNX knockdown cells degrade MHC class I heavy chains with kinetics similar to control cells (compare lanes 1-3 with 4-6 and 7-9 with 10-12).
**US2 cells**
Chase: 0 20 40

**pRS**
Pulse 12 min
IP: anti-HC

**pRS-C1**

**US11 cells**
Chase: 0 20 40

**pRS**
Pulse 12 min
IP: anti-HC

**pRS-C1**
Figure 3. Retroviral knockdowns of PDI and Ero1α.

A) Immunoblot for PDI on 5 μg of SDS lysate prepared from each knockdown cell line. MG-U373 cells were transduced and selected with empty pRS or pRS expressing PDI knockdown constructs. pRS-P1 and pRS-P2a were used in subsequent experiments. The immunoblot for β-actin serves as a protein loading control.

B) Immunoblot for Ero1α on 30 μg of SDS lysate prepared from each knockdown cell line. MG-U373 cells were transduced and selected with empty pRS or pRS expressing Ero1α knockdown constructs. pRS-E2a and pRS-E3 were used in subsequent experiments.
A) PDI knockdowns

<table>
<thead>
<tr>
<th>pRS</th>
<th>P1</th>
<th>P2a</th>
<th>P2b</th>
<th>P3a</th>
<th>P3b</th>
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Blot: anti-PDI

B) Ero1α knockdowns

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<tr>
<th>pRS</th>
<th>E1</th>
<th>E2a</th>
<th>E2b</th>
<th>E3</th>
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Blot: anti-Ero1
DNA constructs could not be sequenced. The β-actin immunoblot serves as a protein loading control. Similarly, an immunoblot for Ero1α demonstrates pRS-E2 and E3 significantly reduced the steady-state levels or Ero1α, whereas E1 had little effect (Figure 3B). The band recognized by Ero1α-specific anti-sera that appears below the prominent Ero1α band has been reported to be Ero1β, which was not targeted by the siRNA constructs (Tsai and Rapoport, 2002). Ero1β is not up-regulated in Ero1α knockdowns.

For subsequent PDI knockdown experiments, U373-MG cells stably expressing the model degradation substrates were infected twice with mixtures of pRS-P1 and pRS-P2. Cells expressing the model substrates were infected twice with pRS-E2 and pRS-E3 for subsequent Ero1α knockdown experiments. The effect of increased expression of the hairpins by multiple infections and the efficacy of two different siRNA constructs compared to one was not directly compared in these experiments, but data from Drosophila and mammalian tissue culture experiments indicate these approaches can enhance the effectiveness of siRNA (C. Dillon, B. Armstrong, pers. comm.).

The PDI knockdown constructs lowered the cellular levels of PDI in U373-MG cells expressing empty control vector (CC), US2 or US11 (Figure 4A). The immunoblot for the mammalian cdc48 homolog p97 serves as a protein loading control. Ero1α knockdown constructs effectively lowered the cellular levels of Ero1α in CC, US2 or US11 U373-MG cells (Figure 4B). The p97 immunoblot serves as a protein loading control. These knockdown cell lines were used for subsequent experiments.

To determine if PDI or Ero1α affect US2-catalyzed dislocation, pulse-chase experiments were performed on US2 expressing U373-MG cells stably transduced with empty pRS or the pRS vectors expressing Ero1α or PDI knockdown constructs (Figure
Figure 4. Knockdown of PDI and Ero1α in US2- and US11-expressing cell lines.

A) Immunoblot for PDI in Control Cells, US2-, and US11-expressing cells. MG-U373 were transduced and selected with empty LNW, LNW-US2 or LNW-US11. Cells were subsequently transduced and selected with empty pRS or pRS-P1 and pRS-P2. Cells were lysed and SDS and immunoblot was performed on 20 µg lysate/lane. Cells transduced with pRS-P1 and pRS-P2 show reduced levels of PDI. The immunoblot for p97 serves as a protein loading control.

B) Immunoblot for Ero1α in Control Cells, US2-, and US11-expressing cells. MG-U373 were transduced and selected with empty LNW, LNW-US2 or LNW-US11. Cells were subsequently transduced and selected with empty pRS or pRS-E2 and pRS-E3. Cells were lysed and SDS and immunoblot was performed on 40 µg lysate/lane. Cells transduced with pRS-E2 and pRS-E3 show reduced levels of PDI. The immunoblot for p97 serves as a protein loading control.
A) pRS-P1,P2:

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Blot: anti-p97

Blot: anti-PDI

B) pRS-E2,E3:

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<th>CC</th>
<th>US2</th>
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Blot: anti-p97 & anti-Ero1
5A). Cells were pulsed for 12 minutes, chased for the indicated times, lysed, and samples were subjected to immunoprecipitation and SDS-PAGE analysis. The three cell lines expressed abundant levels of the viral protein (lanes 1, 5, 9). Immunoprecipitations were performed for the MHC class I heavy chain at the 0, 20, and 40 minute chase points (lanes 2-4, 6-8, 10-12). The data show that significantly reduced levels of Ero1α or PDI had little effect on the rate of US2-catalyzed dislocation of MHC class I heavy chains.

To address the role of these chaperones on US11-catalyzed dislocation, a pulse-chase experiment was performed on US11 expressing U373-MG cells (Figure 5B). US11 cells were stably transduced with empty pRS or pRS expressing Ero1α or PDI knockdown siRNA constructs. Immunoprecipitations for US11 demonstrate all three cell lines expressed high levels of the viral protein (lanes 1, 5, & 9). Pulse-chase analysis demonstrated that decreased cellular concentrations of Ero1α or PDI did not significantly affect the rate of US11-catalyzed dislocation of MHC class I heavy chains compared to untreated control cells (lanes 2-4, 6-8, 10-12).

*Role of CNX, Ero1α, and PDI in TCRα degradation*

The role of the ER chaperones CNX, Ero1α, and PDI in the degradation of the model ER substrate TCRα was addressed by pulse-chase analysis (Figure 6). U373-MG cells stably expressing TCRα were stably transduced with empty pRS or pRS expressing the siRNAs of interest. Cells were metabolically labeled for 20 minutes, chased for the indicated times, lysed, and subject to immunoprecipitation and SDS-PAGE analysis. The cell lines expressing the knockdown constructs display similar rates of degradation of
Figure 5. US2- and US11-catalyzed degradation of MHC class I heavy chains is unaffected in PDI and Ero1α knockdown cell lines.

A) Pulse-chase of US2-catalyzed degradation in the knockdown cell lines. MG-U373 cells expressing US2 were transduced and selected with empty pRS, pRS-E2 and pRS-E3, or pRS-P1 and pRS-P2. Cells were metabolically labeled for 12 min and chased for 0, 20, or 40 min., lysed in SDS and subject to immunoprecipitation and SDS-PAGE. Lanes 1,5, and 9 are IPs for US2 from the 0 chase point, indicating the three cell lines express equivalent levels of US2. The three cell lines degrade MHC class I heavy chains with similar kinetics.

B) Pulse-chase of US11-catalyzed degradation in the knockdown cell lines. MG-U373 cells expressing US11 were transduced and selected with empty pRS, pRS-E2 and pRS-E3, or pRS-P1 and pRS-P2. Cells were metabolically labeled for 12 min and chased for 0, 20, or 40 min., lysed in SDS and subject to immunoprecipitation and SDS-PAGE. Lanes 1, 5, and 9 are IPs for US11 from the 0 chase point, indicating the three cell lines express equivalent levels of US11. The three cell lines degrade MHC class I heavy chains with similar kinetics.
**US2 cells**

A)

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<th>pRS-E2,E3</th>
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<tr>
<td>Chase: (min)</td>
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Pulse 12 min  
IP: anti-US2 & anti-HC

**US11 cells**

B)

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<th>pRS-P1,P2</th>
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Pulse 12 min  
IP: anti-US2 & anti-HC
Figure 6. TCRα degradation proceeds normally in the knockdown cell lines.

MG-U373 cells were transduced and selected with LNm-TCRα. Cells were subsequently transduced and selected with empty pRS, pRS-C1 (CNX KD), pRS-E2 and pRS-E3 (Ero1 KD), or pRS-P1 and pRS-P2 (PDI KD). Cells were metabolically labeled for 20 min and chased for 0, 1, 2, or 3 hours, lysed in SDS and samples were subject to immunoprecipitation for TCRα and SDS-PAGE. The degradation of TCRα proceeds at similar rates in the four cell lines. The decreased recovery of TCRα in the CNX knockdown cell line was consistently observed.
Pulse 20 min
IP: anti-TCRα
TCR\(\alpha\) as the control cell line, indicating decreased cellular levels of the selected chaperones did not significantly affect the degradation of TCR\(\alpha\).

Interestingly, recovery of TCR\(\alpha\) was decreased in the CNX knockdown cells compared to the other three cell lines. In addition, a radio-labeled band corresponding to the size of deglycosylated TCR\(\alpha\) appears below the ER form of TCR\(\alpha\) only in the CNX knockdown cells. TCR\(\alpha\) expressing cells treated with the glucosidase-I inhibitor castanospermine also exhibit relative destabilization of the glycoprotein compared to untreated cells (P. Stern, unpub. obs.). Castanospermine prevents TCR\(\alpha\) from interacting with CNX, and thereby may enhance degradation of the glycoprotein. This destabilization in the CNX cell line gives an indication that the knockdown constructs do have an effect on cellular functions.

Although the absolute levels of the chaperone of interest are markedly decreased, there is no protocol to determine if activities of the cellular pathways dependent on these chaperones are compromised. In an attempt to develop a functional readout for decreased expression levels of these chaperones, several strategies were employed.

The synthesis of MHC class I molecules proceeds through a well-characterized, highly-regulated assembly process. The heavy chains first interact with CNX, followed by sequential association with a number of ER chaperones, including the oxidoreductase Erp57. The assembly rate of free MHC class I heavy chains into properly-conformed trimers was compared between control U373-MG cells and cells expressing stable knockdown constructs targeting CNX, Ero1\(\alpha\), or PDI. If the ER environment for MHC class I folding is compromised by the knockdowns, the rate of assembly of the properly-folded trimer will be decreased. Properly folded MHC class I molecules acquire
reactivity to the monoclonal antibody W6/32. Cells were pulsed for 4 minutes and
chased for 0, 4, 8, and 12 minutes. The rate of assembly of W6/32-reactive material was
identical in all four cell lines (Figure 7).

In addition, several attempts were made to isolate a disulfide-linked intermediate
with PDI. If PDI reduces disulfides of dislocation substrates and if Ero1-alpha is
essential for reducing the PDI-substrate intermediate, as has been shown for cholera
toxin, then perhaps Ero1-alpha knockdown cell lines would permit visualization of this
transient intermediate. To this end, control cells or cells expressing US2 or US11 and the
knockdown constructs to Ero1α or PDI were metabolically labeled for 20 minutes, then
treated with the thiol reactive compound N-ethyl-maleimide (NEM). NEM reacts with
free thiols and should, conceivably, trap any disulfide-linked intermediates between PDI
and MHC class I heavy chains by preventing dithiol reduction between PDI and its
substrate. This method should allow visualization of disulfide-linked intermediates if
dislocated heavy chains do indeed interact with PDI during the dislocation process. Cells
were lysed and subjected to immunoprecipitation for PDI. The isolated material was then
subjected to re-immunoprecipitation for MHC class I heavy chains. No heavy chain-PDI
interaction was visualized in US2- or US11-expressing, nor in the cells expressing the
knockdown constructs (data not shown). However, this is an imperfect experiment, as no
positive control is available to demonstrate that a PDI-substrate intermediate can be
isolated.
Figure 7. MHC class I trimer assembly is unaffected in the knockdown cell lines.

Pulse-chase analysis of assembly of W6/32-reactive material in the knockdown cell lines. MG-U373 cells were transduced and selected with empty pRS, pRS-C1, pRS-E2 and pRS-E3, or pRS-P1 and pRS-P2. Cells were metabolically labeled for 4 min and chased for 0, 4, 8, or 12 min. Cells were lysed in NP-40 lysis buffer and samples were subject to immunoprecipitation with W6/32 and SDS-PAGE analysis. Assembly of the W6/32-reactive trimer is unaffected in the chaperone knockdown cell lines.
pRS
min 0 4 8 12
pRS-C1
min 0 4 8 12
pRS-E2,E3
min 0 4 8 12
pRS-P1,P2
min 0 4 8

HC-
Pulse 4 min
IP: W6/32
Discussion

Pharmacologic inhibition of ER glucosidases supported a model for ER degradation that was not dependent on CNX, but deletion of CNX had not been formally tested. Work by Sifers indicates that CNX plays a pivotal role in the selection process in the ER that targets misfolded polypeptides for destruction (Cabral, et al., 2000). Specifically, persistent association of the substrate with CNX results in dislocation and proteasomal degradation. This model was based on results obtained from observing the degradation of the soluble terminally-misfolded substrate α-1-anti-trypsin NHK. The results presented here indicate CNX does not play an essential role in the disposal of membrane protein from the ER. The disposal of soluble substrates may rely on pathways distinct from the pathways responsible for degrading membrane proteins.

In contrast, the data from the TCRα experiments support a model that CNX sequesters substrates from degradation by the quality control mechanisms present in the ER. Perhaps CNX delays the destruction of the glycoprotein to allow time for TCRα to form a productive interaction with its binding partner TCRβ. The lower molecular weight band at the 0-minute chase point may represent a population of TCRα that has been dislocated and deglycosylated. Alternatively, this population may never have been glycosylated, due to the effects of decreased CNX levels on glycoprotein processing. Further experiments are needed to explore these possibilities.

The dislocation of cholera toxin to the cytosol has been likened to the dislocation of ER degradation substrates. The role of the gene products essential for cholera toxin dislocation had not been addressed previously in a system utilizing bona fide ER dislocation substrates. These results indicate that significant reductions in the cellular
concentrations of PDI or Ero1α, deemed essential for cholera toxin dislocation, have little effect on the rate of dislocation of the model substrates examined. Cholera toxin is dislocated to the cytosol, but not degraded. Perhaps the toxin utilizes a cellular pathway that is distinct from the ER disposal route that the model degradation substrates traverse.

In addition, the experiments presented herein and the cholera toxin experiments were performed under significantly different conditions. The data implicating PDI and Ero1α in cholera toxin dislocation were performed in an in vitro system utilizing proteoliposomes reconstituted with select recombinant proteins. This system is less complex than experiments performed in intact cells, but may also overestimate the contribution of single factors to the process being investigated.

Alternatively, the siRNA strategies employed to address the role of these ER chaperones may be limited by its efficacy, as siRNA can significantly reduce, but not completely ablate protein expression. Lowering the expression level of a protein by several fold may have a profound effect on the cellular activities dependent on the targeted protein. Alternatively, the integrity of a cellular pathway, although dependent on a protein’s activity, may be intact at significantly reduced levels of the essential factor. As a means of compensation, the cell may adapt to lowered levels of a particular protein by decreasing turnover of the targeted protein or by up-regulating compensatory factors.

Cellular pathways often possess factors with redundant functions that are able compensate for loss of a member. For example, calreticulin may fully compensate for loss of CNX. The host of oxido-reductases present in the ER may compensate for PDI deficiency, and Ero1β may replace the functions of Ero1α, although Ero1β expression was not increased in the Ero1α knockdown cell lines. In support of the significance of
the knockdowns to cellular activities, the PDI knockdown cells grew at half the normal growth rate and were morphologically distinct from wild-type U373-MG cells.

In addition, it is difficult to assess how profound the effect of the knockdown constructs is on the targeted proteins. This is due to the fact that few, if any, cellular reactions have been defined that are dependent on CNX, PDI, or Ero1α. One obvious assay is the dislocation of cholera toxin in the PDI and Ero1α knockdown cell lines. However, a fraction of Ero1α and PDI still remains in the knockdown cell lines and in the best case scenario cholera toxin would still be dislocated, but at a decreased rate. The experiment would be fatally confounded if other cellular factors can compensate for PDI or Ero1α.
References.


Chapter 3

The role of the mammalian Unfolded Protein Response in the dislocation of membrane proteins
Abstract

In yeast, the Unfolded Protein Response (UPR) is essential for efficient dislocation of substrates from the ER to the cytosol for degradation by the proteasome. The role of the UPR in mammalian cells was addressed utilizing the US2- and US11-catalyzed destruction of MHC class I molecules and the degradation of TCRα as model substrates. The ATF6 pathway was disrupted by expression of a transcriptionally inactive version of ATF6 that competes with the endogenous molecule for DNA binding. Stable expression of the ATF6 mutant by retroviral transduction for several days was highly toxic, but did not significantly affect the degradation rates of the model substrates. The IRE1α-XBP-1 pathway of the UPR was inhibited by expression of a cytoplasmic truncation of IRE1α that disrupts efferent signaling from the ER. Long-term expression of this inhibitor was well-tolerated and the degradation rates of the model substrates was not significantly affected. Thus, unlike yeast, mammals have evolved signaling pathways regulating the degradative capacity of the ER that are distinct from the pathways regulating the cargo-carrying capacity of the ER.
**Introduction** UPR

Most membrane and secretory proteins are translocated into the lumen of the endoplasmic reticulum (ER) where they must fold into their proper conformation to pass through ER quality control prior to exit (Ellgaard, et al., 1999). The ER contains a number of chaperone molecules that assist the nascent polypeptide in adopting its proper tertiary and quaternary structure (Ellgaard and Helenius, 2003). Those polypeptides that cannot pass quality control are retained in the ER and dislocated from the ER to the cytosol for destruction by the proteasome (Brodsky and McCracken, 1999a). The flux of proteins through the secretory pathway is tightly regulated. If the quantity of secretory proteins were to exceed the folding and degradative capacity of the ER, this could result in organelle dysfunction and jeopardize cellular viability (Oyadomari, et al., 2002). The mammalian ER has developed several means to respond to changes in secretory load and ensure cellular homeostasis.

The cellular response to the stress of accumulation of misfolded proteins in the ER is termed the Unfolded Protein Response (UPR) (Mori, et al., 1996). In yeast, the transcriptional response to ER stress is mediated by the ER-resident transmembrane kinase IRE1p (Cox, et al., 1993). Upon accumulation of misfolded polypeptides in the lumen, IRE1p activates the transcription factor HAC1p that, in turn, induces expression of ER chaperones, as well as proteins that facilitate ER-associated degradation and remodeling of the secretory pathway (Casagrande, et al., 2000), (Travers, et al., 2000). The activity of IRE1p is regulated by its oligomerization state. It is maintained in a monomeric, inactive state by the association of BiP with the luminal portion of IRE1p. As unfolded proteins accumulate, BiP is titrated away from IRE1p, allowing IRE1p to
dimerize, auto-trans-phosphorylate the cytoplasmic domain and activate an intrinsic endoribonuclease activity (Shamu and Walter, 1996). This cleaving activity excises an intron from HAC1 mRNA, thereby stabilizing and activating the transcription factor (Kawahara, et al., 1998).

Mammals possess a paralog to yeast IRE1, termed IRE1α (Tirasophon, et al., 2000). Mammalian cells respond to ER stress by activating IRE1α-mediated splicing of XBP-1, the mammalian HAC1 homolog (Lee, et al., 2002). IRE1α activation is regulated by its oligomerization state, which is regulated by the concentration of free BiP in the ER (Bertolotti, et al., 2000). In unstressed cells, BiP levels are controlled by the action of ATF6, a membrane bound transcription factor that promotes transcription of ER chaperones (Haze, et al., 1999). Through BiP regulation, the responsiveness of the IRE1α pathway is modulated by ATF6. In addition to buffering IRE1α activity by up-regulating BiP, in unstressed cells ATF6 is essential for transcription of the IRE1α target mRNA encoding XBP-1 (Yoshida, et al., 2001).

Experimentally, the IRE1α pathway can be disrupted by introduction of a mutant IRE1α that lacks its cytoplasmic kinase and ribonuclease domain (Liu, et al., 2002). This molecule is still capable of dimerizing with the endogenous IRE1α in the lumen of the ER, but downstream signaling cannot occur. Similarly, the ATF6 pathway can be disrupted by introduction of a truncated ATF6 molecule that possesses a DNA-binding domain but lacks a transcription transactivation domain (Gotoh, et al., 2002). Expression of a mutant of ATF6 consisting of amino acid residues 190-370 results in an impotent transcription factor that can bind the ATF6 DNA consensus sites and compete with the
endogenous ATF6 for gene activation (Wang, et al., 2000). Inhibiting the ATF6 pathway also results in disruption of the IRE1α pathway by preventing XBP-1 transcription.

One model of regulation proposes that ATF6 regulates a minor subset of UPR responsive genes, primarily lumenal chaperones, and by this attenuates the IRE1α pathway (Yoshida, et al., 2003). The IRE1α pathway is only activated when the secretory load in the ER requires a global response, including increasing the degradative capacity and remodeling the secretory pathway to adapt to the stress. By this model, the capacity for the cell to efficiently degrade terminally misfolded proteins from the ER would require an intact IRE1α signaling pathway.

Glycoproteins that are unable to pass ER quality control are dislocated from the lumen of the ER to the cytosol (Tortorella, et al., 1998). This step-wise process first targets and then extracts the protein from the membrane into the cytosol. Once dislocated, the polypeptide is exposed to the action of peptide-N-glycanase which removes high mannose glycans (Jarosch, et al., 2002), (Hirsch, et al., 2003). The deglycoyslated substrate is then rapidly degraded by the proteasome and can be visualized experimentally by inclusion of a proteasome inhibitor in the reaction. Several model substrates have been utilized to define this dislocation process.

One model ER degradation substrate is TCRα, a membrane-spanning glycoprotein constituting half of the intact T cell receptor complex (Huppa and Ploegh, 1997). The protein has two lysines in the membrane-spanning portion, rendering it unstable in the absence of its binding partner TCRβ. TCRα is dislocated from the ER and degraded with a half-life of 1-2 hours.
The Human Cytomegalovirus (HCMV) proteins US2 and US11 have evolved to efficiently eliminate MHC class I antigen presentation and thereby allow infected cells to evade immune surveillance by cytotoxic T cells (Tortorella, et al., 2000). This task is accomplished by inducing the dislocation of MHC class I molecules from the ER to the cytosol with a half-life of 10 minutes.

The essential role of IRE1 in regulating degradation of ER proteins in yeast is well established (Casagrande, et al., 2000), (Travers, et al., 2000). The role of IRE1α in regulating ER-associated degradation of membrane proteins in mammalian cells has not been addressed experimentally. To this end, mutant ATF6 or mutant IRE1α were introduced by retroviral transduction into cells stably expressing the aforementioned model degradation substrates to assess the role of the UPR in efficient ER-associated degradation.
Materials & Methods

DNA Constructs

The multiple cloning site (mcs) of the retroviral vector pLNCX (Clontech, Palo Alto, CA) was expanded from three to eight unique sites to generate the vector LNm. The Woodchuck Post-transcriptional Response Element (WPRE) was inserted into the ClaI site located 3' to the expanded mcs in LNm to produce the vector LNW. The WPRE mediates nuclear export of unspliced transcripts driven by the LTR and CMV promoter in LNm, thus increasing protein expression. The neomycin resistance gene of LNW was replaced with green fluorescent protein (GFP) to produce the vector LGW.

The truncation mutant of ATF6 (amino acid residues 191-273) was amplified by polymerase chain reaction (PCR) from HeLa cDNA using the following primers (5'- ATGAAGACTCAAACAAACTCC, 3'-TTAACTAGGGACTTTAAGCC). The PCR product was cloned into the vector pcDNA 3.1 (Invitrogen) for in vitro translation. The full-length cDNA of ATF6 was a generous gift from Ron Prywes (Columbia U.). The full-length and truncation mutant cDNA of IRE1α was a generous gift from Randall Kaufman (U. Mich.). The truncation mutants of ATF6 and IRE1α were cloned into the retroviral vector LGW. The cDNA for TCRα was cloned into LNm and the cDNAs for US2 and US11 were cloned into LNW. The 3X UPRE-luciferase reporter construct was a gift from Ron Prywes (Wang, et al., 2000). The luciferase gene was replaced with GFP to generate the 3X UPRE-GFP reporter vector.

The mammalian expression vector expressing Moloney retrovirus Gag and Pol genes and the vector expressing retroviral envelope VSV-G were gifts from Richard Mulligan (Children's Hospital, Boston, MA).
Cell lines, reagents, and antibodies

U373-MG astrocytoma cells were transduced with LNm expressing TCRα or LNW expressing US2 or US11. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum and 5% calf serum and 0.5μg/ml geneticin (Gibco, Frederick, MD). Mouse 3T3 and HEK 293 cells were maintained in DMEM supplemented with 5% FCS and 5% CS. The anti-heavy chain serum (Tortorella, et al., 1998), anti-TCRα serum (Huppa and Ploegh, 1997), W6/32 (Parham, 1979), AF8 (Rajagopalan, et al., 1994), anti-US2 (Wiertz, et al., 1996a), anti-US11 (Wiertz, et al., 1996b) were used as described. Anti-KDEL was purchased from Stressgen (San Diego, CA). The full-length p97 protein was expressed in bacteria. Rabbits were immunized with the recombinant protein and polyclonal antisera was obtained. Tunicamycin was purchased from Sigma Chemical Co (St. Louis, MO). ZL₃VS was synthesized and used as described (Bogyo, et al., 1997). In vitro translations were performed using the TnT Coupled Reticulocyte Translation System (Promega). FACS analysis was performed on a FACScan (Becton Dickinson, San Jose, CA). FACS sorting was performed at the Dana Farber Cancer Center Cell Sorting Facility.

Metabolic labeling of cells and pulse-chase analysis

Cells were detached from their substrate by trypsin treatment, followed by starvation in methionine/-cysteine-free DMEM for 45 min at 37°C. Cells were metabolically labeled with 500 μCi/ml of [³⁵S] methionine/ cysteine (1200 Ci/mM; NEN-Dupont, Boston, MA)/ml at 37°C for the times indicated. In pulse-chase experiments, cells were
radiolabeled as above and were chased for the times indicated in DMEM containing non-
radiolabeled methionine (2.5 mM) and cysteine (0.5mM). Cells were then lysed in NP-
40 lysis buffer (10 mM Tris pH 7.8, 150mM NaCl, 5 mM MgCl₂, 0.5% NP-40) 
supplemented with 1.5 µg/ml aprotinin, 1µM leupeptin, 2mM phenylmethylsulfonyl 
fluoride (PMSF) followed by immunoprecipitation (see below). For cells lysed in 1% 
SDS, the final concentration of SDS was adjusted to 0.067% with NP-40 lysis mix prior 
to immunoprecipitation.

**Immunoprecipitation**

Following cell lysis, debris was removed by centrifugation at 15,000g for 5 min. Non-
specific binding proteins were removed from the cell lysates by the addition of 3µl/ml 
normal rabbit serum, 3 µl/ml normal mouse serum and formalin-fixed, heat-killed 
*Staphylococcus aureus* for 1 h at 4°C. Immunoprecipitation was performed by 
incubation with antiserum for 45 min at 4°C, followed by addition of *S.aureus* for 45 min 
at 4°C. The pelleted *S.aureus* were washed four times with washing buffer (0.5% NP-40 
in 50 mM Tris pH 7.4, 150 mM NaCl and 5 mM EDTA). The pellet was resuspended in 
SDS sample buffer (4% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.025% 
bromophenol blue in 62.5 mM tris pH 6.8) and the released materials were subjected to 
12.5% SDS-PAGE.

**Generation of cell lines by retroviral transduction**

To produce VSV-G pseudotyped retrovirus, HEK 293 cells were plated in HEK 293 
media to yield 50% confluent monolayers the following day. Cells were transiently
transfected with GagPol, VSV-G and retroviral vectors expressing the cDNA of interest at a ratio of 3:2:5 using Fugene 6 (Roche) following the manufacturer's guidelines. The media was changed 16-20 hours after transfection. The following day, supernatant was collected and used to infect target cells.

For infection, target cells were plated the day before infection in 6-well dishes at 2x10^7/well. Retroviral supernatants were filtered through 0.8-μm syringe filters and diluted 1:2 with DMEM+10% FCS plus 4μg/ml (final conc) hexadimethrine bromide (Sigma Chemical Co.) to produce the retroviral cocktail. The U373 media was aspirated and 1ml of infectious cocktail added to each well of plated U373 cells. Plates were centrifuged in a Beckman Allegra 6KR centrifuge for 2 h at 1000 x g at 30°C. After 2 h, plates were moved to a humidified CO₂ incubator overnight. The following day, the cocktail was replaced with DMEM+10% FCS. Selection was imposed 2 days post infection.
Results

cDNA-based inhibitors and activators of the UPR

To investigate the role of the UPR in the efficient degradation of model membrane substrates from the ER, experimental control of UPR signaling is essential. The UPR is regulated by two ER-proximal signaling pathways that induce distinct, as well as overlapping, sets of genes. The ATF6 response is constitutively active and is mediated by cleavage of a 50 kilodalton (kDa) cytoplasmic transcription factor from a 90 kDa ER-transmembrane precursor (Ye, et al., 2000). The released fragment enters the nucleus and activates transcription of target genes. The active transcription factor is composed of a DNA binding domain, which binds to ER Stress Element (ERSE) sites in target genes, and a transcriptional transactivation domain, which recruits co-factors to activate transcription (Yoshida, et al., 1998). A truncation mutant of the active cleaved fragment lacking the transactivation domain can still bind ERSE sites, but it suppresses gene activation by acting as a competitive inhibitor of endogenous ATF6 (Gotoh, et al., 2002). This strategy was utilized to eliminate ATF6-dependent signaling.

The ATF6 truncation mutant (ATF6tm) was generated from HeLa cell cDNA and the polypeptide was visualized by in vitro translation (Figure 1A). The translation products of cDNA clones 1 and 2 ran at the expected molecular weight of 22 kDa, whereas clones 3 and 4 produced defective products. Clone 2 was used for subsequent studies. The ATF6tm does act as an inhibitor of UPR signaling (Figure 1B). HEK 293 cells were transiently transfected with a GFP reporter plasmid in which GFP transcription is under the control of three tandem UPRE DNA elements (3X-UPRE-GFP).
Figure 1. The ATF6tm construct is translated and inhibits UPR induction.

A) IVT of ATF6m constructs. The nucleotide sequence corresponding to amino acids 191-373 of ATF6 was isolated by PCR from HeLa cell cDNA and cloned into pcDNA 3.1. From IVT of 4 clones, clone 1 and 2 produce a translated product of the expected size of 22kDa. Clone 2 was utilized in further experiments.

B) ATF6tm inhibits activation of a UPR reporter. Schematic of UPR-GFP reporter in which three tandem UPRE sites transcriptionally regulate GFP. HEK-293 cells were transiently transfected with 2μg reporter plasmid and 2μg control plasmid (Ctrl) or 2μg ATF6tm-expressing plasmid. 48 hours post-transfection, cells were treated with DMSO carrier or 2 μg/ml tunicamycin (Tun) to induce ER stress. 10 hours post-induction, cells were subject to FACS analysis to determine mean fluorescent intensity (MFI) of GFP. ATF6tm prevents induction of GFP synthesis by tunicamycin.
A) \textit{In vitro} translation of ATF6tm

clone: 1 2 3 4

B) 3X UPRE    GFP ORF

\begin{center}
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MFI GFP &  \\
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Ctrl & \\
ATF6tm & \\
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\begin{itemize}
\item DMSO
\item Tunicamycin
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The Unfolded Protein Response Element (UPRE) is a UPR response element in genes that binds XBP-1. UPRE is distinct from the ERSE that binds ATF6. As XBP-1 transcription is dependent on ATF6, ablating ATF6 activity results in inactivation of XBP-1 dependent processes, including UPRE transactivation (Yoshida, et al., 2001). For cells containing the 3X-UPRE-GFP construct, induction of ER stress by tunicamycin treatment for 10 hours increased the mean fluorescence intensity (MFI) as assessed by FACScan from 50 to 100 units. This increase is due to induction of XBP-1 by ER stress and subsequent transactivation of the GFP reporter. In cells co-transfected with ATF6tm, tunicamycin had no effect on GFP MFI compared to untreated control cells, indicating that ATF6-dependent transcription was disrupted.

The second ER-proximal signaling pathway regulating the UPR is the IRE1α/XBP-1-dependent response. Upon accumulation of unfolded proteins in the ER, the chaperone BiP is titrated away from the luminal domain of IRE1α. The free luminal portions can then dimerize, which activates the kinase and subsequently the ribonuclease activities in the cytoplasmic portions. The ribonuclease activity produces a splice shift in the XBP-1 transcript, resulting in translation of a highly active transcription factor that binds to and activates transcription of genes that contain UPREs. Deletion of the cytoplasmic portion of IRE1α (IREΔ) results in a transmembrane protein that can still dimerize with the endogenous protein but eliminates efferent signaling by preventing trans-phosphorylation and subsequent activation (Tirasophon, et al., 2000).

Disruption of IRE1α activity should result in a defect in XBP-1 splicing. The unspliced XBP-1 transcript produces a highly unstable protein of approximately 30 kDa
that can only be visualized in the presence of proteasome inhibitors. The spliced transcript produces a protein of 55 kDa. Treatment of HEK-293 cells with proteasome inhibitor allowed visualization of the 30 kDa band and treatment with TM resulted in loss of the 30 kDa form (Fig 2A, lanes 1 & 2), presumably due to splicing of the XBP-1 transcript to generate the 55 kDa form. The reagents for visualizing human XBP-1 are poor and the 55 kDa species cannot be visualized under these conditions. In HEK-293 cells transiently transfected with IREΔ and treated with tunicamycin, the 30 kDa band persisted (lane 3), indicating the splicing of XBP-1 message and subsequent loss of 30 kDa form to the 55 kDa form was disrupted.

The ATF6-dependent response has been determined to account for 30% of the chaperone induction observed upon treatment with TM, whereas the XBP-1-dependent response accounts for the remaining 70% of the increase (Yoshida, et al., 2001). U373-MG cells stably transduced with IREΔ were deficient in induction of BiP with respect to wild-type cells that have an intact XBP-1 response upon treatment with tunicamycin (Figure 2B, compare lanes 2 & 3). The BiP signal was practically absent in cells that had not been treated with tunicamycin (lanes 1 & 4). An immunoprecipitation for the cytosolic protein p97 serves as a loading control.

Pharmacologic agents such as tunicalycin and DTT are efficient inducers of the UPR, but their effects can be pleiotropic. To complement inhibition studies, constructs expressing the transcriptionally active cleaved form of ATF6 (ATF6act) and the active spliced form of XBP-1 (XBP-1spl) were obtained from other investigators and shown to be active in the 3X-UPRE-GFP reporter assay (data not shown).
Figure 2. IREA inhibits XBP-1 splicing and Bip induction by tunicamycin.

A) Immunoblot for XBP-1 in HEK-293 cells transfected with IREA. Cells were transfected with empty LGW or LGW-IREA and treated for 8 hours with 2 µg/ml tunicamycin to induce ER stress and XBP-1 splicing. LGW-IREA prevented loss of the 30 kDa band corresponding to unspliced XBP-1 (compare lane 2 & 3).

B) IREA diminishes tunicamycin-induced Bip induction. MG-U373 were transduced with empty LGW or LGW-IREA and sorted for GFP+ cells. Cells were treated with 1.5 µg/ml tunicamycin for 8 hours, metabolically labeled for 30 min and lysed in SDS. Samples were subject to immunoprecipitation with anti-KDEL antibody and anti-p97 antisera and SDS-PAGE analysis. LGW-IREA significantly diminishes tunicamycin-induced Bip expression (compare Bip lanes 2 & 3). The p97 IP serves as a protein loading control.
A) Vector: LGW  LGW  LGWIREA

Tunicamycin: -  +  +

Unspliced XBP-1

Blot: anti-XBP-1

B) Tunicamycin: LGW  LGW-IREA

Bip

Pulse 30 min
IP: anti-KDEL & anti-p97
Role of the UPR in degradation of TCRα

TCRα is a transmembrane glycoprotein that, in the absence of its binding partner TCRβ, is dislocated from the ER to the cytosol with a half-life of about 90 minutes. To examine the role of the UPR in TCRα degradation, HEK 293 cells were transiently transfected with 2 μg of vector expressing TCRα and 3 μg of each UPR inhibitor ATF6tm and IREA, 6 μg of empty vector, or 3 μg of each UPR activator ATF6act and XBP-1spl. Forty-eight hours post-transfection, cells were metabolically labeled, chased for the indicated times, lysed, and subjected to immunoprecipitation and SDS-PAGE analysis. Despite manipulations of UPR activity, TCRα is degraded with similar kinetics in all three treatments (Figure 3A). It is not surprising that the UPR activators did not affect TCRα degradation, since tunicamycin and other ER stress inducers have been shown to have marginal effects on TCRα degradation. However, it was surprising that inhibition of the UPR did not affect TCRα degradation in 293 cells, as the UPR is essential for degradation of ER proteins in S. cerevisiae.

One caveat was that perhaps an essential factor for protein dislocation was transcriptionally suppressed by the UPR inhibitors but still remained 48 hours after transfection. This is a reasonable possibility as some ER proteins have been reported to have half-lives on the order of days (T. Rapoport, pers. comm.). To address this possibility of a long-lived factor, the inhibitor construct was stably introduced into the cells of interest. U373-MG cells stably expressing TCRα were transduced with retroviral vectors co-expressing GFP (LGW) and either ATF6tm or IREA. GFP co-expression was utilized throughout these experiments to assess transduction efficiency. All experiments were performed on cells that demonstrated at least 75% transduction.
Figure 3. The UPR does not affect degradation of TCRα.

A) Activating or inhibiting the UPR does not affect TCRα degradation in HEK-293 cells. HEK-293 cells were transiently transfected with 2μg vector expressing TCRα. “UPR↓” cells were co-transfected with 3 μg vector expressing ATF6tm and 3 μg vector expressing IREΔ. “–” cells were co-transfected with 6 μg empty vector. “UPR↑” cells were co-transfected with 3 μg wild-type ATF6 and 3 μg vector expressing spliced XBP-1. Cells were metabolically labeled for 15 min and chased for 0, 2, or 4 hours and lysed in SDS. Samples were subject to immunoprecipitation for TCRα and SDS-PAGE analysis. The rate of TCRα degradation is not significantly affected by activation or inhibition of UPR signaling.

B) Inhibiting either arm of the UPR does not affect TCRα degradation in MG-U373 cells. MG-U373 cells were stably transduced and selected with LNm-TCRα. Cells were subsequently transduced with empty LGW, LGW-IREΔ, or LGW-ATF6tm. Cells were metabolically labeled for 15 min and chased for 0, 1, 2, or 3 hours and lysed in SDS. Samples were subject to immunoprecipitation for TCRα and SDS-PAGE analysis. The rate of TCRα degradation is not significantly affected by inhibition of the UPR.
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Chase:  

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15 min pulse  

IP: anti-TCRα  

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B)  

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15 min pulse  

IP: anti-TCRα
efficiency for the relevant constructs. Inhibition of the UPR for four days by either construct did not influence degradation of TCRα in U373-MG cells (Figure 3B).

Prolonged culture (3 weeks) of IREA transduced U373-MG yielded similar results. Interestingly, prolonged expression of ATF6tm in of U373-MG cells was highly toxic.

U373-MG cells stably transduced with IREA grew normally up to four weeks post-infection (the longest time-point assayed). In contrast, the viability of cells stably expressing ATF6tm dropped precipitously after four days of culture. To further examine this observation, U373-MG cells were transduced with either empty LGW or LGW expressing ATF6tm or IREA. Cells were subject to FACS analysis to assay the percentage of GFP-positive cells surviving at indicated times post-infection. Cells co-expressing ATF6tm exhibited a marked reduction in GFP-positive cells at four and six days post-infection compared to cells expressing GFP alone or co-expressing IREA (Figure 4). The cause of this loss in viability is presumably due to unchecked ER stress, as both UPR pathways are inhibited by ATF6-dependent inhibition, but the nature of the death remains unanswered. Although the cells rounded and detached from the tissue culture plate, attempts to assay for apoptotic markers (DNA laddering, Annexin V exposure) were inconclusive. Incubation with caspase 8 inhibitor had no effect on viability and incubation with caspase 3 inhibitor appeared to delay, but not prevent, cell death (data not shown). Cells stably expressing ATF6tm likely die by unchecked ER stress, but the route to death remains undefined.
Figure 4. Inhibition of the ATF6, but not IREα, pathway is toxic.

Graph of percentage of GFP+ cells following transduction with inhibitors of the ATF6 or IREα UPR pathway. MG-U373 cells were transduced with empty LGW, LGW-IREΔ, or LGW-ATF6tm. Cells were subject to FACS analysis 2, 4 and 6 days post-infection. ATF6tm expression significantly reduced cell survival by 6 days post-infection.
Cell survival post-infection

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Role of the UPR in US2- and US11-catalyzed degradation

The kinetics of TCRα degradation are quite slow when compared to the action of the HCMV glycoproteins US2 and US11 on MHC class I molecules. Each of these two viral molecules can catalyze the dislocation of MHC class I heavy chains from the ER with a half-life of about ten minutes. These viral molecules may use cellular pathways distinct from TCRα and from each other to accomplish heavy chain dislocation. In addition, U373-MG cells constitutively expressing US11 have increased concentrations of the ER chaperones BiP and GP96 when compared to control cells or cells expressing US2 (data not shown). This observation raised the intriguing possibility that US11 may utilize cellular components regulated by the UPR to degrade MHC class I molecules.

To address the role of the UPR in US2-catalyzed degradation of MHC class I molecules, U373-MG cells stably expressing US2 (US2 cells) were transduced with empty LGW and LGW expressing ATF6tm or IREΔ. Four days after infection, cells were metabolically labeled for 12 minutes, chased for the indicated times, lysed, subjected to immunoprecipitation and SDS-PAGE analysis. The three cell lines expressed comparable levels of US2 (Figure 5, lanes 1, 5 & 9). Moreover, inhibition of UPR signaling by ATF6tm or IREΔ did not affect US2-catalyzed degradation of MHC class I molecules. The rate of disappearance of radio-labeled heavy chains was comparable in all three cell lines, with the majority being degraded by the 20 minute chase point. A similar experiment performed with cells stably expressing US11 demonstrated similar results (Figure 6). Cells were subjected to pulse-chase and immunoprecipitation analysis. The three cell lines expressed similar levels of US11
Figure 5. Inhibition of the UPR does not affect US2-catalyzed degradation of MHC class I heavy chains.

Pulse-chase analysis of US2-catalyzed degradation of heavy chains in MG-U373 cells. MG-U373 cells were stably transduced and selected with LNW-US2. Cells were subsequently transduced with empty LGW, LGW-IREΔ, or LGW-ATF6tm. Cells were metabolically labeled for 12 min, chased for 0, 20, or 40 min and lysed in SDS. Lysates were subject to immunoprecipitation for US2 and MHC class I heavy chains and SDS-PAGE analysis. IPs for US2 (lanes 1, 5, & 9) demonstrate the three cell lines express similar levels of US2. The rate of heavy chain disappearance is similar in the three cell lines.
Pulse 12 min
IP: anti-US2 & anti-HC
Figure 6. Inhibition of the UPR does not affect US11-catalyzed degradation of MHC class I heavy chains.

Pulse-chase analysis of US11-catalyzed degradation of heavy chains in MG-U373 cells. MG-U373 cells were stably transduced and selected with LNW-US11. Cells were subsequently transduced with empty LGW, LGW-IREΔ, or LGW-ATF6tm. Cells were metabolically labeled for 12 min, chased for 0, 20, or 40 min and lysed in SDS. Lysates were subject to immunoprecipitation for US11 and MHC class I heavy chains and SDS-PAGE analysis. IPs for US11 (lanes 1, 5, & 9) demonstrate the three cell lines express similar levels of US11. The rate of heavy chain disappearance is similar in the three cell lines.
Pulse 12 min
IP: anti-US11 & anti-HC
(lanes 1, 5 & 9). Thus, at four-days post-infection, the inhibitors of the UPR have no affect on the rate of US11-catalyzed destruction of MHC class I molecules.

Inhibition of UPR signaling for several days did not affect degradation of MHC class I molecules catalyzed by US2 or US11 (Figure 5, 6 and data not shown). The formal possibility still remained that a long-lived factor may persist after several days of UPR inhibition and that sustained suppression of the UPR may render some demonstrable defect in degradation. To this end, US2 and US11 cells transduced with the LGW-IREΔ virus were sorted by flow cytometry and cultured for two weeks, after which these cell lines were assayed for degradation of MHC class I molecules. The toxicity of the ATF6tm construct made this experiment untenable.

Unpublished data reported that cells could adapt by an unknown mechanism to stable expression of IREΔ and mount a full UPR response despite the presence of inhibitory constructs (D. Ron, pers. comm.). After two weeks of stable expression of IREΔ, US2 cells were defective in UPR responsiveness as assessed by BiP induction in response to tunicamycin treatment (Figure 7). Cells were metabolically labeled for 30 minutes, lysed and subjected to immunoprecipitation and SDS-PAGE analysis. Without tunicamycin treatment, BiP was poorly visualized. In the presence of tunicamycin, BiP was potently induced as evidenced by increased recovery of the radio-labeled polypeptide during the 30 minute pulse. Cells stably expressing IREΔ for two weeks demonstrated significantly reduced induction of BiP protein, indicative of sustained disruption of the IRE1α-XBP-1 dependent response. The immunoprecipitation for p97 serves as protein loading control. Similar results were obtained for cell lines stably expressing US11 (data not shown).
Figure 7. Sustained expression of IREΔ effectively inhibits tunicamycin-induced Bip induction.

Metabolically labeled MG-U373 cells transduced with LGW-IREΔ exhibit reduced induction of Bip by tunicamycin. MG-U373 were stably transduced with LGW or LGW-IREΔ and sorted for GFP+ cells. Two weeks after transduction, cells were pulsed for 30 min and lysed in SDS. Lysates were subject to immunoprecipitation for Bip with anti-KDEL antibody and p97 with anti-p97 antisera and analyzed by SDS-PAGE. Tunicamycin-induced Bip expression is decreased in cells expressing IREΔ. The IP for p97 serves as a protein loading control.
Pulse 30 min
IP: anti-KDEL & anti-p97
Long-term expression of IREA does not significantly affect the rates of US2- or US11-catalyzed destruction of MHC class I molecules (Figures 8 & 9). After sustained inhibition of the IRE1α-dependent pathway for two weeks, degradation proceeds normally for both US2- and US11-catalyzed reactions. Cells stably expressing US2 or US11 were transduced with LGW or LGW-IREA, sorted, pulsed for 12 minutes, chased for the indicated times, lysed and samples were processed as previously reported. Cells expressed similar levels of the viral protein (lanes 1 & 5, both Figures). US2 and US11 cell lines stably expressing IREA for two weeks exhibit the same rate of MHC class I destruction as the untreated control cell lines. The long-term expression of IREA had no significant effect on the degradation of TCRα in U373-MG cells (data not shown). Thus, neither the ATF6 nor the IRE1α-XBP-1 arms of the UPR appear to be essential for the efficient dislocation of membrane-associated ER proteins.
Figure 8. Sustained expression of IREA does not affect US2-catalyzed degradation of MHC class I heavy chains.

Pulse-chase analysis of US2-catalyzed degradation of heavy chains in MG-U373 cells. MG-U373 cells were stably transduced and selected with LNW-US2. Cells were subsequently transduced with empty LGW or LGW-IREA and sorted for GFP+ cells. Cells were metabolically labeled for 12 min, chased for 0, 20, or 40 min and lysed in SDS. Lysates were subject to immunoprecipitation for US2 and MHC class I heavy chains and SDS-PAGE analysis. IPs for US2 (lanes 1 & 5) demonstrate the two cell lines express similar levels of US2. The rate of heavy chain disappearance is similar in the two cell lines.
Pulse 12 min
IP: anti-HC & anti-US2
Figure 9. Sustained expression of IREΔ does not affect US11-catalyzed degradation of MHC class I heavy chains.

Pulse-chase analysis of US11-catalyzed degradation of heavy chains in MG-U373 cells. MG-U373 cells were stably transduced and selected with LNW-US11. Cells were subsequently transduced with empty LGW or LGW-IREΔ and sorted for GFP+ cells. Cells were metabolically labeled for 12 min, chased for 0, 20, or 40 min and lysed in SDS. Lysates were subject to immunoprecipitation for US11 and MHC class I heavy chains and SDS-PAGE analysis. IPs for US2 (lanes 1 & 5) demonstrate the two cell lines express similar levels of US11. The rate of heavy chain disappearance is similar in the two cell lines.
Pulse 12 min
IP: anti-HC & anti-US11
Discussion

Through the use of cDNA based inhibitors of the two signaling pathways constituting the mammalian UPR, several novel conclusions can be made. First, neither the ATF6-dependent nor the XBP-1-dependent responses of the UPR are essential for the efficient degradation of membrane proteins from the ER. Second, the ATF6 response, but not the IRE1α-XBP-1 response, is essential for cellular viability. ER-stress has been shown to induce programmed cell death, but the factors regulated by ATF6 that suppress cell death have not been identified (Oyadomari, et al., 2002). BiP is a likely candidate, as BiP over-expression has been shown to suppress ER-stress mediated apoptosis (Rao, et al., 2002).

Inhibition of the UPR also resulted in significantly reduced retroviral titers. Infectious viral particles are produced by co-transfection of the retroviral vector expressing the gene of interest with vectors expressing the accessory viral structural and replicative components. VSV-G is the retroviral envelope protein used in these studies to confer infectivity of human cells. VSV-G is inserted in the ER and traffics through the secretory pathway to the plasma membrane, where the viral particles are assembled and bud into the supernatant. Overexpression of VSV-G has been reported to be a potent inducer of the UPR (R. Mulligan, pers. comm.).

In this retrovirus system, vectors co-expressing GFP and either ATF6tm or IREΔ produced exceedingly poor viral titers when compared to the GFP control, presumably due to the inability of the virus producer cells to up-regulate UPR-dependent secretory components essential to virus production. Other laboratories have experienced this dilemma with virus production (D. Ron, pers. comm.). However, co-transfection of the
retroviral vectors expressing the UPR inhibitors with vectors expressing the active forms of XBP-1 and ATF6 resulted in efficient virus production. Although the evidence is indirect, this reduction and subsequent restoration of virus production illustrates the efficacy of the constructs employed in these studies.

Recently, Mori and co-workers identified the XBP-1 response as essential for the degradation of the terminally misfolded lumenal ER protein alpha-1-anti-trypsin (Null Hong Kong variant (NHK)) (Yoshida, et al., 2003). Degradation of NHK is dependent on EDEM, an ER-mannosidase-like protein implicated in glycoprotein turnover in the ER. EDEM is exclusively regulated by the transcriptional activity of spliced XBP-1. Disruption of XBP-1 signaling by genetic deletion of IRE1α (IRE1α -/-) results in marked stabilization of the soluble NHK protein. Interestingly, transfection of a vector expressing EDEM into the IRE1α -/- cells fully restores degradation of the soluble mutant protein. These results demonstrate that XBP-1 regulates a specific co-factor (EDEM) essential for the recognition of soluble ER degradation substrates and subsequent recruitment to the dislocation machinery. However, unlike yeast, an XBP-1-independent pathway regulates the machinery responsible for extraction and degradation of ER substrates.

Given that an intact IRE1 response is essential for the efficient degradation of ER proteins in S. cerevisae, it is interesting to speculate how mammalian regulation may have diverged. From previously published data, it appears the homologous pathway in mammals regulates the expansion of the ER and the cargo carrying capacity of this organelle. Experiments examining the development of naïve B cells into plasma cells have identified XBP-1 as essential for this process (Reimold, et al., 2001). XBP-1
appears to be the major factor regulating ER expansion and chaperone induction, which is a role it shares with its yeast paralog. However, it appears mammalian regulation has separated the ER expansion and cargo-carrying requirements from the disposal requirements of the ER. This is not surprising in the context of cellular specialization in multi-cellular organisms. Highly differentiated plasma cells and pancreatic acinar cells require significant secretory capacity without an apparent need for a concomitant increase in disposal capacity. The relative uniformity the yeast life cycle and the demands on the yeast ER dictate a more coincident regulation of these two capacities.

If the degradation of membrane proteins from the mammalian ER is not regulated by BiP sequestration from IRE1α and the resulting XBP-1 response, then what could be the sensor that detects and signals an increased need for degradation of ER substrates? The sensor likely lies in the multitude of substrate targeting and dislocating proteins that constitute the “black box” of mammalian ER-associated degradation. Several factors have been identified in the dislocation process, but no other transcription factors have been identified that are regulated by ER stress.
References


Chapter 4

The cytosolic domain of a class I MHC molecule dictates differential susceptibility to US2- or US11-mediated dislocation

Adapted from:


DNA constructs were developed in collaboration with M.J. Kim and D. Tortorella. Figure 6 was generated by M. Lorenzo. Figure 7 was generated by H.L. Ploegh.
Abstract

Human cytomegalovirus glycoproteins US2 and US11 induce proteasomal destruction of the same substrate, MHC class I molecules, by facilitating their extraction (dislocation) from the ER into the cytosol. Mutations of the cytosolic domain of MHC class I identified clear differences between US2 and US11 in their requirements to efficiently mediate dislocation of their substrates. US11 dislocated only MHC class I molecules that possess a cytosolic domain of approximately 30 amino acids. Truncation or addition of 10 residues or more rendered class I molecules refractory to US11-mediated dislocation. In contrast, US2 dislocated MHC class I molecules with cytosolic domains shortened to 10 amino acids or lengthened to 25kDa by fusion of GFP to the C-terminus. US2 and US11 therefore utilize different mechanisms to degrade MHC class I molecules.
Introduction

The endoplasmic reticulum (ER) is the point of entry for most membrane and secretory proteins. The environment of the ER facilitates nascent polypeptides in achieving their properly folded conformations. As a consequence of the flux of proteins through the ER and the inevitability of protein misfolding (Schubert, et al., 2000), the ER has means to counteract the deleterious effects of folding misfits and thus prevents perturbation of its function (Bonifacino and Weissman, 1998).

Proteins that cannot adopt a proper folded conformation may be extracted from the ER and delivered to the cytosol for subsequent proteasomal degradation (Wiertz, et al., 1996a). This process, referred to as retrotranslocation, or dislocation, is an integral step of the ER quality control machinery and is used for both general quality control of misfolded polypeptides, as well as a means of regulating polypeptides involved in anabolic and developmental pathways (Plemper, et al., 1997), (Hampton, et al., 1996), (Nishikawa, et al., 2001).

Much effort has been directed at identifying the protein complexes responsible for ubiquitination and retrotranslocation of ER substrates (Hiller, et al., 1996), (Knop, et al., 1996), (Tiwari and Weissman, 2001). In the aggregate, the published data is consistent with multiple independent pathways for degradation of proteins from the ER. Discrimination based on membrane topology and the soluble or membrane-bound nature of the dislocation substrate are attractive possibilities (Swanson, et al., 2001)(Bays, et al., 2001).
The mammalian immune system has evolved several mechanisms to survey for the presence of intracellular pathogens (Ploegh, 1995). The primary means of defense against intracellular invaders such as viruses involves class I MHC antigen presentation. Class I MHC molecules present intracellular antigens in the form of 8-10 residue peptides. Class I MHC molecules are assembled in the ER and consist of a glycosylated heavy chain (HC) that associates first with a light chain (beta-2-microglobulin) and subsequently with an antigenic peptide to form a stable trimeric molecule. This trimer is then competent to egress from the ER to the cell surface for recognition by T cells.

Members of the herpes virus family have evolved numerous tactics to counteract surveillance by their host’s immune system. One family member of the β-herpesviruses, human cytomegalovirus (HCMV) has at least four genes that directly disrupt MHC class I mediated antigen presentation: US3, US6, US2 and US11 (Jones, et al., 1995). The single-pass type I transmembrane glycoproteins US2 and US11 mediate destruction of the MHC class I molecules, thereby preventing the surface expression of MHC class I (Wiertz, et al., 1996a), (Tortorella, et al., 1998).

The HCMV gene products US2 and US11 reside in the ER where they bind MHC class I molecules and catalyze the extraction of the class I heavy chain from the ER to the cytosol for destruction by the proteasome (Wiertz, et al., 1996b). The process of dislocation in mammalian cells was first identified in the US2/US11-mediated destruction of class I. This system has demonstrated the stepwise process of dislocation, deglycosylation of the glycosylated ER protein, and degradation by the proteasome. In the absence of any other HCMV gene products, each of these viral proteins can mediate destruction of class I molecules. HCMV has been proposed to utilize US2 and US11 to
co-opt the host’s ER quality control machinery for its own ends. These viral molecules can thus provide insight into the cellular mechanisms of targeting and membrane extraction of substrates degraded from the ER.

The viral molecules, each a glycosylated type I membrane protein of about 200 amino acids, mediate dislocation of a common substrate with similar kinetics. A substrate targeted for degradation by two different means provides a unique opportunity to dissect steps in the degradation process that may be either shared between the two or unique to each. Evidence is emerging that these two viral molecules have markedly different structural and co-factor requirements to successfully accomplish the same task (Furman, et al., 2002). For instance, US2 only induces degradation of MHC class I heavy chains that have adopted the properly folded tertiary structure, whereas US11 causes degradation of free, unfolded heavy chains. In addition, US2 and US11 appear to induce accumulation of markedly different ubiquitinylated MHC class I intermediates (Shamu, et al., 2001).

What elements affect the efficiency with which these viral molecules target MHC class I molecules for destruction? Class I molecules lacking the 30 residue cytoplasmic tail are refractory to degradation by US2 or US11, although they retain the ability to interact with both US2 or US11 (Story, et al., 1999). Few experiments have addressed the role of the protein substrate in the degradation process, and how (or if) structural elements of the degraded molecule may affect its fate. We sought to identify how the structure of the cytoplasmic tail of MHC class I molecules may influence the ability of US2 or US11 to mediate destruction of class I molecules. Intriguingly, US2 and US11 differ markedly in their substrate constraints. This fact, coupled to the unique properties
of each of the viral proteins, supports a model in which these viral molecules interact
with distinct quality control pathways to mediate destruction of MHC class I molecules.
Materials and Methods

DNA Constructs

Truncation mutants, chimeric A2 MHC class I heavy chain molecules and chimeric US2 and US11 were generated by PCR and confirmed by DNA sequence analysis. Truncation mutants were generated using oligonucleotides encoding the corresponding C-terminal acid amino acid followed by a stop codon. The hemagglutinin epitope was generated by synthetic oligonucleotides. Chimeric molecules were generated so as to not introduce extraneous coding sequence using a unique PCR strategy. Briefly, regions (coding sequences corresponding to luminal, transmembrane or cytoplasmic regions) from each cDNA of interest were amplified by PCR using Pfu polymerase (Roche). Products were 5'-phosphorylated by T4 polynucleotide kinase (New England Biolabs) and corresponding regions were ligated together. A second round of PCR was performed using PCR primers to generate the full-length chimera. The PCR products of the proper size were excised from agarose gel and verified by DNA sequence analysis and transfection.

HLA-A2 mutant heavy chains were generated by PCR. The mutant molecules were terminated at the following amino acid number (aa 1 = initiator methionine): HC_{310^-} aa 356; HC_{320^-} aa 346; HC_{330^-} aa 335; HC_{310HA^-} aa 356 + 10 aa HA-tag; HC_{330HA^-} aa 335 + 10 aa HA-tag; HC_{335HA^-} full length + 10 aa HA-tag. The HA-tag sequence consists of residues AYPYDVPDYA and was cloned as a synthetic oligonucleotide. The A2-GFP was ligated in frame to green fluorescent protein in the vector N1 (Clontech, Palo Alto)
Cell lines, reagents, and antibodies

U373-MG astrocytoma cells transfected with the US2 or US11 cDNA were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum and 5% calf serum. HC_{A10}, HC_{A20}, HC_{A30}, HC_{A101A}, HC_{A30HA}, HC_{365HA}, and HC_{365GFP} were maintained in DMEM supplemented with 5% FCS, 5% CS, and 0.5μg/ml geneticin (Gibco, Frederick, MD). The HEK 293 GPG retroviral packaging cell line was a generous gift from Dr. Richard C. Mulligan (Children's Hospital, Boston, MA). HEK 293 GPG were maintained in DMEM supplemented with 5% FCS, 5% CS and 1.0 μg/ml doxycycline (Sigma Chemical Co.). The anti-heavy chain serum (Tortorella, 1998), W6/32 (Parham et al., 1979) and 12CA5 were used as described. ZL3VS was synthesized as described (Bogyo, et al., 1997).

Metabolic labeling of cells and pulse-chase analysis

Cells were detached from their substrate by trypsin treatment, followed by starvation in methionine-/cysteine-free DMEM for 45 min at 37°C. Cells were metabolically labeled with 500 μCi/ml of [35S] methionine/cysteine (1200 Ci/mM; NEN-Dupont, Boston, MA)/ml at 37°C for the times indicated. In pulse-chase experiments, cells were radiolabeled as above and were chased for the times indicated in DMEM containing non-radiolabeled methionine (2.5 mM) and cysteine (0.5mM). Cells were then lysed in NP-40 lysis buffer (10 mM Tris pH 7.8, 150mM NaCl, 5 mM MgCl₂, 0.5% NP-40) supplemented with 1.5 μg/ml aprotinin, 1μM leupeptin, 2mM phenylmethylsulfonyl fluoride (PMSF) followed by immunoprecipitation. For cells lysed in 1% SDS, the final
concentration of SDS was adjusted to 0.067% with NP-40 lysis mix prior to immunoprecipitation.

**Immunoprecipitation**

Following cell lysis, debris was removed by centrifugation at 15,000g for 5 min. Non-specific binding proteins were removed from the cell lysates by the addition of 3μl/ml normal rabbit serum, 3 μl/ml normal mouse serum and formalin-fixed, heat-killed *Staphylococcus aureus* for 1 h at 4°C. Immunoprecipitation was performed by incubation with antiserum for 45 min at 4°C, followed by addition of *S.aureus* for 45 min at 4°C. The pelleted *S.aureus* were washed four times with washing buffer (0.5% NP-40 in 50 mM Tris pH 7.4, 150 mM NaCl and 5 mM EDTA). The pellet was resuspended in SDS sample buffer (4% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.025% bromophenol blue in 62.5 mM tris pH 6.8) and the released materials were subjected to 12.5% SDS-PAGE.

**Generation of cell lines by retroviral transduction**

MHC class I heavy chain cDNAs were cloned into retroviral vector LNCX (Clontech, Palo Alto, CA). To produce VSV-G pseudotyped retrovirus, HEK 293 GPG were plated in HEK 293 media to yield 50% confluent monolayers the following day. Cells were washed twice in DMEM supplemented with 10% FCS lacking doxycycline and were transiently transfected with LNCX expressing the cDNA of interest using Fugene 6 (Roche) following the manufacturer’s guidelines. The media was changed once daily and the 96 hour supernatant was used to infect MG-U373 cells.
For infection, MG-U373 cells were plated the day before in 6-well dishes at $2 \times 10^5$ well. Retroviral supernatants were filtered through 0.8-μm syringe filters and diluted 1:2 with DMEM+10% FCS plus 4μg/ml (final conc) hexadimethrine bromide (Sigma Chemical Co.) to produce the retroviral cocktail. The U373 media was aspirated and 1ml of infectious cocktail added to each well of plated U373 cells. Plates were centrifuged in a Beckman Allegra 6KR centrifuge for 2 h at 1000 x g at 30°C. After 2 h, plates were moved to humidified CO₂ incubator overnight. The following day, the cocktail was replaced with DMEM+10% FCS. Selection was imposed 2 days post infection.
**Results**

*US2- and US11-mediated degradation of MHC class I carboxy-terminal truncation mutants*

The class I MHC heavy chain is a type I membrane glycoprotein with a cytoplasmic tail of 30 residues. Class I molecules that lack the cytoplasmic tail are refractory to US2 and US11 mediated dislocation (Story, et al., 1999). To define more precisely the structural determinants for dislocation, we generated MHC class I heavy chain mutants with progressive truncations of the cytoplasmic tail (Figure 1) and examined these mutants for their susceptibility to US2- and US11-mediated degradation. The mutants consist of the wild type heavy chain lacking the C-terminal 10 (HC\(_{\Delta 10}\)) or 20 (HC\(_{\Delta 20}\)) amino acids from its cytoplasmic domain.

We introduced the HC\(_{\Delta 10}\) or HC\(_{\Delta 20}\) mutant heavy chains by retroviral transduction into U373-MG cells that stably express either US2 or US11. We then performed a pulse-chase experiment to assess the susceptibility of HC\(_{\Delta 10}\) or HC\(_{\Delta 20}\) to degradation mediated by US2 or US11 (Figure 2).

In U373 cells, the slower migrating endogenous class I heavy chain (HC) and both truncation mutants are stable throughout the 60-minute chase (Figure 2, lanes 1-6). In US2 expressing cells, both truncation mutants HC\(_{\Delta 10}\) and HC\(_{\Delta 20}\) are susceptible to US2-mediated dislocation and subsequent cytosolic degradation, as evidenced by the loss of recovery of the mutant molecules during the chase (Figure 2, lanes 7-12). On overexposure of the autoradiogram, the endogenous heavy chain, expressed at lower levels than the truncation mutant, disappears with similar kinetics (data not shown). When the chase is performed in the presence of the proteasome inhibitor ZL\(_3\) VS, the
Figure 1. Mutant heavy chains

Schematic of MHC class I heavy chains used in following experiments. The wild-type cytosolic portion is 35 amino acids. The transmembrane domains are represented by the shaded regions and the hemaglutinin epitope tags by the black regions.

Sequence of the wild-type cytoplasmic tail of HLA-A2

- R R K • S S D R K G G S Y S • Q A A S S D S A Q G • S D V S L T A C K V -COOH

__________________________________________Δ10

_____________________________________________Δ20

—Δ30

Sequence of the hemaglutinin epitope

- A Y P Y D V P D Y A -COOH
Figure 2. C-terminal truncations of the class I heavy chains are resistant to US11-but not US2-mediated degradation.

U373, US2-U373, and US11-U373 expressing $HC_{\alpha10}$ or $HC_{\alpha20}$ were metabolically-labeled with for 15 min and chased up to 60 min. Cells were lysed and endogenous and mutant MHC class I heavy chains were immunoprecipitated from the lysates with anti-heavy chain antisera. The endogenous heavy chains and truncation mutants were stable in U373 cells (lanes 1-6). In US2 expressing cells, the mutant heavy chains were degraded throughout the chase (lanes 7-12). In US11 expressing cells, the endogenous heavy chains were rapidly degraded but the mutant heavy chains were stabilized (lanes 13-18).
Chase (min) 0 30 60

U373 cells

Lane 1 2 3

HC Δ20

HC Δ20

US2 cells

Lane 7 8 9

HC Δ20

HC Δ20

US11 cells

Lane 13 14 15

HC Δ20

HC Δ20

HC Δ10

HC Δ10

HC Δ10

HC Δ10

Pulse: 15 min
Immunoprecipitation: anti-HC
endogenous deglycosylated wild-type and mutant deglycosylated heavy chain accumulate in the cytosol (data not shown).

In contrast to US2, neither the HC$_{\Delta10}$ nor HC$_{\Delta20}$ mutants were destabilized in the presence of US11 (Figure 2, lanes 13-18). However, the endogenous HC continued to be degraded in a US11-dependent manner. This failure of US11 to degrade these molecules was not due to the inability of HC$_{\Delta10}$ or HC$_{\Delta20}$ to fold properly, as both molecules could be immunoprecipitated with the conformation-specific monoclonal antibody W6/32 (data not shown). Therefore, US2 and US11 differ in the requirements for structural determinants in the cytoplasmic domain of MHC class I molecules to effectuate dislocation from the ER to the cytosol.

**US2- and US11-mediated degradation of chimeric MHC class I molecules**

Class I MHC heavy chains lacking 20 C-terminal residues (HC$_{\Delta20}$) were efficiently degraded in a US2 dependent manner, but class I heavy chains lacking 30 residues (HC$_{\Delta30}$) were resistant to the effects of US2. To determine whether the susceptibility of HC$_{\Delta20}$ to US2 was dependent on a particular amino acid sequence in the cytoplasmic tail, we generated a chimeric class I molecule in which we appended the hemagglutinin (HA) epitope recognized by the monoclonal antibody 12CA5 to the C-terminus of HC$_{\Delta30}$ (HC$_{\Delta30\text{HA}}$, Figure 1). This mutant molecule has a cytoplasmic tail distinct from the wild-type sequence but has the same number of residues as HC$_{\Delta20}$.

The HC$_{\Delta30\text{HA}}$ mutant is stable in U373 cells throughout the chase period (lanes 1-3, 7-9). In US2-expressing cells, HC$_{\Delta30\text{HA}}$ is susceptible to US2 mediated dislocation and is degraded with kinetics similar to wild-type heavy chain (Figure 3, lanes 4-6, 10-12).
Figure 3. **US2 has no sequence preference for the shortened cytosolic tail of class I heavy chains.**

U373 and US2-U373 cells expressing HC_{Δ30HA} were pulse-labeled with [^{35}S] methionine for 15 min and chased up to 60 min, lysed, and subjected to immunoprecipitation with anti-HA and anti-heavy chain antisera. HC_{Δ30HA} was stable in U373 cells (lanes 1-3) similar to endogenous heavy chains (lanes 7-9). HC_{Δ30HA} was destabilized in the presence of US2 (lanes 4-6) with kinetics similar to the endogenous heavy chain (lanes 10-12).
Pulse 15 min
Immunoprecipitation: anti-HA

Pulse 15 min
Immunoprecipitation: anti-HC
HC_{Δ30HA} accumulates as a deglycosylated cytosolic intermediate when the cells were treated with the proteasome inhibitor ZL3VS (data not shown). Therefore, the rapid dislocation of MHC class I by US2 is not dependent on any particular amino acid sequence present in the cytoplasmic tail of the class I molecule, but instead dislocation relies on a relatively small, sequence independent domain by which the class I molecule may be extracted.

We used a similar strategy to determine if US11 requires any particular sequence in the C-terminal 10 amino acids of the MHC class I heavy. Since HC_{Δ10} is not susceptible to US11 mediated dislocation, we appended the HA sequence to this mutant (HC_{Δ10HA}), restoring the number of residues in the cytoplasmic portion to that of the wild-type heavy chain. U373 and US11 expressing cells were pulsed for 15 minutes and chased for 0, 45, and 90 minutes, lysed and heavy chains were immunoprecipitated with anti-HA and anti-HC serum. The immunoprecipitates were analyzed by SDS-PAGE. The HC_{Δ10HA} molecules is stable in wild-type cells throughout the 90 minute chase point (Figure 4, lanes 1-3, 7-9). In US11 expressing cells, HC_{Δ10HA} is rapidly targeted and dislocated by US11 (lanes 4-6, 10-12). The mutant heavy chain is dislocated to the cytosol and the deglycosylated intermediate can be visualized by addition of ZL3VS to the chase medium. (data not shown) Thus, US11 does not require any particular amino acid sequence but, similar to US2, a sequence-independent domain suffices to engage the dislocation machinery and remove class I MHC from the ER.

We have shown that US2 requires at least 10 amino acids and US11 at least 30 amino acids in the cytoplasmic tail of MHC class I molecules to mediate dislocation (Figure 1). Having defined the lower limit for the length of the cytoplasmic tail, we next
Figure 4. US11 has no sequence preference for the C-terminal 10 residues of the full-length heavy chain.

U373 and US11-U373 cells expressing $\text{HC}_{\Delta 10\text{HA}}$ were pulse-labeled with $^{35}$S methionine for 15 min and chased up to 90 min, lysed, and subjected to immunoprecipitation with anti-HA and anti-heavy chain antisera. $\text{HC}_{\Delta 10\text{HA}}$ was stable in U373 cells (lanes 1-3) similar to endogenous heavy chains (lanes 7-9). $\text{HC}_{\Delta 10\text{HA}}$ was destabilized in the presence of US11 (lanes 4-6), as were the endogenous heavy chains (lanes 10-12). Endogenous heavy chains were degraded with slower kinetics due to competition by the over-expressed mutant heavy chain.
Pulse 15 min
Immunoprecipitation: anti-HA

Pulse 15 min
Immunoprecipitation: anti-heavy chain
sought to identify an upper limit for the length of the carboxy-terminal tail for dislocation mediated by these viral molecules. We appended the HA sequence to the C-terminus of wild-type heavy chain (HC$_{365HA}$), extending the cytoplasmic tail by 10 residues. Wild-type, US2-expressing and US11-expressing cells were radio-labeled, chased, lysed and subject to immunoprecipitation with anti-HA antibodies, followed by analysis by SDS-PAGE. HC$_{365HA}$ is stable in U373 cells throughout the 40-minute chase period. (Figure 5, lanes 1-2, 7-8) In US2 expressing cells, US2 mediates degradation of HC$_{365HA}$ as well as the endogenous HC molecule. (lanes 3-4, 9-10) In contrast, US11 is incapable of mediating dislocation of HC$_{365HA}$ but can still efficiently degrade the endogenous HC. (lanes 5-6, 11-12)

In similar fashion to HC$_{365HA}$, we generated an MHC class I molecule with green fluorescent protein (GFP) fused to the C-terminus (A2-GFP). This fusion protein was stably introduced into U373-MG, US2-expressing, and US11-expressing cells. The GFP moiety was properly folded, as stable cell lines exhibited green fluorescence. Cells were metabolically labeled, chased, lysed and subject to immunoprecipitation with anti-GFP and sequentially with anti-heavy chain serum. In wild type cells, the A2-GFP molecule was stable through the 30-minute chase (Figure 6, lanes 1-4). In US2 expressing cells, US2 was capable of efficiently dislocating A2-GFP (lanes 5-8) and the endogenous HC (lanes 13-16) to the cytosol, generating the appropriate deglycosylated intermediate in the presence of the proteasome inhibitor ZL$_2$VS. In contrast to US2, US11 is incapable of mediating dislocation of A2-GFP (lanes 9-12) but efficient at mediating dislocation of the endogenous HC (lanes 17-20).
Figure 5. C-terminal extension of the class I heavy chains are resistant to US11- but not US2-mediated degradation.

U373, US2-U373, and US11-U373 expressing HC<sub>365HA</sub> were pulse-labeled with <sup>[35S]</sup>methionine for 15 min and chased up to 40 min. Cells were lysed and the endogenous and mutant MHC class I heavy chains were immunoprecipitated from the lysates with anti-HA and anti-heavy chain antisera. The C-terminal extended mutants were stable in U373 cells (lanes 1-2) as were the endogenous heavy chains (7-8). In US2 expressing cells, the mutant heavy chains were degraded throughout the chase (lanes 3-4), as well as the endogenous heavy chains (9-10). In US11 expressing cells, the mutant heavy chains were stabilized (lanes 5-6) but the endogenous heavy chains continued to be degraded (lanes 11-12).
Pulse 15 min
Immunoprecipitation: anti-HA
Figure 6. US2 but not US11 can degrade class I heavy chains with a C-terminal GFP moiety.

U373, US2-U373, and US11-U373 expressing A2-GFP were pulse-labeled with $[^{35}S]$ methionine for 15 min and chased for 30 min. Cells were lysed and endogenous and mutant MHC class I heavy chains were immunoprecipitated from the lysates with anti-GFP and anti-heavy chain antisera. The endogenous heavy chains and A2-GFP fusions were stable in U373 cells (lanes 1-4). In US2 expressing cells, the A2-GFP fusions were degraded throughout the chase (lanes 5-6) as were endogenous heavy chains (lanes 13-14) and in the presence of proteasome inhibitor the dislocated intermediate accumulated (lanes 7-8 and 15-16). In US11 expressing cells, A2-GFP fusions were stable (lanes 9-12). The endogenous heavy chains were degraded in US11 cells (lanes 17-18) and the cytosolic intermediate was visualized upon incubation with proteasome inhibitor (lanes 19-20).
Cell line: U373 US2 US11
50 μM ZL3 VS: 
Chase time (min): 0 30 0 30 0 30 0 30 0 30
IP: anti-GFP 70-
sequential IP: anti-HC 50-
A2-GFP
A2-GFP-CHO
HC
HC-CHO
These data demonstrate that US2 tolerates significant perturbations in the length and sequence of the cytoplasmic tail of MHC class I and still dislocates C-terminally extended class I molecules. In contrast, US11 mediated dislocation of class I is constrained by the length of the cytoplasmic tail, but, similar to US2, it is rather indifferent to a particular amino acid sequence in the tail.
Discussion

US2 and US11 have markedly different length requirements for the cytoplasmic tail of MHC class I molecules to mediate dislocation. US11 is more constrained in relation to US2, and degrades only class I molecules with a cytoplasmic tail consisting of approximately 30 residues, but in a sequence independent manner. US2, however, will degrade a class I molecule with an extended cytoplasmic domain, including the addition of a large globular GFP molecule, as well as a class I molecule in which the cytoplasmic tail has been replaced by 10 residues of alternate sequence. The different substrate requirements imposed by US2 and US11 argue strongly for differential recruitment of some unknown factor(s) that mediates the retrotranslocation process. Consistent with our data, we propose a “groove” versus “pocket” model for substrate targeting by US2 and US11 (Figure 7). This model of the viral molecules utilizing disparate pathways is consistent with other data. The cytoplasmic tail of US11 is dispensable for binding and dislocation of MHC class I. In contrast, the tail of US2 is not necessary for class I binding but essential for US2 mediated destruction of MHC class I (Furman, et al., 2002).

Why has HCMV evolved two different proteins that seem to mediate the same function? One scenario is that US2 and US11 have evolved to interact with distinct sets of the highly polymorphic MHC products in the human population. However, no data have so far identified differences in the affinity of these viral molecules for various human class I alleles. Alternatively, these two seemingly redundant molecules may have been retained by the virus because they differ in their efficacy to down-regulate MHC class I molecules in different cell types. US11 is more effective than US2 at degrading MHC class I molecules in primary dendritic cells (Rehm, et al., 2002).
Figure 7. Model for differential recruitment

US2 and US11 must recruit different factors to mediate dislocation of class I heavy chains. US2 is indifferent to the length of the cytosolic tail of class I. US11 is highly sensitive to the tail length.
US2 mechanism: insensitive to tail length

US11 mechanism: measures tail length
Alternatively, these viral molecules may have evolved to more effectively degrade MHC class I molecules under different stress conditions, such as in the presence of inflammatory mediators. In this scenario, US2 and US11 would interact with distinct pathways in the ER quality control process.

As for the distinct quality control pathways with which these viral proteins may interact and the functional consequences, at present we can only speculate. Perhaps the difference lies in a single protein that may bridge class I molecules to the ER degradation machinery. Alternatively, US2 and US11 may interact with functionally and mechanistically distinct ER degradation pathways.

Studies in yeast and mammalian cells have identified proteins involved in the targeting, retrotranslocation, and degradation of ER proteins. The ER chaperones calnexin, Bip, and protein disulfide isomerase (PDI) have been implicated in the initial steps of the targeting process (McCracken and Brodsky, 1996), (Brodsky, et al., 1999). Biochemical and mutagenesis experiments suggest that the translocon is the conduit through which polypeptides are dislocated from the ER to the cytosol (Zhou and Schekman, 1999). Neither the role of ER-resident chaperones nor that of the translocon has been found to apply to all types of dislocation suggested in the literature. In several cases, the involvement of ubiquitin conjugation in some aspect of the dislocation reaction has been documented (Hampton and Bhakta, 1997), (Biederer, et al., 1997). Ubiquitin and ubiquitin-conjugation enzymes such E2 and E3 ubiquitin ligases are believed to be important players in the degradation of ER proteins.

The yeast protein complex Npl4-Ufd1-Cdc48 and its mammalian homolog have been implicated in dislocation (Bays, et al., 2001), (Ye, et al., 2001). This complex
appears essential for extracting ubiquitinated substrates from the ER membrane in yeast, and data from mammalian systems are consistent with this role for the homologous complex in higher eukaryotes. The Npl4-Ufd1-Cdc48 complex recognizes poly-ubiquitinated substrates which are presumably mobilized for exit from the ER membrane by the ATPase activity of Cdc48 (Jarochs, et al., 2002). This complex may thus define a proximal step for presenting degradation substrates to the cytosolic proteasome.

The “groove” and “pocket” adaptors of US2 and US11 may link with identical components that mediate ubiquitination and present substrates to the Npl4-Ufd1-Cdc48 complex for extraction and subsequent proteasomal degradation. Alternatively, these viral proteins may utilize distinct pathways that have few factors in common. The results that US2 degrades only properly folded MHC class I molecules whereas US11 can degrade free heavy chains (J. Loureiro, submitted) leads to speculation that US2 and US11 may interact with components involved in regulated versus non-regulated degradation of ER substrates.
References


Chapter 5

Identification of functional domains that mediate US11-catalyzed dislocation of class I MHC molecules

DNA constructs used to produce Figures 1-6 were developed in collaboration with Domenico Tortorella.
Abstract

The HCMV glycoproteins US2 and US11 catalyze the rapid dislocation of MHC class I molecules from the ER to the cytosol. Chimeric molecules were generated by swapping the lumenal domains of the viral proteins and these constructs were assayed for their ability to dislocate MHC class I molecules. Although the chimeric molecules could bind to their target, the chimeras could not catalyze dislocation. In a similar approach, chimeric molecules were constructed by exchanging the lumenal or cytoplasmic domain of the dislocation substrate HLA-A2 with the respective domains of the viral proteins. Most of these constructs were stable when expressed alone or in conjunction with their complementary molecules. Only one molecule, the fusion of the cytoplasmic tail of HLA-A2 to the lumen and transmembrane region of US11, was dislocated with rapid kinetics. In further studies, ER-inserted GFP with the transmembrane domain of US11 and cytoplasmic tail of HLA-A2 was rapidly dislocated to the cytosol. This rapid dislocation was dependent on the glutamine residue present in the transmembrane of US11. The glutamine has also been shown to be essential for US11-catalyzed destruction of MHC class I molecules, indicating the GFP chimera faithfully recapitulates US11-catalyzed degradation in a single molecule.
Introduction

Glycoproteins undergo quality control in the endoplasmic reticulum (ER) before progressing through the secretory pathway (Ellgaard, et al., 1999). ER-resident chaperones and other components in the ER environment facilitate signal sequence processing, disulfide bond formation, membrane insertion and glycosylation. This environment aids the nascent polypeptide in adopting its proper tertiary and quaternary structure (Braakman, et al., 1991). Proteins unable to properly fold or multimerize are retained in the ER and eventually discharged to the cytosol for subsequent degradation by the proteasome (Brodsky and McCracken, 1999a), (Plumper, et al., 1999). The steps of recognition, targeting, and dislocation of the folding misfits are being elucidated and likely involve multiple disposal pathways (Bays, et al., 2001), (Swanson, et al., 2001).

Viruses have co-opted this quality control pathway for their own ends (Tortorella, et al., 2000). As the ER is the site of synthesis for the majority of immunologically-relevant cell-recognition molecules, viral proteins target this organelle to disrupt such recognition. The HCMV gene products US2 and US11 catalyze the dislocation of MHC class I molecules from the ER to the cytosol for destruction by the proteasome (Wiertz, et al., 1996a), (Wiertz, et al., 1996b). These viral molecules require no other viral factors to accomplish this task, and thus co-opt the endogenous quality control machinery to disrupt MHC class I antigen presentation.

Most experimental systems for studying protein degradation from the ER rely on model substrates that possess molecular lesions that do not allow them to pass quality control in the ER (Sifers, et al., 1988), (Huppa and Ploegh, 1997). US2 and US11 provide a unique system to study ER-associated degradation, as they “bridge” a substrate
otherwise competent to pass ER quality control to the degradation machinery and thereby induce the protein's destruction. A similar strategy is employed by the HIV protein Vpu to degrade the glycoprotein CD4 (Margottin, et al., 1998). Efforts to define the cellular components that potentiate the ability of US2 and US11 to degrade MHC class I molecules will better define the components of the ER quality control machinery.

Many cellular proteins can be partitioned into discrete domains, each possessing a specific function. A scheme emerges where a protein may be composed of multiple functional "modules" and a particular combination will generate a protein with a particular function based on protein association, localization, and enzymatic activity (Harris and Lim, 2001), (Karandikar and Cobb, 1999). In a similar fashion, domains regulating the traffic and intracellular localization of proteins within a cell are often discrete regions in the cytoplasmic or transmembrane domain (Teasdale and Jackson, 1996).

There is evidence that US2 and US11 can be partitioned into discrete domains, each mediating a different activity. The crystal structure of the lumenal domain of US2 in association with the lumenal portion of MHC class I was solved (Gewurz, et al., 2001), demonstrating the points of contact mediating association between the lumenal portions of the viral molecule and its cellular partner. Similarly, a mutant US11 molecule lacking its transmembrane and cytoplasmic domain co-immunoprecipitates in complex with MHC class I molecules (D. Tortorella, unpub. res.). Although the lumenal domains of both viral molecules bind their substrate, US2 and US11 likely mediate MHC class I degradation through distinct mechanisms. For example, the cytoplasmic tail of US2 is
essential to mediate destruction of MHC class I molecules, whereas the cytoplasmic tail of US11 is dispensable for this activity (Furman, et al., 2002).

To better define how these viral proteins may be arranged in discrete functional domains, we generated reciprocal swaps of corresponding domains between US2 and US11 to investigate if these chimeric molecules degrade the endogenous MHC class I molecules. This scheme would determine if the domains of US2 and US11 complement each other and, potentially, if the molecules utilize the same cellular factors. In addition, we generated reciprocal swaps between US2 or US11 and the MHC class I molecule HLA-A2. In this scenario, all sequence information for mediating association and dislocation is present, but it is arranged in a trans rather than cis configuration. The data support a model in which US2 and US11 each utilize a distinct degradative pathway. In addition, the combination of regions from the viral molecule and MHC class I molecule is necessary and sufficient for substrate targeting and degradation.
Materials and Methods

Cell lines, reagents, and antibodies

U373-MG astrocytoma cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, 5% calf serum. When appropriate, cells were selected in 0.5 mg/ml neomycin and/or 0.4 μg/ml puromycin. The HEK 293 GPG retroviral packaging cell line was a generous gift from Dr. Richard C. Mulligan (Children's Hospital, Boston, MA). HEK 293 GPG were maintained in DMEM supplemented with 5% FCS, 5% CS and 1.0 μg/ml doxycycline (Sigma Chemical Co, St. Louis, MO). The anti-heavy chain serum (Tortorella, et al., 1998), anti-TCRα serum (Huppa and Ploegh, 1997), W6/32 (Parham, 1979), the calnexin-specific monoclonal AF8 (Rajagopalan, et al., 1994), anti-US2 (Wiertz, et al., 1996a), anti-US11 (Wiertz, et al., 1996b), and anti-PDI (Fiebiger, et al., 2002) were used as described. Full-length protein of GFP was expressed in bacteria. Rabbits were immunized with the recombinant proteins and polyclonal antisera were obtained. ZL3-VS was synthesized and used as described (Bogyo, et al., 1997).

Metabolic labeling of cells and pulse-chase analysis

Cells were detached from their substrate by trypsin treatment, followed by starvation in methionine-/cysteine-free DMEM for 45 min at 37°C. Cells were metabolically labeled with 500 μCi/ml of [35S]methionine/cysteine (1200 Ci/mM; NEN-Dupont, Boston, MA)/ml at 37°C for the times indicated. In pulse-chase experiments, cells were radiolabeled as above and were chased for the times indicated in DMEM containing non-radiolabeled methionine (2.5 mM) and cysteine (0.5 mM). Cells were then lysed in NP-
40 lysis buffer (10 mM Tris pH 7.8, 150mM NaCl, 5 mM MgCl₂, 0.5% NP-40) supplemented with 1.5 µg/ml aprotinin, 1µM leupeptin, 2mM phenylmethylsulfonyl fluoride (PMSF) followed by immunoprecipitation. For cells lysed in 1% SDS, the final concentration of SDS was adjusted to 0.067% with NP-40 lysis mix prior to immunoprecipitation.

**Immunoprecipitation**

Following cell lysis, debris was removed by centrifugation at 15,000g for 5 min. Non-specific binding proteins were removed from the cell lysates by the addition of 3µl/ml normal rabbit serum, 3 µl/ml normal mouse serum and formalin-fixed, heat-killed *Staphylococcus aureus* for 1 h at 4°C. Immunoprecipitation was performed by incubation with antiserum for 45 min at 4°C, followed by addition of *S.aureus* for 45 min at 4°C. The pelleted *S.aureus* were washed four times with washing buffer (0.5% NP-40 in 50 mM Tris pH 7.4, 150 mM NaCl and 5 mM EDTA). The pellet was resuspended in SDS sample buffer (4% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.025% bromophenol blue in 62.5 mM tris pH 6.8) and the released materials were subjected to 12.5% SDS-PAGE.

**Generation of cell lines by retroviral transduction**

MHC class I heavy chain cDNAs were cloned into retroviral vector pLNCX (Clontech, Palo Alto, CA). To produce VSV-G pseudotyped retrovirus, HEK 293 GPG were plated in HEK 293 media to yield 50% confluent monolayers 24 hours later. Cells were washed twice in DMEM supplemented with 10% FCS lacking doxycycline and were transiently
transfected with LNCX expressing the cDNA of interest using Fugene 6 (Roche) following the manufacturer's guidelines. The media was changed once daily and the 96-hour supernatant was used to infect U373-MG cells.

For infection, U373-MG cells were plated the day before in 6-well dishes at 2x10⁷ well. Retroviral supernatants were filtered through 0.8-µm syringe filters and diluted 1:2 with DMEM+10% FCS plus 4µg/ml (final conc) hexadimethrine bromide (Sigma Chemical Co.) to produce the retroviral cocktail. The U373 media was aspirated and 1ml of infectious cocktail added to each well of plated U373 cells. Plates were centrifuged in a Beckman Allegra 6KR centrifuge for 2 h at 1000 x g at 30°C. After 2 h, plates were moved to humidified CO₂ incubator overnight. The following day, the cocktail was replaced with DMEM+10% FCS. Selection was imposed 2 days post infection.

**Subcellular fractionation**

GFPc cells were metabolically labeled for 12 min and chased for 60 min. Each time point was washed twice in 50 mM Tris pH 7.5, 250 mM sucrose (homogenization buffer). The cells were resuspended in 1 ml homogenization and passed 6 times through a ball bearing homogenizer. The homogenate was centrifuged at 1000 g for 5 min; the pellet fraction was resuspended in NP-40 lysis mix to give 1k fraction. The supernatant was centrifuged 150,000 g using a TLA 100.2 rotor in a Beckman centrifuge. The supernatant was diluted to a final 1X NP-40 lysis mix. The pellet was resuspended in 1X NP-40 lysis mix.
Results

Domain swaps between US2 and US11

A plethora of data has demonstrated that the HCMV glycoproteins US2 and US11 catalyze destruction of MHC class I molecules, but the mechanism by which this activity is executed remains a mystery. Presumably, different domains of the viral molecules mediate association with MHC class I molecules and targeting to the degradation machinery in the ER. To address this idea, chimeric molecules were constructed in which the lumenal or cytoplasmic portions of US2 or US11 were exchanged with each other or with their target HLA-A2. The chimeric molecules were made to provide insight into which domains may be important for substrate binding or targeting and how these domains may interact.

Although US2 and US11 catalyze the same reaction, there is little data as to whether they utilize shared or distinct degradation pathways. In an attempt to define regions of the viral molecules that could complement each other, chimeras were made in which the lumenal portions of US2 and US11 were reciprocally exchanged. The chimera consisting of the lumenal domain of US2 fused to the transmembrane and cytoplasmic domain of US11 is named 2-11, and the reciprocal molecule is termed 11-2. These constructs were stably introduced into U373-MG cells and clonal cell lines were generated. 2-11 and 11-2 cells were metabolically labeled for 15 minutes and chased for 30 minutes. Cells were lysed in denaturing detergent and subjected to immunoprecipitation for the molecules of interest. The 2-11 molecule was stable through the 30-minute chase point, as were the endogenous heavy chains (Figure 1A). The 11-2 molecule was stable and did not catalyze degradation of the endogenous heavy chain.
Figure 1. Chimerias between US2 and US11 are stable and do not destabilize MHC class I heavy chains.

Pulse-chase of heavy chains in MG-U373 cells expressing 2-11 or 11-2 constructs. Single cell clones of MG-U373 stably transfected with constructs expressing 2-11 or 11-2 were metabolically labeled for 10 min and chased for 0 or 30 min. Cells were lysed in SDS and subjected to immunoprecipitation for MHC class I heavy chains and US2 simultaneously. The mixed IPs were analyzed by SDS-PAGE analysis.

A) Neither the MHC class I heavy chains nor 2-11 were destabilized by the 30 minute chase point.

B) Neither the MHC class I heavy chains nor 11-2 were destabilized by the 30 minute chase point.
A) 2-11 cells

Chase: 0 30 (min)

HC
- 25
- 40

2-11
- +CHO
- -CHO

Pulse 10 min
IP: anti-HC & anti-US2

B) 11-2 cells

Chase: 0 30 (min)

HC
- 25
- 40

11-2

Pulse 10 min
IP: anti-HC & anti-US11
molecules (Figure 1B). Thus, the luminal portions of the viral molecules cannot complement one another to degrade MHC class I molecules.

**Luminal domain swaps**

A second set of experiments addressed if the topological domains of the viral and host molecules present in cis on a single polypeptide could mediate dislocation if present in a trans configuration. The luminal domains of US2 and HLA-A2 were exchanged to yield 2lum-A2 and A2lum-2. These constructs were co-expressed in U373-MG cells. Cells were metabolically labeled and chased for the indicated times. The A2lum-2 molecule reacted with antisera specific for unfolded heavy chains (lanes 1-3) but this chimeric molecule folded quite poorly, as evidenced by failure to react with the conformation specific antibody W6/32 (Figure 2, lanes 4-6 & data not shown). The 2lum-A2 molecules reacted with antisera to US2 and co-immunoprecipitated with the properly folded endogenous MHC class I molecule. This association is consistent with previous results indicating the luminal portion of US2 mediates association with MHC class I molecules.

Neither chimeric molecules nor the endogenous heavy chains were destabilized when expressed alone or when co-expressed in the same cells (data not shown). One caveat is US2 seems to bind only to properly folded MHC class I molecules (J. Loureiro, submitted). The poor folding of A2lum-2, as evidenced by lack of recovery with W6/32, may mean the domains of the two chimeric molecules associate poorly, which could account for the observed lack of degradation. The increase in molecular weight of the
Figure 2. Co-expression of A2lum-2 and 2lum-A2.

Pulse-chase of MHC class I heavy chains, A2lum-2, and 2lum-A2 in MG-U373 cells. Single cell clones of MG-U373 stably transfected with constructs expressing A2lum-2 and 2lum-A2 were metabolically labeled for 10 min and chased for 0, 20, or 40 min. Cells were lysed in NP-40 lysis buffer and subjected to immunoprecipitation for unfolded MHC class I heavy chains, folded W6/32-reactive material and US2. The IPs were analyzed by SDS-PAGE. Neither of the chimeric molecules were destabilized, nor were the MHC class I heavy chains.
A2lum-2: **A2** tm **US2**

US2lum-A2: **US2** tm **A2**

<table>
<thead>
<tr>
<th>IP</th>
<th>Chase (min)</th>
<th>W6/32</th>
<th>anti-US2</th>
</tr>
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<tr>
<td>anti-HC</td>
<td>0 20 40</td>
<td>0 20 40</td>
<td>0 20 40</td>
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<tr>
<td>W6/32</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>anti-US2</td>
<td>0 20 40</td>
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</table>

Pulse 10 min
folded endogenous heavy chains (compare lanes 4 & 6) is due to elaboration of the N-linked glycan as the heavy chain trafficked through the Golgi.

Similar constructs were made in which the lumenal portions of US11 and HLA-A2 were exchanged to produce 11lum-A2 and A2lum-11. These chimeric molecules were co-expressed in U373-MG cells. Cells were metabolically labeled, chased for the indicated times, lysed and subjected to immunoprecipitation. The A2lum-11 molecule reacted with antisera to free heavy chains (Figure 3, lanes 1-3) as well as antibodies to the properly folded MHC class I molecule (lanes 4-6 & data not shown). The 11lum-A2 molecule was recognized by antisera to US11 (lanes 7-9) and weakly co-immunoprecipitated with the properly folded MHC class I molecules (lanes 5-6). 11-A2lum may be associated with the endogenous heavy chains or with the A2lum-11 molecules. Neither chimeric molecule nor the endogenous heavy chains were destabilized by expression alone or when co-expressed in the same cell (Figure 3 & data not shown).

Interestingly, the rate at which MHC class I molecules exit from the ER, as evidenced by conversion to the higher molecular weight species, appeared markedly reduced in cells expressing 11lum-A2 (lanes 4-6 & data not shown). This is consistent with previous data that the luminal portion of US11 can significantly slow the egress of MHC class I molecules from the ER (B. Lilley, pers. comm.).

* Cytoplasmic domain swaps

The next approach was to exchange the cytoplasmic portions of the viral molecules and their substrates to determine if these domains could potentiate degradation
Figure 3. Co-expression of A2 lum-11 and 11 lum-A2.

Diagram of chimeric molecules and pulse-chase-chase of MHC class I heavy chains, A2 lum-11, and 11 lum-A2 in MG-U373 cells. Single cell clones of MG-U373 stably transfected with constructs expressing A2 lum-11 and 11 lum-A2 were metabolically labeled for 10 min and chased for 0, 20, or 40 min. Cells were lysed in NP-40 lysis buffer and subjected to immunoprecipitation for unfolded MHC class I heavy chains, folded W6/32-reactive material and US11. The IPs were analyzed by SDS-PAGE. Neither of the chimeric molecules were destabilized, nor were the MHC class I heavy chains.
A2lum-11: A2 tm US11
US11lum-A2: US11 tm A2

**IP:**
- anti-HC
- W6/32
- anti-US11

**Chase (min):**
- 0
- 20
- 40

**Pulse 10 min**
in a *trans* configuration. The cytoplasmic domains of US2 and HLA-A2 were swapped to generate 2-A2tail and A2-2tail. These chimeric molecules were co-expressed in U373-MG cells. Cells were metabolically labeled, chased for the indicated times, lysed and subjected to immunoprecipitation. A2-2tail reacted with antisera to the free heavy chains (Figure 4, lanes 1 & 2) and could form properly-folded W6/32-reactive conformers (lane 5). 2-A2tail reacted with antisera to US2 (lanes 3 & 4) and co-immunoprecipitated with W6/32 reactive material (lane 5). Comparing the intensity of the bands on the autoradiogram, it appears 2-A2tail bound to W6/32-reactive A2-2tail (lane 5, compare intensity of HC and A2-US2tail). Neither chimeric molecules nor the endogenous heavy chains were destabilized when the chimeras were expressed alone or simultaneously (Figure 4 & data not shown).

The cytoplasmic tails of HLA-A2 and US11 were exchanged to produce A2-11tail and 11-A2tail. These chimeric molecules were co-expressed in U373-MG cells. Cells were metabolically labeled, chased for the indicated times, lysed, and subjected to immunoprecipitation. The A2-11tail molecule reacted with antisera to the free heavy chains (Figure 5, lanes 1& 2) as well as the antibody W6/32 (data not shown), and this molecule was stable through the 60-minute chase point. 11-A2tail reacted with antisera to US11 and was significantly destabilized by the 60-minute chase point, as evidenced by decreased recovery of labeled material from the lysate. The destabilization of 11-A2tail was investigated further.

U373-MG cells stably expressing 11-A2tail were metabolically labeled and chased for the indicated times in the presence or absence of the proteasome inhibitor ZL3VS. The 11-A2tail molecule was rapidly degraded, with the majority of the
Figure 4. Co-expression of A2-US2tail and 2-A2tail.

Diagram of chimeric molecules and pulse-chase of MHC class I heavy chains, A2-US2tail, and 2-A2tail in MG-U373 cells. Single cell clones of MG-U373 stably transfected with constructs expressing A2-2tail and 2-A2tail were metabolically labeled for 10 min and chased for 0 or 40 min. Cells were lysed in NP-40 lysis buffer and subjected to immunoprecipitation for unfolded MHC class I heavy chains, folded W6/32-reactive material and US2. The IPs were analyzed by SDS-PAGE. Neither of the chimeric molecules were destabilized, nor were the MHC class I heavy chains.
Figure 5. Co-expression of A2-11tail and 11-A2tail.

Diagram of chimeric molecules and pulse-chase of MHC class I heavy chains, A2-11tail, and 11-A2tail in MG-U373 cells. Single cell clones of MG-U373 stably transfected with constructs expressing A2-11tail and 11-A2tail were metabolically labeled for 10 min and chased for 0 or 60 min. Cells were lysed in SDS and subjected to immunoprecipitation for unfolded MHC class I heavy chains and US11. The IPs were analyzed by SDS-PAGE. The A2-US11tail molecule was stable through the chase. The 11-A2tail molecule was destabilized as evidenced by loss of recovery at the 60-min chase point.
A2-US11tail: A2 tm US11

11-A2tail: US11 tm A2

IP: anti-HC anti-US11
Chase: 0 60 0 60 (min)

Pulse 15 min
Figure 6. 11-A2tail is unstable and dislocated from the ER to the cytosol.

Pulse-chase of 11-A2tail in the absence and presence of proteasome inhibitor. Single cell clones of MG-U373 cells stably transfected with a construct expressing 11-A2tail were metabolically labeled for 15 min and chased for 0, 20, or 40 min in the absence (-) or presence (+) of the proteasome inhibitor ZL3VS. Lysates were subjected to immunoprecipitation for US11 and SDS-PAGE analysis. The glycosylated form of 11-A2 tail (+CHO) was converted to the deglycosylated form (-CHO) during the chase. The minor bands of uncleaved signal sequence form appears above the major bands.
11-A2tail cells

ZL₃VS: - +

min: 0 20 40 0 20 40

11-A2tail

Pulse 15 min
IP: anti-US11
radiolabeled molecules present at the 0 time point having disappeared by the 20 minute
time point (Figure 6, lanes 1-3). In the presence of proteasome inhibitor, the higher
molecular weight radiolabeled species were converted into a lower molecular weight
form (lanes 4-6), indicative of dislocation from the ER to the cytosol and subsequent
removal of the N-linked glycan.

Multiple US11-antisera-reactive radiolabeled forms can be visualized in the
presence of proteasome inhibitor. The two major forms consist of the N-glycosylated and
deglycosylated molecules. The minor species present just above these major species
contain the uncleaved signal sequence that directs the polypeptide for ER insertion.
Aberrant signal sequence processing has been observed for US11 and the effect is more
robust for its mutants in which the transmembrane and cytoplasmic domains have been
removed (Rehm, et al., 2001). This processing defect has also been observed for US11
molecules containing single amino acid substitutions in the transmembrane region (B.
Lilley, pers. comm.).

Minimal regions for dislocation

Given the instability of the 11-A2tail molecule and assuming that the luminal
portion of US11 is responsible solely for associating with its substrate, I speculated the
transmembrane region of US11 juxtaposed to the tail of HLA-A2 synergized to mediate
dislocation from the ER. To formally test this hypothesis, a cDNA was constructed
consisting of the coding sequence of the H2-Kb signal sequence fused to GFP followed
by the transmembrane of US11 and the cytoplasmic tail of HLA-A2. This construct was
stably transduced into U373-MG cells. Cells were metabolically labeled, chased for the
indicated times, lysed and subjected to immunoprecipitation. The GFP chimera (GFPc) was rapidly degraded, with the majority being destroyed by the 40 minute chase point (Figure 7). In contrast, ER-inserted soluble GFP with a C-terminal KDEL ER-retention motif has a half-life greater than 2 hours (data not shown), indicating GFP is not inherently unstable in the ER.

GFPc is not glycosylated, so pulse-chase analysis followed by subcellular fractionation was employed to determine if the GFP chimera is dislocated from the ER to the cytosol. Cells were metabolically labeled for 15 minutes and chased for 60 minutes in the presence of proteasome inhibitor. Cells were lysed by ball bearing homogenization and separated by centrifugation into pellet and soluble supernatant fractions. The GFP chimera was present at the 0 and 60 minute time points (Figure 8A, lanes 1 & 2). At the 0-time point, GFPc was present exclusively in the pellet fraction (present in lane 3, not in lane 4). At the 60-minute time point, some of the ER associated material had moved to the soluble fraction, indicating dislocation form the ER to the cytosol (compare lanes 4 & 6). The controls for proper separation of membrane and soluble cellular components by our fractionation protocol confirm GFPc was dislocated to the cytosol. Calnexin (CNX), a transmembrane ER-chaperone, was present only in the pellet fractions at the 0 and 60 minute time points (compare lanes 9 to 11 and 13 to 15). The soluble ER-chaperone protein disulfide isomerase (PDI) was present in the pellet and supernatant fractions. The controls show the membrane fraction did not contaminate the soluble fraction, and therefore, GFPc present in the soluble fraction had been dislocated from the ER. Thus, GFPc is an ER dislocation substrate.
Figure 7. GFPc is rapidly degraded from the ER.

ER-inserted GFP with the transmembrane of US11 and cytoplasmic tail of HLA-A2 (GFPc) was degraded in MG-U373 cells. MG-U373 cells were stably transduced and selected with LNW-GFPc. Cells were pulsed for 12 min and chased for 0, 20, or 40 min, lysed in SDS and subjected to immunoprecipitation for GFP and SDS-PAGE analysis. GFPc was degraded during the chase period.
GFPc cells

Chase: 0 20 40
(min)

Pulse 12 min
IP: anti-GFP
Figure 8. GFPc is dislocated form the ER to the cytosol.

Pulse-chase and sub-cellular fractionation of cells expressing GFPc. MG-U373 cells expressing GFPc were metabolically labeled for 12 min and chased for 0 or 60 min. Cells were lysed in a ball bearing homogenizer and lysates were centrifuged at 1000g (1k lanes). 1k supernatants were centrifuged at 150,000g to yield pellet (P) and supernatant fractions (S). Fractions were resuspended in SDS buffer. Lysates were subject to immunoprecipitation for GFP, CNX, or PDI and analyzed by SDS-PAGE. GFPc was absent from the soluble fraction at the 0-min time point, but accumulated in the soluble fraction by the 60-min time point (compare lanes 4 & 6). The membrane fraction containing CNX does not contaminate the soluble fraction (lanes 11 & 15).
The next step determined if degradation of GFPc was dependent on the same factors that influence US11 degradation of MHC class I molecules. Mutation of the glutamine residue to leucine in the transmembrane domain of US11 abrogates degradation of MHC class I molecules (B. Lilley, submitted). This same Q to L mutation was introduced into the transmembrane of GFPc and this construct was transduced into U373-MG cells. Pulse-chase, immunoprecipitation and SDS-PAGE analysis were performed. The Q to L amino acid substitution rendered GFPc refractory to degradation compared to GFPc with the wild-type US11 transmembrane sequence (Figure 9A). A graph of the percentage of labeled polypeptide recovered at each time point in the autoradiogram above indicates the half-life of GFPc was approximately 20 minutes, whereas the half-life of the Q to L transmembrane mutant was more than 60 minutes. Thus, GFPc is a bona fide ER dislocation substrate that faithfully recapitulates US11-dependent degradation.
Figure 9. Rapid GFPc dislocation is dependent on the glutamine residue in the US11 transmembrane domain.

Pulse-chase of GFPc and GFPc Q→L.

A) MG-U373 were stably transduced and selected with LNW-GFPc or LNW-GFPc Q→L. Cells were metabolically labeled for 12 min and chased for 0, 15, 30 or 60 min, lysed in SDS and subjected to immunoprecipitation for GFP and SDS-PAGE analysis. GFPc Q→L was degraded with slower kinetics than GFPc.

B) Graph of rates of disappearance for GFPc and GFPc Q→L. GFPc is degraded with a half-life of 20 min, while the half-life of GFPc Q→L is approximately 60 min.
A)  

<table>
<thead>
<tr>
<th>Chase: (min)</th>
<th>GFPc</th>
<th>GFPc Q→L</th>
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<tbody>
<tr>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
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<tr>
<td>30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td></td>
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</tbody>
</table>

Pulse 12 min  
IP: anti-GFP

B)  

![Graph showing the percentage remaining over time for GFPc and GFPc Q→L](image)

% remaining

<table>
<thead>
<tr>
<th>minutes</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>60</th>
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<tr>
<td>GFPc</td>
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<td>40</td>
</tr>
<tr>
<td>GFPc Q→L</td>
<td>100</td>
<td>90</td>
<td>80</td>
<td>60</td>
</tr>
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</table>
Discussion

The basis for these experiments was the assumption that US2 and US11 can be dissected into discrete, and separable, functional modules which mediate substrate binding or recruitment to the ER dislocation machinery. Although the lumenal domains of US2 and US11 bind MHC class I molecules, these domains cannot functionally complement each other to degrade their target substrates. This observation lends support to the hypothesis that these two viral molecules utilize separate ER disposal pathways to disrupt antigen presentation. Previous data have demonstrated that these two immuno-evasins require markedly different structural elements in the cytoplasmic tail of MHC class I molecules (chapter 3). In addition, US2 degrades only properly folded MHC class I molecules, whereas US11 degrades free heavy chains (J. Loureiro, pers. comm.). *In toto*, the weight of the evidence favors a model in which US2 and US11 utilize distinct mechanisms to degrade MHC class I molecules.

US11 is indeed composed of discrete functional modules. The lumenal portion is sufficient to mediate association with MHC class I molecules (D. Tortorella, unpub. obs.) and the cytoplasmic tail is dispensable for US11-mediated dislocation. These data implicate the transmembrane region, perhaps by virtue a glutamine residue positioned in the lipid bilayer, for causing the association with a component(s) of the ER dislocation machinery. Although MHC class I molecules are destroyed, US11 remains in the ER and is remarkably stable. Thus, US11 binds and recruits MHC class I molecules to a factor(s) that targets the cytoplasmic tail of MHC class I molecules for destruction.

The rapid degradation of 11-A2tail and GFPc seem to support this model in which the transmembrane of US11 recruits factors that recognize and dislocate the cytoplasmic
tail of MHC class I molecules. Of the ER degradation signals characterized thus far, this
is the first example of a bipartite signal. The bipartite signal nicely illustrates the step-
wise nature of the dislocation process. The substrate is first targeted to the dislocation
machinery, then recognized and extracted.

The glutamine in the transmembrane has been implicated in the recruitment
process, but elements of the cytoplasmic tail of MHC class I are implicated as well. A
report from Wiertz et al. states the tyrosine residue in the cytoplasmic tail of MHC class I
molecules is essential for US11-mediated degradation. Experiments with US11-
catalyzed degradation of mouse H2-K\(^b\) containing tyrosine to alanine substitutions in the
cytoplasmic tail support this contention (P. Stern, unpub. data). Future experiments
utilizing the GFPc molecule will address the role of specific residues in the cytoplasmic
and transmembrane regions that affect the activity of the bipartite signal for degradation,
or degron.

The bipartite nature of the degradation signal is intriguing. Each element is
innocuous in isolation and seemingly does not destabilize ER proteins, as MHC class I
and US11 are independently stable molecules. Yet, when juxtaposed, these domains
catalyze rapid dislocation of the polypeptide. It is intriguing to speculate on the cellular
disposal pathway responsible for such rapid, and seemingly specific, substrate
degradation. The tyrosine requirement in the tail implicates cellular regulation of
signaling molecules, perhaps mis-expressed oncogenes or other tyrosine-based cell-
surface signal transduction molecules. However, the role of the tyrosine in MHC class I
molecules, which have no known intra-cellular signaling activity, remains to be defined.
References


Chapter 6

Discussion and Future Directions
Discussion and Future Directions

The degradation of proteins from the endoplasmic reticulum presents an interesting cell-biology puzzle. The mechanisms that recognize, recruit, and extract a protein substrate from the limiting membrane have significant implications for human health and for the pathologic conditions that may arise when these processes are disrupted. Multiple disposal pathways must exist to efficiently eliminate the host of mammalian proteins that transit through the secretory pathway. Comparison of US2- and US11-mediated destruction of MHC class I heavy chains highlights the heterogeneity in the mechanisms that enable dislocation of membrane proteins from the ER. Discussed below are the results of studies presented in this thesis and further pursuits the data engender.

Chaperone Knockdowns

The cellular chaperones PDI and Ero1α have been implicated in the unfolding and dislocation of cholera toxin from the ER. As this process has been likened to the retrograde transport of model ER degradation substrates, we examined the role of these chaperones in US2- and US11-mediated dislocation of MHC class I heavy chains utilizing a retroviral siRNA approach. Neither the knockdowns of these two chaperones nor the knockdowns of calnexin were found to have a significant effect on the rate of dislocation of the model substrates examined. A possible explanation for these results could be that the dislocation process relies on one of these chaperones for it to proceed efficiently and the siRNA approach cannot sufficiently reduce the level of the targeted
gene product. The lack of an assay to assess chaperone activity will continue to hamper siRNA studies.

The ideal method to assay the importance of cellular factors in dislocation would be a semi-permeabilized cell system. A version of this system has been used successfully to address US11-catalyzed ubiquitination of MHC class I heavy chains (Shamu, et al., 2001). The permeabilized cell system allows antibody-depletion of specific factors, as well as reconstitution of the dislocation reaction with engineered substrates and cofactors, such as modified ubiquitin and dominant-negative-acting mutant molecules.

The role of Sec61α and associated factors as the conduit for the dislocation reaction is generally accepted, often for lack of other candidates. Many components of the translocon are essential to cell survival. A straightforward siRNA approach targeting individual components to identify elements necessary for the dislocation reaction would likely be fruitless. However, concomitant knockdown of a translocon component and replacement by a mutant transgene could prove an effective method. Several regions of yeast Sec61p have been identified as important for dislocation to proceed [Wilkinson, 2000 #115]. Similar regions of mammalian Sec61α could be constructed and introduced into US2/US11 cells in combination with Sec61α knockdown constructs. This approach could also be applied to other components of the translocon to identify elements essential to the dislocation process.

The UPR

Previous work from our lab identified the UPR in S. cerevisiae as essential for efficient dislocation of ER substrates (see Appendix). Addressing the role of the UPR in
the dislocation of proteins from the mammalian ER required inhibition of cellular stress response pathways that may be essential to cell survival. Initial experiments determined that long-term expression of UPR inhibitors was lethal. The generation of cell lines by stable transfection and clonal expansion was not feasible. The demands of such experiments required a stable introduction and selection of the gene-based UPR inhibitors into a large population of cells, which consequently required advances in the gene delivery techniques greater than what was currently available in the Ploegh lab.

By re-engineering a commercially available retroviral vector, we developed a versatile, high-expression vector with GFP as the selectable marker, allowing rapid assessment and isolation of transduced cells (see Materials & Methods). Introduction of gene-based inhibitors of the UPR resulted in several novel conclusions. The ATF6, but not IRE1α, pathway is essential for cell survival, but neither pathway is essential for the ER dislocation reaction to proceed efficiently. This last result stands in marked contrast to results obtained in yeast.

These data support a model in which mammals have evolved separate regulatory pathways for the cargo-carrying and degrading capacity of the ER. The cargo-carrying capacity is regulated by IRE1α/XBP-1. The question remains as to how the mammalian ER controls its degradative capacity. Several molecules have been implicated as downstream effectors in the mammalian UPR. IRE1α, in addition to cleaving XBP-1 to generate a splice shift, may act on another unidentified transcript. Alternatively, the phosphorylated cytoplasmic domain of activated IRE1α may interact with TRAF-type molecules to initiate signal transduction events. The phosphorylated cytoplasmic domain
of IRE1β reportedly binds TRAF2. TRAF activation could up-regulate the components necessary for the dislocation reaction.

The essential role of ATF6 in cell survival is intriguing. Perhaps the spliced form of XBP-1 rescues the cell from the effect of ATF6 inhibition? ATF6 reportedly regulates about 40 genes (D Ron, pers. comm.), none of which are considered major cell survival genes such as Bcl-2 family members or caspase inhibitors. ATF6, most likely, promotes survival by increasing the cellular concentration of BiP, which inhibits ER-stress signal-transduction effectors, such as IRE1α and PERK. When BiP levels decrease below a critical threshold, unchecked ER-stress signaling would activate the apoptotic program. If BiP levels are responsible for the ATF6 effect, transfection of BiP or XBP-1 should rescue cells from this fate.

**MHC class I tail mutants**

The ability of the HCMV glycoproteins to dislocate MHC class I heavy chains from the ER to the cytosol was first identified in 1996. A wealth of data has since accumulated defining the similarities between these two viral immunoevasins, yet there has been a dearth of data detailing the differences. The data presented in this thesis identify marked differences in the ability of US2 and US11 to degrade MHC class I heavy chains with mutated cytoplasmic domains. US11 has a strict preference for the wild-type length of 30 amino acids in the tail of MHC class I molecules. In contrast, US2 is relatively indifferent to the length of the tail and is able to degrade MHC class I heavy chains with tails as short as 10 amino acids or as long as several hundred amino acids (by fusion of GFP).
The US2 and US11 molecules can degrade MHC class I heavy chains absent any other viral factors, implicating the endogenous ER quality control machinery as the major effector in the dislocation reactions. Presumably, the luminal domains of US2 or US11 bind the luminal portions of MHC class I molecules, which allows the complex to be recognized by ER quality control as "misfolded" and the MHC class I heavy chains to be dislocated. Whether US2 and US11 utilize distinct or identical degradation pathways to accomplish the dislocation reaction remains to be determined. From a teleological point of view, it seems unlikely a virus subject to the rigors of selection by the mammalian immune system over an evolutionary time-scale would retain two proteins possessing identical activities and utilizing identical mechanisms. US2 and US11 perform similar functions, but likely accomplish these tasks by disparate means we are as of yet unable to define.

The susceptibility to degradation of the MHC class I tail mutants are particularly interesting because the results seem to indicate that US2 and US11 do utilize distinct mechanisms to dislocate MHC class I molecules. All of the tail mutant molecules still effectively bind US2 and US11, so the differences in susceptibility to dislocation lies not in the interaction with viral molecule and substrate, but possibly with the substrate and downstream effectors. The data support a model whereby US11 recruits MHC class I molecules to the dislocation machinery that is capable of only degrading membrane proteins that expose a cytoplasmic domain of approximately 30 amino acids. In contrast, the dislocation machinery recruited by US2 is capable of processing substrates of at least 10 amino acids to seemingly any length.
The physical interactions between the substrate and effector molecules that dictate different susceptibilities to degradation are practically impossible to define given the current knowledge in the field. Only in the last few years has the dislocation reaction been parsed into discrete steps, but the molecular biology of the reaction remains largely undefined. The simplest scenario is depicted by the “groove” versus “pocket” model in Chapter 4, Figure 7. Although depicted solely to summarize the data presented, US2 and US11 must recruit different factors to dislocate MHC class I heavy chains. Continued efforts to identify the factors involved in US2- and US11-mediated degradation will further define the cellular components mediating dislocation of membrane substrate from the ER.

Chimeric molecules

Chimeric molecules were constructed in which the luminal portions of US2 and US11 were exchanged to identify if reciprocal swaps of the viral molecules could still effectively dislocate MHC class I molecules. However, I found the luminal portions of US2 and US11 cannot complement the activity of each other. This result supports a model where these viral molecules utilize distinct degradation pathways. However, the difference may be that US2 and US11 bind opposite sides of the MHC class I molecule and the viral chimeras are sterically hindered. This possibility is difficult to assess until the factors that interact with US2 and US11 to potentiate the dislocation reaction are identified.

A similar set of experiments exchanged the luminal or cytoplasmic domain of the substrate HLA-A2 with the corresponding regions of US2 and US11 to identify discrete
functional domains of the viral molecules. As all amino acid sequence information of the wild-type molecules was conserved in the chimeras, this approach aimed to identify regions of the proteins that could synergize to potentiate dislocation.

Most of these chimeric molecules were stable when expressed in isolation or when expressed together. The chimeras still bound their complementary molecule, but dislocation was not observed. However, I identified a molecule that contained all the necessary signals for dislocation within a single polypeptide. The molecule in which the cytoplasmic tail of US11 was replaced with the cytoplasmic tail of HLA-A2 was dislocated from the ER with rapid kinetics. The ER-inserted GFP molecule with the transmembrane domain of US11 and the cytoplasmic tail of HLA-A2 (GFPc) was rapidly dislocated from the ER. GFPc dislocation was sensitive to the presence of a glutamine residue in the transmembrane domain. This same mutation is essential for US11-catalyzed destruction of MHC class I molecules, indicating GFPc faithfully recapitulates US11-dependent degradation.

The GFPc molecule should greatly facilitate dissecting the US11-dependent dislocation reaction, as the complexity of the system has been greatly reduced. Previous studies concerning US11-dependent dislocation of MHC class I molecules were hampered by the required interaction of two polypeptides of considerable size. GFPc significantly reduces this complexity. The relevant regions essential for dislocation (the transmembrane and tail) span just under 50 amino acids. An alanine-scan approach to identify important residues would be feasible in a protein region this size. Mutagenesis and other manipulations of the GFPc molecule could identify regions of US11 or MHC class I that contribute to the dislocation reaction. However, the utility of GFP as a
screening reagent aids in identifying the cellular factors that facilitate US11-dependent dislocation.

Cells stably expressing GFPc do not exhibit green fluorescence when examined by eye and are marginally fluorescent when assessed by FACS. The GFPc Q→L molecule is obviously green when examined by eye and is approximately 10 times brighter than GFPc by FACS. Cells expressing GFPc that are defective in some aspect of the dislocation reaction should exhibit enhanced fluorescence and thus be easily isolated from the background population. In this way, several approaches could be used.

The Ploegh lab has recently obtained a siRNA library that targets all known de-ubiquitinating enzymes. Although the role of de-ubiquitination in the dislocation reaction is not obvious, perturbations of one aspect of the ubiquitin pathway may have profound effects on other areas. The relative ease of the experiment, transient transfections followed by FACS analysis, makes this approach difficult to dismiss.

A less biased approach than the siRNA library is random gene-disruption methods of GFPc expressing cells, followed by FACS analysis. A retrovirus-based poly-adenylation trap construct could efficiently select for cells with insertions only in expressed genes. This method decreases the high background associated with random integration methods. The virus carries its own promoter and drug-resistance cassette that is only expressed if the virus integrates upstream of an active poly-adenylation signal. The disrupted gene can then be identified by PCR. The diploid genome in mammals presents a technical hurdle for this technique, as it is highly unlikely this technique will disrupt both copies of a gene. Thus, only cellular factors involved in dislocation that are sensitive to haplo-insufficiency could be identified.
A cross-linking approach could identify factors that directly interact with GFPc. The transmembrane of US11 and cytoplasmic tail of HLA-A2 can act autonomously to dislocate the luminal polypeptide to which they are attached. As the residues in the polypeptide essential for the dislocation reaction are identified (perhaps by alanine scanning), the non-essential amino acids could be replaced with residues amenable to covalent modification by cross-linking reagents, provided dislocation was not disrupted by these replacements. Sulphydryl- and amino-specific reagents are commercially available, as well as photo-activatable reagents. The luminal domain could be replaced with a protein engineered to facilitate protein purification from cell lysates. For example, a histidine tag would allow protein purification on a nickel column and a protease cleavable region would allow specific elution of the construct from the material immobilized on the column. Incorporation of cross-linking sites throughout the substrate could covalently modify interacting cellular proteins in the lumen, membrane, or cytosol. The complex could be purified on a column and the eluate subject to mass spectrometry to identify the interacting proteins.

Conclusions

The cellular factors that execute the dislocation of transmembrane polypeptides from the ER to the cytosol remain elusive. These factors are neither regulated by the cellular response to unfolded proteins in the ER, nor are they the predominant ER chaperones that assist membrane glycoprotein folding and disulfide bond formation. The model degradation system utilizing the US2- and US11-catalyzed destruction of MHC class I heavy chains implicates multiple disposal pathways in the dislocation of ER
substrates. For US11-dependent dislocation, the domains essential to the dislocation
reaction have been identified and further studies should illuminate the specific residues,
as well as the cellular factors, necessary for the process to proceed. The use of viral
molecules to dissect the cellular pathways they usurp continues to yield unforeseen insights
into the inner workings of their mammalian hosts.

References

Polyubiquitination Is Required for US11-dependent Movement of MHC Class I Heavy
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Appendices
Degradation of Proteins from the ER of *S. cerevisiae* Requires an Intact Unfolded Protein Response Pathway

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Summary

To dissect the requirements of membrane protein degradation from the ER, we expressed the mouse major histocompatibility complex class I heavy chain H-2Kb in yeast. Like other proteins degraded from the ER, unassembled H-2Kb heavy chains are not transported to the Golgi but are degraded in a proteasome-dependent manner. The overexpression of H-2Kb heavy chains induces the unfolded protein response (UPR). In yeast mutants unable to mount the UPR, H-2Kb heavy chains are greatly stabilized. This defect in degradation is suppressed by the expression of the active form of Hac1p, the transcription factor that upregulates UPR-induced genes. These results indicate that induction of the UPR is required for the degradation of protein substrates from the ER.

Introduction

The folding of nascent proteins in the endoplasmic reticulum (ER) is often assisted by ER-resident chaperones. Exposure of cells to agents that alter the redox potential of the ER or that interfere with proper protein glycosylation changes the local folding environment, with attendant accumulation of unfolded proteins in the ER. In both yeast and mammalian cells, proteins that cannot proceed properly along the secretory pathway are destroyed. The misfolded or unassembled proteins are shuttled from the ER to the cytosol, in a process termed dislocation, where they are subsequently destroyed by the proteasome (reviewed by Bonifacino and Weissman, 1998).

Major histocompatibility (MHC) class I molecules, which present peptides at the cell surface for immune recognition, serve as useful models to study ER quality control in mammalian cells. The MHC class I complex is a heterotrimer composed of a type I membrane glycoprotein heavy chain (44 kDa), a soluble light chain, β2m-microglobulin (β2m, 10 kDa), and a short (8- to 10-amino acid) peptide. In the absence of the β2m, the MHC class I complex fails to assemble, and the heavy chain is arrested in the ER and destroyed by the ER degradation pathway (Ploegh et al., 1979; Hughes et al., 1997).

Eukaryotic cells sense the accumulation of misfolded proteins in the ER and respond by upregulating the synthesis of ER-resident chaperones via a signaling pathway called the unfolded protein response (UPR). The UPR is conserved from yeast to mammals, and many key components of the pathway have now been identified (reviewed by Chapman et al., 1998). In yeast, accumulation of misfolded proteins in the ER activates Ire1p, an ER-resident transmembrane protein with both kinase and RNase activity (Cox et al., 1993; Mori et al., 1993; Sidrauskis and Walter, 1997). Activated Ire1p then cleaves the mRNA encoded by the HAC1 gene, removing an intron. HAC1 mRNA is translated by tRNA ligase, producing the HAC1 mRNA, which encodes the transcription factor, Hac1p, that induces expression of genes encoding ER-resident chaperones (Cox and Walter, 1996; Sidrauskis et al., 1996; Sidrauskis and Walter, 1997). Expression of intron-less versions of HAC1 mRNA fully induces the UPR even in the absence of Ire1p (Cox and Walter, 1996).

Hac1p upregulates expression of genes that contain an unfolded protein response element (UPRE) upstream of their promoter (Mori et al., 1992). Chaperones such as Kar2p (the yeast homolog of BiP), protein disulfide isomerase (PDI), and peptidyl-prolyl-cis-trans isomerase (FKBP26) are upregulated by the UPR to allow survival of stress in the ER that is lethal in the absence of *IRE1* or *HAC1* (reviewed by Chapman et al., 1998). Do UPR-induced proteins contribute to repair of damaged or misfolded polypeptides or keep unfolded proteins from aggregating and poisoning the ER? Do they enhance the degradation of proteins by helping eliminate them from the ER? While the UPR must provide benefits to the cell, the mechanism by which the UPR assists stressed cells is not clear.

We wanted to examine the possible link between the degradation of misfolded/misassembled ER proteins and the role of the UPR in recovery from stress. Because the stability of MHC class I heavy chains depends on the presence of β2m and peptide, we reasoned that expression of a mouse MHC class I heavy chain (H-2Kb) alone in yeast would yield a type I membrane glycoprotein that would be targeted for degradation. Indeed, H-2Kb heavy chains expressed in yeast are destroyed by the ER degradation pathway. The overexpression of these heavy chains induces the UPR. We found that disrupting the UPR by the deletion of the *IRE1* gene impairs the degradation of H-2Kb heavy chains and of CYH, a well-characterized mutant secretory protein.
also degraded by the proteasome. This defect in degradation is suppressed by constitutively inducing the UPR via expression of Hacp. These results suggest that induction of the UPR is required for the efficient degradation of protein substrates from the ER.

Results

H-2K<sup>α</sup> Heavy Chains Are Degraded by the ER Quality Control Process

To study degradation of MHC class I heavy chains from the ER, the H-2K<sup>α</sup> heavy chain was expressed in yeast under control of the yeast Gal 1/10 promoter on a CEN/ARS plasmid (pK6). To obtain efficient insertion of the class I heavy chains into the yeast ER, the endogenous H-2K<sup>α</sup> signal sequence was replaced with the signal sequence from the mating pheromone α factor (Kurjan and Herskowitz, 1982). As is the case in mice, the H-2K<sup>α</sup> heavy chains are glycosylated at two sites in yeast, as demonstrated by digestion with endoglycosidase H (data not shown), suggesting that H-2K<sup>α</sup> is inserted properly into the ER membrane. Stability of the H-2K<sup>α</sup> heavy chains in yeast was assessed by pulse-chase analysis. Unlike H-2K<sup>κ</sup> immunoprecipitated from mouse EL-4 cells, which matures and is exported to the cell surface, H-2K<sup>α</sup> heavy chains expressed in yeast are unstable and have a half-life of about 15 min (Figure 1A). ER function was not obviously impaired in yeast cells expressing H-2K<sup>α</sup> because, after a short period of H-2K<sup>α</sup> induction, a control secretory protein, the protease CPY, was transported to the vacuole and processed to its mature form (Figure 1A). Since CPY is a stable, secretory protein, it serves as a control in experiments in which we examine the degradation of H-2K<sup>α</sup>. In these experiments, H-2K<sup>α</sup> is expressed in the absence of the other subunits of the MHC class I complex. It is therefore likely to be recognized as misassembled and targeted for destruction.

Proteins that are degraded from the ER are destroyed by the proteasome without the need for vesicular traffic between the ER and Golgi. To examine whether degradation of H-2K<sup>α</sup> conforms to this scheme, we examined the fate of H-2K<sup>α</sup> in cells carrying the sec12-1 allele. The sec12-1 mutation prevents ER-to-Golgi transport by preventing vesicle formation at the restrictive temperature (Barlowe and Scheiman, 1993). This is best seen by following the maturation of CPY. In sec12-1 cells at the restrictive temperature, CPY does not mature properly as indicated by the failure to acquire the additional mannosos normally attached in the Golgi apparatus or to undergo proteolytic processing in the vacuole (Figure 1B). Under the same conditions, degradation of H-2K<sup>α</sup> continues even when vesicular traffic from the ER to the Golgi is blocked (t<sub>1/2</sub> of 15 min in sec12-1 strain versus t<sub>1/2</sub> of 10 min in wild-type at 37°). Thus, the degradation of H-2K<sup>α</sup> heavy chains does not require their transport from the ER to the Golgi.

Proteasome activity is also required for the degradation of H-2K<sup>α</sup> heavy chains. We expressed H-2K<sup>α</sup> heavy chains in a control strain (yhi29/w) and in a strain carrying mutations in proteasome subunits that reduce proteasomal activity (pre1-1 and pre4-1, strain yhi29/14; Hilt et al., 1993). H-2K<sup>α</sup> heavy chains were more stable in the proteasome-compromised strain (half-life > 1 hr) than in the wild-type strain (half-life about 15 min) (Figure 1C). Taken together, these data indicate that H-2K<sup>α</sup> heavy chains are recognized and degraded in a manner similar to that described for other substrates of degradation from the ER, such as class I heavy chains and TOR α in mammalian cells and Sec61p and CPY in yeast (Finger et al., 1993; Biederer et al., 1996; Hughes et al., 1997; Hoppa and Ploegh, 1997).

Expression of H-2K<sup>α</sup> Induces the UPR in Wild-Type Yeast

Treatment of cells with reagents such as DTT and tunicamycin, which affect the folding of most proteins in the ER, induces the UPR. Is the overexpression of a
single protein that is recognized as unfolded/misassembled sufficient to induce the UPR? The UPR is often monitored by assessing the production of β-galactosidase expressed from a UPR-lacZ reporter gene (Cox et al., 1993). We compared β-gal levels in cells that bear the UPR-lacZ reporter and that express H-2Kk to levels in control cells that do not express H-2Kk, and observed no difference between these strains in several independent experiments. Treatment with tunicamycin induced β-galactosidase expression to similar levels in both strains (data not shown).

A more direct and sensitive method to detect the UPR is to examine mRNA levels of genes known to be induced by the UPR, such as KAR2 and ERO1. We took advantage of the availability of DNA microarray assays to examine whether the expression of H-2Kk increased the transcription of genes also induced by the UPR. mRNA was harvested from wild-type cells that either express H-2Kk or bear a control plasmid. The mRNA was fluorescently labeled and hybridized to a microarray of all yeast open reading frames (DeRisi et al., 1997). We detected a 3-fold increase in KAR2 and ERO1 mRNA, and a 3- to 4-fold increase in LHS1 and PDI1 mRNA in cells expressing H-2Kk heavy chains as compared to that harvested from control cells. Treatment of cells with tunicamycin causes a 20-fold increase in the amount of KAR2 and ERO1 mRNA and a greater than 7-fold increase in the amount of LHS1 and PDI1 mRNA compared to untreated cells. Other genes known to be upregulated by the UPR were also upregulated by H-2Kk expression. The complete data set from this experiment is available at http://genome-www.stanford.edu/upr. We conclude that expression of H-2Kk heavy chains induces the UPR, but to a lower extent than that observed when global misfolding in the ER is imposed by tunicamycin treatment.

The Degradation of Misfolded ER Proteins Is Impaired in a Strain Lacking Functional Ire1p

The UPR is induced in order for a cell to cope with the accumulation of unfolded proteins in the ER. Although it seems reasonable to suggest that accumulation of unfolded proteins would somehow be linked to their degradation, the involvement of the UPR in the degradation of proteins from the ER has not been explored. Because H-2Kk heavy chain is a bona fide substrate for protein degradation from the ER and induces the UPR, we examined the stability of H-2Kk heavy chains in a strain deleted for IRE1 and therefore unable to carry out the UPR. H-2Kk heavy chains have a much longer halflife in Δire1 cells (t1/2 > 1 hr) than in the control, IRE1 cells (t1/2 about 15 min) (Figure 2A, compare lanes 1–5 with 6–10). Furthermore, the degradation of human HLA-A2 heavy chains (70% sequence identity to H-2Kk heavy chains) produced in yeast is similarly reduced in a Δire1 strain (M. Lorenzo, personal communication).

To test whether Ire1p is required for the degradation of soluble proteins as well as membrane proteins, we examined the degradation of CPY+ , a misfolded soluble protein known to be destroyed by the ER quality control process (Finger et al., 1993; Hiller et al., 1996). We truncated CPY+ at residue 423 (T-CPY+) to permit clear resolution of the mutant protein from endogenous CPY by SDS-PAGE. When expressed in IRE1 cells, T-CPY+ is sensitive to endo H, indicating that it is inserted into the ER and glycosylated as expected (data not shown). Furthermore, T-CPY+ is degraded at approximately the same rate as full-length CPY+ (t1/2 = 25 min; Finger et al., 1993). However, when it is expressed in Δire1 cells, T-CPY+ is stabilized (t1/2 > 1 hr; Figures 2C and 2D). We also find that CPY+ is stabilized in Δare1 cells that have the prc1-1 allele (encoding CPY+) as the only copy of the PRC1 gene (data not shown). These data indicate that the IRE1 gene is required for the proper removal of T-CPY+ and CPY+ from the yeast ER. These experiments also show that the ER quality control apparatus can accurately distinguish misfolded T-CPY+ from folded CPY+.

The growth rates of Δare1 cells and IRE1 cells are identical for the first 10 hr of H-2Kk expression (Figure 3). Three hours after induction of H-2Kk, at the start of the pulse-chase experiments, the number of viable Δare1 cells per unit of optical density, as assayed by colony formation on glucose-containing media, is the same as that observed for IRE1 cells (data not shown). Also, as determined by Kar2p immunofluorescence, the morphology of the ER of both these strains seems normal (data not shown). Furthermore, the maturation of CPY in the Δare1 cells proceeds at a rate similar to CPY in the IRE1 cells, indicating that transport out of the ER is not affected by the short-term (less than 4 hr) expression of H-2Kk (Figure 2A and data not shown). Thus, the defect in degradation of H-2Kk is not due to a general infertility of strains that lack IRE1.

However, when H-2Kk was expressed for more than 10 hr in Δare1 cells, their growth slowed as compared to that of IRE1 cells or of Δare1 cells transformed with a control plasmid (Figure 3). After 36 hr of H-2Kk expression, only 3% of the Δare1 cells were viable. Greater than 95% of IRE1 cells expressing H-2Kk and Δare1 cells with the control plasmid were viable (data not shown). Thus, IRE1 is required for cells to grow properly when a single overexpressed protein misfolds and accumulates in an otherwise stress-free environment. This suggests that synthetic lethality with the Δare1 mutation is a very sensitive assay of ER stress.

If the defect in degradation observed in the Δare1 strain is due to an inability to initiate or sustain the UPR, then the expression of active Hac1p should suppress this defect. The Δare1 strains expressing H-2Kk or T-CPY+ were transformed with a plasmid (pHac) that expresses intron-less hac mRNA, which causes constitutive induction of the UPR (Cox and Walter, 1994). These strains show a rate of degradation of H-2Kk and T-CPY+ almost identical to that in IRE1 cells (Figure 2A, lanes 11–15, and 2C). When IRE1 cells are transformed with pHac, the rate of degradation of H-2Kk heavy chains increases approximately 1.25-fold (data not shown). Therefore, the degradation defect of both H-2Kk and T-CPY+ in Δare1 cells is not due to a lack of Ire1p per se, but to an inability to initiate the UPR.

Overexpression of KAR2 Does Not Suppress the Degradation Defect in Δare1 Cells

The expression of Kar2p is induced by the UPR, and Kar2p has been suggested to be involved in the degradation of CPY+ and pro-α factor (Plesmer et al., 1997;
Brodsky et al., 1999). Furthermore, Δire1 cells have a lower steady-state level of Kar2p in the ER. Is the degradation defect observed in Δire1 cells simply due to a scarcity of Kar2p? Is overexpression of Kar2p sufficient to suppress the degradation defect observed in Δire1 cells? We transformed Δire1 cells that express H-2Kb with either a control plasmid or plasmid bearing the KAR2 gene under the control of the GAL 1/10 promoter (pKar2). Overexpression of Kar2 in the Δire1 strain yields a level of Kar2p similar to that found in wild-type cells treated with tunicamycin and twice as great as Δire1 cells transformed with a control plasmid (Figure 4A). Despite this increase in Kar2p levels, the rate of degradation of H-2Kb remained essentially unaltered (Figure 4B). Overexpression of Kar2p in wild-type cells harboring pKb did not affect the degradation rate of H-2Kb (data not shown). We conclude that the defect in degradation of H-2Kb is not simply due to a reduced amount of Kar2p in the ER of Δire1 cells.

Discussion

Degradation of proteins that fail to attain their proper folded state is an essential element of quality control (Kopito, 1997). At first glance, it seems contradictory that the ER, in which newly synthesized proteins must fold de novo, has mechanisms for the elimination of misfolded polypeptides. The new proteins must not be destroyed before they can fold, yet terminally misfolded proteins must be cleared from the ER. In recent years,
it has become clear that the proteolysis of misfolded or misassembled proteins in the ER occurs in the cytosol by the proteasome (Ward et al., 1985; Biederer et al., 1996; Hampton et al., 1996; Hillier et al., 1996; Wiertz et al., 1986). Thus, there is first the need to recognize the misfolded proteins in the ER, then deliver them to the cytosol for proteolysis. How is such recognition accomplished? We have begun to address this question by examining the link between the degradation of misfolded or misassembled proteins and the pathway by which proteins are known to be recognized as unfolded in the ER, the UPR.

MHC class I molecules are type I membrane proteins, whose folding and assembly have been well studied. In the absence of its two smaller subunits, β2m and peptide, the free class I heavy chain is commonly considered misfolded and retained in the ER (Ploegh et al., 1979). We show that mouse class I heavy chains expressed in S. cerevisiae are degraded rapidly, in a proteasome-dependent manner and without the need for transport to the Golgi. The H-2Kb heavy chains expressed in yeast thus follow a pathway of degradation very similar to that of class I heavy chains in the absence of β2m in mammalian cells (Hughes et al., 1997). Likewise, although H-2Kb is not native to yeast, it seems to be destroyed by the same pathway as are misfolded yeast proteins such as CPV* and Sec61-1p (Finger et al., 1993; Biederer et al., 1996).

The UPR improves the survival of cells exposed to agents that cause misfolded proteins to accumulate in the ER (Cox et al., 1993). The UPR is often induced experimentally by treating cells with agents that cause a global misfolding of proteins in the ER. Here we examined the effects of the overexpression of a single protein that accumulates in the ER of wild-type cells. The expression of H-2Kb heavy chains induced the UPR to a lesser extent than did treatment with tunicamycin. This induction of the UPR, as assayed by DNA microarray, does not occur in cells that cannot mount the UPR due to deletion of the Ire1 gene (R. C. and M. D., unpublished results). Another indication that misassembled class I heavy chains induce the UPR is that their prolonged overexpression in the Δire1 strain slows growth and eventually leads to their death. That misassembled heavy chains induce the UPR is consistent with the observation that CPV* induces the UPR when protein degradation from the ER is impaired (Knoop et al., 1996).

We conclude that the UPR is not an all-or-none response to stress but one that is tunable to the amount of misfolding that occurs in the ER. Such ability to modulate the UPR probably allows the cell to conserve energy in times of only minor stress.

How the UPR aids cellular survival during ER stress remains unclear. It is possible that the increased amount

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**Table 1. Yeast Strains Used**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference or Source</th>
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<tbody>
<tr>
<td>LTY 108</td>
<td>lys2-801, his 3-11, leu2-3112, ura3-52, Mata</td>
<td>Solomon Lab collection</td>
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<tr>
<td>LTY 109</td>
<td>lys2-801, his 3-11, leu2-3112, ura3-52, ade2-1, Mata</td>
<td>Solomon Lab collection</td>
</tr>
<tr>
<td>CKY 199</td>
<td>sec12-1, leu2-3112, ura3-52, his3-11, Mata</td>
<td>Kaiser Lab collection</td>
</tr>
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<td>yh629/w</td>
<td>leu2-3112, ura3-52, his3-11, Mata</td>
<td>Hilt et al., 1993</td>
</tr>
<tr>
<td>yh629/14</td>
<td>prot1-4, leu2-3112, ura3-52, his3-11, Mata</td>
<td>Hilt et al., 1993</td>
</tr>
<tr>
<td>JC102</td>
<td>his 3-11, ura3-52, ade2-1, can1-100, trp1-1, leu2-3112::LEU1::UPRE-lacZ, Mata</td>
<td>Shamu and Walter, 1996</td>
</tr>
<tr>
<td>CS308</td>
<td>his 3-11, ura3-52, ade2-1, can1-100, trp1-1, leu2-3112::LEU1::UPRE-lacZ, Δire1::TRP1, Mata</td>
<td>Shamu and Walter, 1996</td>
</tr>
<tr>
<td>RCY5</td>
<td>leu2-3122, ura3-52, his 3-11, ade2-1, Δire1::TRP1, Mata*</td>
<td>Spore from CS308X LTY 108 diploid</td>
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<tr>
<td>RCY8</td>
<td>leu2-3122, ura3-52, his 3-11, ade2-1, Mata*</td>
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</tbody>
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*The status of the TRP1 locus of RCY5 and RCY8 was not determined.
of chaperones in the ER simply prevents unfolded proteins from aggregating and clogging the ER. By binding to chaperones, these misfolded proteins would be given time to fold and exit the ER to the Golgi. However, the UPR might also help clear the ER of aberrantly folded polypeptides through the activation of the dissolution and degradation machinery. Our data suggest that the UPR is involved not only in keeping unfolded proteins from aggregating, but also in the actual removal and destruction of unfolded proteins that accumulate in the ER, while leaving properly folded proteins untouched.

The exact mechanism for activation of dislocation/degradation by the UPR is unknown. Increased expression of Kar2p in L. tarentolae strains does not restore efficient H-2K\(^{\beta}\) degradation. Kar2p is just one of the many genes upregulated by the UPR. We also find that DERT, a gene known to be involved in the degradation of misfolded ER proteins (Knap et al., 1996), is upregulated (see http://genome-www.stanford.edu/urp). When the UPR senses unfolded proteins in the ER, it may activate components of the degradation machinery to better dispose of these proteins. Perhaps transcription of genes that encode components of the dislocation/degradation machinery is directly induced by the UPR. Alternatively, gene products whose expression is induced by the UPR may somehow activate the ER clearance machinery indirectly. Many of the genes we have found by DNA microarray assay to be upregulated by the UPR are not known to function in quality control or protein degradation. By examining the roles of these genes, novel components of the ER clearance and proteolysis machinery may be identified.

**Experimental Procedures**

**Cells, Plasmids, and Media**

Yeast strains used are listed in Table 1. Media were prepared as described in Sherman (1991); minimal media also contained 100 \(\mu\)g/ml insulin. Unless indicated otherwise, all cells were cultured and labeled at 30°C. Where added, tunicamycin (Boehringer Mannheim) was at 1.25 \(\mu\)g/ml. The plasmid pHac1, encoding the intron-less form of HAC1, was a gift of Dr. P. Walter (USCF; PB8938). The plasmid pHacK2 was a gift of Dr. K. D. Witzup (University of Illinois, Urbana). On this 2 \(\mu\)m plasmid, pKAK2 is under the control of the GAL1/10 promoter. The plasmid expressing CPY* (pCPY*) from its endogenous promoter was a gift of Dr. D. Wolf (Institut für Biochemie der Universität Stuttgart, Germany). Truncated CPY (tCPY) was made by cutting pCPY* at a unique BglII site and inserting a linker containing an in-frame stop codon instead of amino acid 424.

The H-2K\(^{\beta}\) construct used in this study consists of the H-2K\(^{\beta}\) heavy chain sequence, minus its normal signal sequence, fused to the signal sequence of the yeast mating factor a. The fusion contains amino acids 1-20 of the a factor precursor (Kurjan and Herskowitz, 1982) fused to amino acids 20-370 of H-2K\(^{\beta}\). A plasmid containing the full-length CDA encoding H-2K\(^{\beta}\) was kindly provided by Dr. E. Joly (Scripps Research Institute). The a factor/H-2K\(^{\beta}\) fusion product, the GAL 1/10 promotor (obtained from Dr. T. Steens, Stanford University), and transcription termination sequences from the yeast ACT1 gene (gift of Dr. D. Botstein, Stanford University) were ligated into the yeast shuttle vector pRS316, resulting in plasmid pgbh.

**Antibodies**

The Ph antibody against mouse H-2K\(^{\beta}\) MHC class I heavy chain was described by Machold et al. (1995). Antibodies against carboxy peptidease Y (CPY) were made using purified CPY (Boehringer Mannheim). Lymphoplated protein was reconstituted at 1 mg/ml in PBS, deoxycholate by endo-H, mixed with an equal volume of Freund's complete adjuvant (GISTO-BRL), and injected into rabbits. The anticalprofase antibody used for Western blotting was described by Oggi and Walter (1993). Anti-\(\beta\)-galactosidase antibody was purchased from Rockland Immunocanals.

**Pulse-Chase Analysis and Immunoprecipitations**

Cells were grown overnight in minimal medium containing raffinose to a density of 0.2-6.0 OD/\(\mu\)l. Before labeling, galactoside was added to the raffinose media to induce expression from the GAl1/10 promoter, and cells were incubated for an additional 3 hr. Cells were labeled for 10 min (unless otherwise indicated in the figure legends) with Express Label \(^{35}\)S-methionine/cysteine (NEL-DuPont) at 300 \(\mu\)Ci/\(\mu\)l at 30°C. At the start of the chase, radiolabeled cells were pelleted and resuspended in 1 ml chase media (with galactose, 2.5 mM methionine, 0.5 mM cysteine). At each time point, samples of 10\(^6\) cells were lysed in 10% TCA, and nonradioactive lysates were prepared as described in Shamu and Walter (1996) and used for immunoprecipitation. Immune complexes were recovered with fixed Staphylococcus aureus bacteria. For the experiments shown in Figure 2C, cells were labeled in media with raffinose as the sole carbon source.

**RNA Preparation, Microarray Manufacture, and Hybridizations**

RNA was isolated from yeast cells by extraction with hot acidic phenol as described (Sherman, 1981). miRNA was collected from total RNA by the FastTracks 2.0 Kit (Invitrogen). DNA microarrays were produced and hybridized as previously described (Eisen and Brown, 1999; see also http://gcmg.stanford.edu/procrown). To quantitate the miRNAs in each sample, Cy5-labeled cDNA was prepared from cells expressing H-2K\(^{\beta}\) or tunicamycin-treated cells, and Cy3-labeled cDNA was prepared from control cells. Microarrays were scanned on a GenePix Scanner (Axon Instruments, Inc.) and analyzed using ScanArray (available at http://rna.stanford.edu/soft ware). Fold induction is normalized to the average change in levels of all the genes on the array. The raw data from these experiments can be found at http://genome-www.stanford.edu/urp.

**Acknowledgments**

We would like to thank Chris Kaiser, Dan Finley, and Sylvia Sanders and the members of the Ploegh, Rapoport, and Walter labs for their reagents and helpful advice. R. C. and H. P. are supported by a grant from the National Institutes of Health (grant R37-A134568). P. S. is funded by a predoctoral fellowship from the Howard Hughes Medical Institute. C. S. is a Special Fellow of the Laskerma and Lymphoma Society of America. M. D. is supported by an MSTP fellowship. P. O. B. is an associate investigator of the Howard Hughes Medical Institute. M. Z. is funded by an NSF grant (MCB-9096241-008) and an NIH grant (R01-A139055-02).

Received October 13, 1999; revised February 25, 2000.

**References**


The UPR Is Required for the Removal of ER Proteins

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Signal peptide cleavage of a type I membrane protein, HCMV US11, is dependent on its membrane anchor

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The human cytomegalovirus (HCMV) US11 polypeptide is a type I membrane glycoprotein that targets major histocompatibility complex (MHC) class I molecules for destruction in a proteasome-dependent manner. Although the US11 signal sequence appears to be a classical N-terminal signal peptide in terms of its sequence and cleavage site, a fraction of newly synthesized US11 molecules retain the signal peptide after the N-linked glycan has been attached and translation of the US11 polypeptide has been completed. Delayed cleavage of the US11 signal peptide is determined by the first four residues, the so-called n-region of the signal peptide. Its replacement with the four N-terminal residues of the H-2Kb signal sequence eliminates delayed cleavage. Surprisingly, a second region that affects the rate and extent of signal peptide cleavage is the transmembrane region close to the C-terminus of US11. Deletion of the transmembrane region of US11 (US11-180) significantly delays processing, a delay overcome by replacement with the H-2Kb signal sequence. Thus, elements at a considerable distance from the signal sequence affect its cleavage.

Keywords: ER subdomains/HCMV US11/post-translational ER processing/signal sequence cleavage/transmembrane anchor

Introduction

Membrane proteins and proteins destined for secretion are targeted to the appropriate intracellular membrane by their signal peptides (Martoglio and Dobberstein, 1998). In eukaryotes, signal peptides are 15–50 amino acids long and are usually located at the N-terminus (von Heijne, 1983). A typical signal peptide is comprised of three distinct regions: a polar N-terminal end (n-region) that may have a net positive charge, a central hydrophobic core (h-region) that consists of 6–15 hydrophobic amino acids, and a polar C-terminal (c-region) end that contains prolines and glycines (von Heijne, 1985). A signal peptide containing the consensus sequence and proper cleavage site ensures that proteins are inserted into the endoplasmic reticulum (ER) membrane and are processed properly.

Mutations within the sequence immediately downstream of the signal peptide affect protein processing, and can result in both inefficient and inaccurate cleavage (Russel and Model, 1981; Folz and Gordon, 1986; Andrews et al., 1988; Wiren et al., 1988). For example, replacement of glutamic acid for leucine at the +2 position of the phage coat protein cleavage site causes inefficient removal of its signal peptide (Russel and Model, 1981). When the prepeptides of human pre-pro-apolipoprotein A-II and pre-pro-parathyroid hormone are deleted, five and six residues, respectively, the generation of an improper N-terminus and a failure to direct the nascent chain to the ER properly are observed (Folz and Gordon, 1986; Wiren et al., 1988). Elements of the nascent chain at greater distances from the signal peptide are not known to affect signal peptide processing.

Shortly after its translation, the signal peptide interacts with signal recognition particle (SRP) and causes translocation arrest (Walter and Blobel, 1981; Walter and Johnson, 1994). SRP is a ribonucleoprotein comprised of a 7S RNA associated with six different polypeptides (Walter and Blobel, 1980, 1982). The 54 kDa subunit of SRP interacts with the signal peptide through a hydrophobic region that promiscuously accommodates signal peptides of different lengths and sequences (Keenan et al., 1998). The SRP-nascent polypeptide chain–ribosome complex is targeted to the ER membrane where SRP binds to the SRP receptor and the ribosome weakly interacts with the translocon (mainly comprised of the Sec61p complex) (Garlich et al., 1992; Kalies et al., 1994; for reviews see Rapoport et al., 1996; Hegde and Lingappa, 1999; Johnson and van Waes, 1999). The signal peptide is then transferred from the SRP into the channel of the translocon, where it directly associates with the Sec61α subunit of the Sec61 complex to promote tight interaction of the ribosome–nascent chain complex with the translocon (Jungnickel and Rapoport, 1995; Mothes et al., 1999; Plath et al., 1998). The signal peptide can also associate with the lipid bilayer and the TRAM protein (Martoglio et al., 1995; Voigl et al., 1996; Mothes et al., 1997), which assists in protein transport through the translocon. The interaction of the signal peptide with the Sec61 complex may also induce the removal of a "gating factor", possibly BiP, from the luminal side of the translocon, to allow access of the nascent polypeptide to the ER lumen (Crowley et al., 1994; Hamman et al., 1998). Chain elongation is re-initiated, followed by signal peptide translocation through the Sec61 channel. The hydrophobic nature of the signal peptide allows its insertion into the ER membrane, followed by signal peptidase cleavage upon luminal exposure of the cleavage site (Blobel and Dobberstein, 1975). This cleavage site is characterized by small uncharged residues at positions −1 and −3 (von Heijne, 1990). After signal peptide cleavage,
chain elongation of the nascent chain continues, while the signal peptide itself can be cleaved further by aminopeptidases or signal peptide peptidase (Lyko et al., 1995; Martoglio et al., 1997).

Signal peptidase is an endopeptidase that resembles other serine proteases (Dalbey and von Heijne, 1992) and performs a similar cleavage reaction for prokaryotic and eukaryotic signal peptidases. The crystal structure of the periplasmic domain of Escherichia coli leader peptidase (Paetzol et al., 1998) reveals important mechanistic aspects of signal peptide cleavage: the catalytic site proposed to be close to the lipid bilayer is surrounded by a hydrophobic region, explaining the requirement for small unchanged, aliphatic residues at the -1 and -3 positions of the cleavage site (Paetzol et al., 1998; von Heijne, 1998). The mammalian signal peptide complex (SPC) is comprised of at least five subunits with molecular masses of 25, 23/22, 21, 18 and 12 kDa (Evans et al., 1986). The non-catalytic subunits of the eukaryotic SPC may function as regulatory subunits for signal peptide recognition and are located in close proximity to the translocon (Meyer and Hartmann, 1997). The Sec61p complex interacts with the 25 kDa subunit of the SPC (SPC25), which suggests a tight interaction between the SPC and the Sec61 complex (Kalies et al., 1998). This interaction may serve to recruit the SPC to the translocation site and thereby enhance the overall translocation efficiency of the nascent polypeptide.

The human cytomegalovirus (HCMV) gene products US11 and US2 target the major histocompatibility complex (MHC) class I molecules for destruction by the proteasome (Wiertz et al., 1996a,b; Tortorella et al., 1998). These viral proteins associate with the class I molecules in the ER and induce the dislocation of the class I heavy chains from the ER, probably via the Sec61p complex, for degradation in the cytosol (Wiertz et al., 1996b). In all likelihood, a similar set of reactions is utilized for the removal and degradation of misfolded and abnormal ER proteins more generally (Bonifacio and Weissman, 1998). The HCMV US11 gene product is an ER-resident type I membrane glycoprotein (Figure 1), the single N-linked glycan attachment site of which is glycosylated quantitatively. The hydrophobic stretch at the N-terminus of US11 is characteristic of a signal peptide, while the hydrophobic stretch at the C-terminal end corresponds to a transmembrane/stop transfer sequence.

Here we report a highly unusual cleavage pattern for the US11 signal peptide. At least a fraction of the US11 signal peptide appears to be cleaved post-translationally. This trait is determined by the US11 signal peptide N-region. What cleavage occurs is also strongly influenced by the US11 transmembrane domain. Delayed cleavage of the US11 signal peptide may reflect the local ER environment in which dislocation takes place.

Results

The HCMV US11 signal peptide is cleaved post-translationally

HCMV US11 is a 215 residue ER-resident protein that targets MHC class I heavy chains for destruction by the proteasome. The detailed mechanism by which the viral gene product accomplishes this is unclear, but is closely coupled to the biosynthesis of the class I and US11 products. We therefore examined whether the biosynthesis of US11 might reveal unique properties of the ER environment in which US11 normally functions. The maturation of US11 was examined in U373-MG cells stably transfected with US11 (US11-215 cells). US11-215 cells were metabolically labeled for 3 min with [35S]methionine and chased for up to 40 min. The US11 protein was recovered from cell lysates by immunoprecipitation using a polyclonal anti-US11 serum (a-US11) and analyzed by SDS-PAGE (Figure 2A). Two species of US11 of distinct mobility were recovered at early time points (Figure 2B, lanes 1 and 2). The faster moving, major species is the ER-resident, mature form of US11 (mUS11-215). It has a mobility indistinguishable from that of US11 recovered from a microsome-supplemented cell-free translation system (D.Tortorella and H.Ploegh, unpublished data).

A precursor-product relationship between the two species was suggested by increased recovery at later chase points of mUS11-215 and decreased recovery of the slower moving species (*) (Figure 2A, lanes 1–4). The identity of the slower moving species (*) was unclear. Is it a distinct form of US11 or is it a protein associated with US11? Both mUS11-215 and the slower moving polypeptide (*) were recovered from SDS-denatured primary
immunoprecipitates in a second round of immunoprecipitation using α-US11 serum (D. Tortorella and H.L. Ploegh, unpublished data). We therefore conclude that the slowly migrating polypeptide is a distinct form of the US11 protein.

The precursor-product conversion observed for the slower moving polypeptide (*) and mUS11-215 does not account fully for the amount of US11 recovered at early chase times. At the early time points of chase, there is a shortfall in the recovery of US11 (Figure 2A, lanes 1–3). This shortfall is not due to the continued incorporation of label during the chase (Figure 2B) and hence must result from the inability to retrieve all US11 at the early time points. Solubilization with the detergent SDS significantly improved recovery of both US11 polypeptides (*) and mUS11-215) at the early time points (D. Tortorella and H.L. Ploegh, unpublished data).

Earlier experiments failed to show the presence of endoglycosidase H (Endo H)-resistant US11 and indicated that US11 was confined to the ER, as confirmed by immunoelectron microscopy (Wiertz et al., 1996a). The primary structure of US11 predicts a single N-linked glycan (CHO) attachment site at position 73 (Asn73–Leu–Ser) (Figure 1). Both polypeptides (*) and mUS11-215) recovered from the US11 immunoprecipitats were susceptible to digestion by Endo H (Figure 2A, lanes 6–10). The difference between these two molecules of US11 cannot be due to an unusual modification of the N-linked glycan and, therefore, must be caused by differences in the polypeptide backbone.

What type of modification could account for the presence of the slower moving species of US11? Based on the observed apparent molecular weight, the slowly migrating species of US11 may still contain the N-terminal signal peptide (spUS11-215). The polypeptide was isolated from [35S]methionine-labeled cells and subjected to 20 cycles of Edman degradation (Figure 2C). The observed peaks of radioactivity fit the position of the methionines at the N-terminal end of the US11 precursor sequence. These results establish that, surprisingly, the slower moving form (*) (Figure 2A) is indeed a glycosylated US11 molecule that has retained its signal peptide.

The US11 signal peptide contains a typical cleavage site

The factor known to influence signal peptide cleavage is the presence of small amino acid side chains at the –1 and –3 position relative to the cleavage site. Does the US11 signal peptide cleavage site indeed contain the consensus

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**Fig. 2.** Two forms of US11 exist early in biosynthesis. (A) US11-215 cells were pulsed for 3 min and chased for up to 40 min. Cells were lysed in 0.5% NP-40 and immunoprecipitated with anti-US11 serum (α-US11). The precipitates were analyzed by SDS–PAGE (12.5%). Two forms of US11 (*) and mature US11-215 (CHO) were recovered from the US11-215 cell lysates (lanes 1–5). Half of the α-US11 precipitates were digested with Endo H (lanes 6–10). (B) Incorporation of [35S]methionine was examined during a pulse–chase experiment of US11-215 cells TCA-precipitable radioactivity (c.p.m.) from [35S]methionine of each time point was plotted against the pulse–chase experiment. An average of each sample is represented at each value. (C) The slower moving US11 polypeptide (*) was subjected to N-terminal radiolabeling. The radioactivity (c.p.m.) from [35S]methionine of each time point was plotted against the Edman cycle number. (D) The n-, b-, and c-regions of the US11 signal peptide are shown. The site of signal peptide cleavage is indicated by an arrow. (E) The N-terminal radiolabeling of the mature form of US11 (mUS11-215) plotted by radioactivity (c.p.m.) from [35S]methionine versus Edman cycle number.
Fig. 3. The delayed cleavage of the US11 signal peptide is determined by its signal sequence and transmembrane/cytoplasmic tail region. (A) The US11 chimeric molecules US11-K1-18,215, US11/A2MCT and wild-type US11-215. (B) Processing of these molecules was examined in stable transfectants of MG-U373 cells using pulse-chase analysis. US11 was recovered from SDS lysates using α-US11 serum and analyzed by SDS-PAGE (12.5%). The signal peptide-containing form of US11 (spUS11-215) and the mature processed form of US11 (mUS11-215) are indicated. (C) The US11-215 molecules recovered from (B) were quantitated by a Molecular Dynamics Storm PhosphorImager. The US11 recovered at each time point is represented as percentage recovery of US11. The US11 recovered at the 8 min chase point was used as the 100% recovery value.

The US11 signal peptide and the transmembrane region contribute to the delayed cleavage of the US11 signal sequence

N-terminal signal peptide cleavage is presumably determined solely by the sequence of the signal peptide itself (Martoglio and Dobberstein, 1998). Changes within the a-, b- or c-region of the signal peptide and the regions directly downstream from the signal peptide affect signal peptide processing (Russel and Model, 1981; Folz and Gordon, 1986; Wier et al., 1988; Izard and Kendall, 1994). Can the US11 signal peptide itself or regions further downstream of the US11 signal sequence, such as the US11 transmembrane region, play a role in signal peptide cleavage? We generated US11-K18-215 (Figure 3A), a chimeric molecule in which the US11 signal peptide was replaced with the signal peptide of the murine MHC class I heavy chain H-2Kb, a type I membrane protein. We also generated US11/A2MCT (Figure 3A), a chimeric molecule in which the transmembrane and cytoplasmic tail of US11 were replaced with the corresponding regions of human MHC class I heavy chain A2. Cleavage of the H-2Kb signal peptide should now generate the N-terminus of mature US11. Pulse-chase analysis of US11-215 cells shows the recovery of spUS11-215 and mUS11 at the early time points and a precursor-product relationship between the two polypeptides (Figure 3B, lanes 1-4, and C). For neither US11-K18-215 nor US11/A2MCT did we observe the presence of a signal sequence-containing precursor (Figure 3B, lanes 5-8 and 9-12). This result suggests that unique features of US11’s signal sequence and transmembrane domain contribute to the persistence of spUS11-215.

The recovery of mUS11-215 and US11/A2MCT increases with time (Figure 3B, lanes 1-4 and 9-12, and C). In contrast, recovery of US11-K18-215 does not significantly change during the chase (Figure 3B, lanes 5-8, and C). We therefore conclude that the US11 signal...
peptide is also responsible for the increased recovery of US11-215 and US11/A2TMCT at the later time points. We suggest that the manner in which the US11 signal peptide initiates contact with the ER may contribute to its solubility properties.

The n-, h-, and c-regions of the US11 signal peptide follow the proposed consensus for a cleavable N-terminal signal peptide. However, the results obtained for the chimeric US11-K<sub>18,215</sub> molecule suggest that the signal peptide itself may account for its delayed cleavage. To characterize further the segment of the US11 signal peptide that is responsible for delayed cleavage, we generated additional chimeras in which the n-region (US11-K<sub>5,215</sub>) or n + h-regions (US11-K<sub>13,215</sub>) of US11 are replaced with the corresponding regions of H-2K<sup>d</sup> (Figure 4A). We transfected US11-215, US11-K<sub>5,215</sub>, US11-K<sub>13,215</sub> and US11-K<sub>18,215</sub> into HEK-293 cells and examined their processing by pulse-chase analysis (Figure 4B). For US11-215, a signal peptide-containing form of US11 and the mature form of US11-215 were evident at early chase times (Figure 4B, lanes 1-3). The two polypeptides showed a precursor-product relationship. For the chimeras US11-K<sub>5,215</sub>, US11-K<sub>13,215</sub> and US11-K<sub>18,215</sub>, removal of the signal peptide is rapid and only the cleaved form of US11 is recovered (Figure 4B, lanes 4-12). We conclude that features within the n-region of the US11 signal peptide contribute to its persistence.

During the chase, there is an increase in recovery of the mature form of US11-K<sub>5,215</sub> and US11-K<sub>13,215</sub> (Figure 4B, lanes 4-9), but not for US11-K<sub>18,215</sub> (Figures 4B, lanes 10-12, and 3B, lanes 5-8, and C). Therefore, the c-region of the US11 signal peptide somehow contributes to recovery of mature US11. While the identity of the c-region does not affect the cleavage of the signal peptide, it does contribute to the recovery of mature US11. Perhaps the c-region is responsible for positioning nascent US11 relative to other components of the translocation machinery. This positioning may affect interactions of US11 with other ER components shortly after its completion, and hence its solubility. In contrast, the presence of the full K<sup>d</sup> signal sequence neither delays signal peptide cleavage nor affects the recovery of US11 from cell lysates.

**The US11 transmembrane region plays a role in US11 signal peptide cleavage**

We next examined the role of the US11 transmembrane region in signal peptide cleavage. Such a role was suggested by the analysis of the US11/A2TMCT chimeric construct (Figure 3). We generated a C-terminal truncation of US11 that lacks the predicted transmembrane segment and the cytoplasmic tail (US11-180) (Figure 1). The
processing of wild-type US11-215 and US11-180 was examined in the appropriate U373-MG transfectants (Figure 5A). US11 recovered at the early chase times from US11-215 cells produced the usual pattern with respect to the precursor–product relationship of spUS11-215 and mUS11-215 (Figure 5A, lanes 1–4). Two major species were recovered from US11-180 cells (** and mUS11-180) (Figure 5A, lanes 5–8). A precursor–product relationship exists for the slower (**) and faster migrating species (mUS11-180) of US11-180. The two polypeptides recovered from the US11-180 transfectants represent distinct forms of the polypeptide backbone and both species of US11-180 are sensitive to Endo H (Figure 5B, compare lanes 1–4 and 5–8).

The slower moving species (**) was isolated from a US11-180 HEK-293 transfectant labeled with [35S]methionine and subjected to 20 cycles of Edman degradation (Figure 5C). The data showed persistence of the signal sequence. The absence of the transmembrane region of US11 thus strongly delays cleavage of its N-terminal signal peptide. An even more pronounced result was observed when US11-180 cDNA was transfected into HEK-293 and COS-1 cells (Figure 7).

mUS11-180 is a soluble protein

The Kyte–Doolittle hydropathy plot of US11 (Figure 1) suggests that the transmembrane region is located between residues 180 and 200. However, the hydrophobic nature of residues 180–200 does not ensure that it is in fact a transmembrane anchor. All attempts at proteolytic removal of the proposed cytoplasmic tail were without success. We performed Na2CO3 extractions to explore stable membrane insertion of US11-215 and US11-180 (Figure 6). US11-215 and US11-180 cells were labeled with [35S]methionine and broken with glass beads in the absence of detergent. Homogenates were then centrifuged at 1000 g to remove large debris, and the supernatant fraction was treated with 100 mM Na2CO3, followed by centrifugation at 150,000 g to sediment the extracted microsomes. US11-215 and US11-180 molecules were immunoprecipitated from detergent extracts prepared from the 1000 g pellet (Figure 6, lanes 1 and 4), the Na2CO3-treated 150,000 g soluble fraction (Figure 6, lanes 2 and 5) and the 150,000 g pellet fraction (Figure 6, lanes 3 and 6). As a soluble, luminal control protein, we used β2-microglobulin (β2m) (Figure 6, lanes 7–12). The US11-215 polypeptide is recovered exclusively from the 150,000 g pellet fraction (Figure 6, lane 3), whereas the bulk of β2m is recovered from the 150,000 g soluble fraction (Figure 6, lane 8). These results confirm that US11-215 is a membrane protein. In contrast, the majority of US11-180 lacking its signal peptide (mUS11-180) and β2m are recovered from the 150,000 g soluble fraction (Figure 6, lanes 5 and 11). These results confirm that mUS11-180 and β2m are soluble, ER luminal proteins.

A small fraction of mUS11-180 is recovered from the 150,000 g pellet fraction (Figure 6, lane 6) and may represent mUS11-180 that continues to associate with the ER membrane shortly after signal peptide cleavage and prior to its release into the ER lumen. Alternatively, a fraction of mUS11-180 may interact with an ER membrane protein in a Na2CO3-resistant manner. As might be expected, the signal peptide-containing form of US11-180 (spUS11-180) remains associated with the membrane fraction even after carbonate extraction (Figure 6, lane 6).

The identity of the signal sequence dictates delayed cleavage of the US11-180 molecule

For reasons of consistency with the data shown earlier, the experiments in Figure 5A were all conducted in U373-MG.
cells stably transfected with the US11-180 cDNA. The delayed cleavage of the signal peptide of US11 is not an aberration of the recipient cell line used for transfection. In fact, when we used either HEK-293 or COS-1 cells in a transient transfection protocol, the persistence of the signal peptide-containing form of both US11-215 (Figure 4B, lanes 1–3) and US11-180 (Figure 7A) was much more pronounced. The relative amount of signal sequence-containing precursor of US11-180 was increased to the extreme, such that in COS-1 cells it is in fact the predominant form of US11-180 at the end of the chase (Figure 7A, lanes 7–12). Our data show that the anomalous behavior of the US11 signal peptide is intrinsic to the US11 molecule. In transfaction experiments exploiting COS-1 cells to express other type I membrane proteins, the persistence of signal peptides was not observed (Huppa and Ploegh, 1997) and to our knowledge has not been reported by others.

We next addressed the contribution of the signal sequence's identity to the delayed cleavage observed for US11-180. We generated a chimeric molecule, US11–K18-180, in which the US11-180 signal peptide is replaced with the H-2Kb signal peptide (Figure 5A). We transfected US11-180 and US11–K18-180 into HEK-293 cells and examined their processing by pulse–chase analysis (Figure 7B). The immunoprecipitates were treated with Endo H to verify glycosylation and ER insertion (Figure 7B, lanes 4–6 and 10–12). For US11-180 carrying the US11 signal peptide, the signal peptide-containing form of spUS11-180 and the mature processed form of US11-180 were observed throughout the chase (Figure 7B, lanes 1–3). In contrast, a single polypeptide with a mobility similar to that of mUS11-180 is recovered from US11–K18-180 transfectants (Figure 7B, lanes 7–9). Delayed cleavage of the US11-180 signal peptide no longer occurs when the US11 signal peptide is replaced with the H-2Kb signal peptide. Not only the US11 transmembrane segment, but also features of the US11 signal sequence itself play a major role in US11 signal peptide cleavage.

**Discussion**

We describe here the unusual properties of the signal sequence of HCMV US11, a type I membrane glycoprotein. Elements contained within the signal sequence's N-terminal segment (Met–Asn–Leu–Val) are responsible for delayed cleavage, such that a fully glycosylated, signal peptide-bearing intermediate is readily detected. In addition, the C-terminal membrane anchor also affects the rate of signal peptide cleavage; a US11 variant lacking its transmembrane/cyttoplasmic tail segment (US11-180) shows an even greater delay in signal peptide cleavage than is seen for full-length US11. This effect is at its most extreme in COS-1 cells, where the glycosylated, signal peptide-containing US11-180 protein (spUS11-180) is the majority of US11 polypeptide that persists. To account for these findings, we propose an extended interaction of the signal peptide and transmembrane segment with the processing apparatus.

Conformity with the consensus parameters within the n-, h- and c-regions of the signal peptide predicts proper cleavage of an N-terminal signal peptide. The US11 signal peptide sequence fits the consensus parameters within the n-, h- and c-regions, yet fails to be cleaved efficiently from the nascent chain. Chimeric molecules in which regions (n, n+n+h or n+h+c) of the US11 signal peptide were replaced with the corresponding regions of the murine class I heavy chain H-2Kb signal peptide demonstrate that it is the n-region of the US11 signal sequence that is most responsible for the delayed cleavage of the US11 signal peptide (Figure 4). An irregular n-region has been observed to affect signal peptide processing; a surfet of positive charges within the n-region of the HIV-1 gp-120 signal sequence probably accounts for its inefficient cleavage (Li et al., 1994, 1996). This aberrant form of gp-120 does not exit the ER and, therefore, cannot be incorporated into a nascent virion. We note that the persistence of the uncleaved signal sequence on gp-120 was never directly shown by sequence analysis.

Regions outside the signal peptide can also influence its cleavage. In pre-pro-apolipoprotein A-II and pre-pro-parathyroid hormone, removal of the propeptide that is immediately downstream of the signal peptide influenced ER protein translocation and proper signal peptide processing (Russel and Model, 1981; Folz and Gordon, 1986; Andrews et al., 1988; Wiede et al., 1988). These changes mostly affect the site of cleavage, shifting it a few residues downstream, while their effect on the rate of signal peptide cleavage was not addressed in any detail. In addition, a mutation at the +2 position of the signal peptide cleavage site of phage coat protein also results in inefficient cleavage (Russel and Model, 1981). All of these mutations are localized immediately downstream of the signal peptide. In contradistinction to such signal sequence-proximal alterations, the transmembrane anchor of US11, at a considerable distance (~160 residues) from the US11 signal sequence, strongly influences signal sequence cleavage. The rate of signal peptide cleavage for the US11 molecule lacking its transmembrane/cyttoplasmic tail region (US11-180) is significantly delayed when compared with that seen for wild-type US11 (Figure 5). Replacement of the US11 signal sequence for that of H-2Kb results in rapid processing of US11 lacking the transmembrane segment, such that signal sequence-containing forms are no longer detected. The unprocessed US11-180 polypeptide is probably in an orientation unfavorable for signal peptide cleavage, and the presence of the US11 transmembrane anchor is clearly required for efficient signal peptide processing (Figure 8).

How can the US11 transmembrane anchor accelerate removal of the US11 signal peptide? The transmembrane domain may interact with the signal peptide and position the signal peptide to facilitate access to the cleavage site. Alternatively, the transmembrane anchor may interact with the SPC and enhance recognition of the US11 signal peptide for reasons of physical proximity. While the specificity of signal peptide cleavage is appreciated in terms of the minimum sequence requirements, cleavage itself is a highly regulated process, the dynamics of which are not well understood. The non-catalytic subunits of the SPC have been cloned and isolated, yet their function remains to be determined. Our results show that regulation of signal peptide cleavage may involve cis-acting elements within the polypeptide that act at considerable distance...
from the actual cleavage site. Such elements could perhaps interact with the non-catalytic subunits of signal peptidase.

Immunoelectron microscopy, the maturation status of its single N-linked glycan and the kinetics with which it catalyzes accelerated destruction of class I molecules all place US11 in the ER. The ER environment of the US11 signal peptide may help determine the unusual signal peptide cleavage pattern that we observe. The site of signal peptide cleavage is in the ER and is postulated to be in close proximity to the translocon (Kahle et al., 1998).

An intrinsic feature of the US11 signal peptide, more specifically the c-region of the signal peptide, may dictate an association with complexes within the ER as judged from the observed cleavage in detergent extractability (Figure 4 and D.Tortorella and H.L.Ploegh, unpublished data). Shortly after signal peptide cleavage, the recovery of the processed form of US11 increases over the chase period. We suggest that these early biosynthetic forms of US11 may reside in specialized regions of the ER.

To address this issue more peripheral to the central claims of this study: is the cleavage pattern of US11's signal sequence related to US11-induced class I degradation? The signal peptide of the chimeric molecule US11-K<sub>518-215</sub> is cleaved rapidly and this molecule readily supports class I destruction (D.Tortorella and H.L.Ploegh, unpublished data). Therefore, the identity of the US11 signal peptide itself is not essential for the ability of US11 to accelerate class I degradation. The signal peptide of the chimera US11/A<sub>2</sub><sup>TmCT</sup> is also cleaved rapidly, but class I heavy chains are not degraded in US11/A<sub>2</sub><sup>TmCT</sup>-expressing cells (D.Tortorella and H.L.Ploegh, unpublished data). Deletion of US11's cytoplasmic tail does not abolish degradation of class I heavy chains (D.Tortorella and H.L.Ploegh, unpublished data), and consequently the identity of the transmembrane segment of US11 should be considered essential to its function.

If our interpretation is correct, then perhaps the interaction of the US11 signal peptide and US11 transmembrane segment would help keep the Sec61 complex and its accessories in a configuration that allows recruitment of the class I heavy chains to the translocon. The recorded efficiency of US11-mediated dislocation suggests that the process is tightly linked, temporally and perhaps physically, to protein translocation into the ER. Thus, close proximity of US11 to the translocation apparatus and efficient gating of the protein channel might account for the speed of the dislocation reaction. Ultimately, this aspect must be related to the properties of US11 itself. The unusual maturation of US11, as described here, may turn out to be an important aspect of how the dislocation apparatus is put in place.

### Materials and methods

#### Cell lines and antibody

U373-MG astrocytoma cells transfected with the US11-215 cDNA were prepared as described (Jones et al., 1995; Kim et al., 1995) and cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum (FCS) and 5% calf serum. US11-201, US11-180, US11-K<sub>518-215</sub> and US11/A2 cells were maintained in DMEM supplemented with 5% FCS, 5% calf serum and 0.5 mg/ml gentamicin (Gibco, Frederick, MD). The human embryonic kidney cell line (HEK-293) was maintained in DMEM supplemented with 5% FCS and 0.5% calf serum. The anti-US11 serum was generated by immunizing rabbits with fragments of US11 (amino acids 18-36, 104-122 and 194-210) conjugated to keyhole limpet hemocyanin (Story et al., 1999). The anti-class I heavy chain serum was generated by immunizing rabbits with bacterially expressed luminal fragment of HLA-A2 and HLA-B27 heavy chains (Tortorella et al., 1998). The anti-β<sub>2m</sub> serum was generated by immunizing rabbits with bacterially expressed human β<sub>2m</sub>.

#### Metabolic labeling of cells and pulse-chase analysis

Cells were detached by trypsin treatment, followed by starvation in methionine/cysteine-free DMEM for 45 min at 37°C. Cells were metabolically labeled with 500 µCi of [35S]methionine/cysteine (1200 Ci/mmol, NEN-Dupont, Boston, MA) at 37°C for the times indicated. In pulse-chase experiments, cells were radiolabeled as above and chased for the times indicated in DMEM containing non-radioabeled methionine (2.5 mM) and cysteine (0.5 mM). Cells were then lysed in NP-40 lysis buffer (10 mM Tris pH 7.8, 150 mM NaCl, 5 mM MgCl<sub>2</sub>, 0.5% NP-40) supplemented with 1.5 µg/µl aprotinin, 1 µM leupeptin, 2 mM phenylmethylsulfonyl fluoride (PMSF) followed by immunoprecipitation (see below). For cells lysed in 1% SDS, the SDS concentration was adjusted, prior to immunoprecipitation, to 0.06% with the NP-40 lysis mix.

#### Immunoprecipitation

Following cell lysis, cell debris was removed by centrifugation at 10 000 g for 10 min. Non-specific binding proteins were removed from the cell lysates by the addition of 3 µl of normal rabbit serum, 3 µl of normal mouse serum and formalin-fixed, heat-killed Staphylococcus aureus for 1 h at 4°C. Immunoprecipitation was performed by incubation with antisera for 45 min at 4°C, followed by the addition of S. aureus for 45 min at 4°C. The pelleted S. aureus were washed four times with washing buffer (0.5% NP-40 in 50 mM Tris pH 7.4, 150 mM NaCl and 5 mM EDTA). The pellet was resuspended in SDS sample buffer (4% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.025% bromophenol blue in 62.5 mM Tris pH 6.8) and the released materials were subjected to 12.5% SDS–PAGE.
cDNA, transfection and Endo H digestion

The cDNA of full-length US11 was cloned from the AD169 HCMV genome. The following primers: 5'- primer, GCCTGCGAACCACACCATCAGGTATGCTCTACGC; Y primer, GCCTCTAGAACCACACCATGCTCGGAAAACATCCAG. The US11 cDNA was cloned into the eukaryotic expression vector pcDNA3.1 (Invitrogen, Carlsbad, CA) using the Xho-I/XbaI restriction site in its poly linker region. US11-1180 was selected from US11 (pcDNA3.1). The chimera molecules: US11/A230TCT (US11 [amino acids 1-178]/pHLaA2 [amino acids 307-365]); US11-K1181 [H-2K * (amino acids 1-5)]; US11 [amino acids 1-5]; US11-K1181 [H-2K * (amino acids 1-21)]; US11 (amino acids 18-215); and US11-K1181 [H-2K * (amino acids 1-21)]/US11 (amino acids 18-180) were generated by initially cloning the US11 fragment and then following by ligation of two of the respective fragments. Using primers specific to the ends of the ligated molecule, it was recloned and inserted into pcDNA3.1. A liposome-mediated transfection (Lipofectamne, Gibco, Fredrick, MD) protocol was performed as described by the manufacturer (4 μg of DNA/20 μl of lipofectamine/10 cm dish of cells). Endo H (New England Biolabs) digestion was performed as described by the manufacturer.

Gel electrophoresis

SDS-PAGE and fluorography were performed as described (Ploegh, 1995). For N-terminal sequencing, the immunoprecipitated US11 protein was resolved by SDS-PAGE and transferred to a PVDF membrane (0.2 μm pore size) in transfer buffer (48 mM Tris-borate, 39 mM glycine, 0.037% SDS, 20% methanol) using a semi-dry blotting apparatus (Buchler Instruments, Kansas, MO).

N-terminal sequence analysis

The PVDF membrane that contained the polypeptide of interest was subjected to automated Edman degradation using an Applied Biosystems Protein Sequencer, Model 477, using ATZ chemistry, at the Biopolymers Laboratory at MIT, Center for Cancer Research. The fractions from each degradation sequencing cycle were collected and counted by liquid scintillation spectrometry.

Na2CO3 treatment

US11-1180 cells were metabolically labeled for 15 min and then washed twice in 50 mM Tris pH 7.5, 250 mM sucrose (homogenization buffer). The cells were resuspended in homogenization buffer and broken by vortexing in the presence of 106 μl glass beads. The homogenate was centrifuged at 10000 g for 5 min. The pellet fraction was resuspended in NP-40 lysis mix (see above) and the supernatant was treated with Na2CO3 (100 mM final) for 30 min at 4°C (Fujiki et al., 1982). The Na2CO3 treated samples were centrifuged at 150 000 g using a TLA 100.2 rotor in a Beckman centrifuge. The 150 000 g high pH supernatant was adjusted to pH 7 with 5 M HCl and diluted to a final 1× NP-40 lysis mix. The 150 000 g pellet was washed twice with homogenization buffer and then resuspended in 1× NP-40 lysis mix. US11 and βm were immunoprecipitated from the 1000 g pellet. 150 000 g superman and the 150 000 g pellet with the respective antibody.

Acknowledgements

We thank Richard Cook of the Biopolymers Laboratory at MIT, Center for Cancer Research for protein sequencing. D.T. is a Charles A King Trust (Boston, MA) Research Fellow. P-S. is supported by HRMI A.R. is supported by the German Research Council (DFG). This work was funded by NIH grant S037-1135456 and a grant by Boehringer-Ingelheim.

References


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Received November 21, 2000; revised February 14, 2001; accepted February 16, 2001