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ACKNOWLEDGEMENTS. We thank A. Habermann and G. Griffiths for the electron-microscopy analysis of $RhoD^{G26V}$-expressing cells; S. Reinsch, E. Stelzer and N. Salmon for advice on confocal microscopy and data processing; A. Giner and M. Stapleton for technical assistance; A. Hall for the $Rac1^{V12}$ plasmid; and J. Burkhardt, M. Glotzer, S. Reinsch, K. Simons, M. Way and W. Witke for critical assessment of the manuscript.

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# Sec61-mediated transfer of a membrane protein from the endoplasmic reticulum to the proteasome for destruction

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**The human cytomegalovirus genome encodes proteins that trigger destruction of newly synthesized major histocompatibility complex (MHC) class I molecules. The human cytomegalovirus gene *US2* specifies a product capable of dislocating MHC class I molecules from the endoplasmic reticulum to the cytosol and delivering them to the proteasome. This process involves the Sec61 complex, in what appears to be a reversal of the reaction by which it translocates nascent chains into the endoplasmic reticulum.**

CYTOLYTIC T cells eradicate virus-infected cells by recognition of virus-derived peptides in a physical complex with MHC class I molecules[1]. In this way, the cell advertises to the immune system that it harbours a pathogen, and invites its destruction.

The selective pressure exerted by the immune system on viruses has led them to acquire, in the course of their evolution, a set of remarkable strategies with which they can elude cytolytic T cells. Certain viruses inhibit surface expression of class I–peptide complexes, thereby preventing destruction of the host cell in which they replicate[2]. In human cytomegalovirus (HCMV)-infected cells, this mechanism involves the rapid degradation of MHC class I molecules[3,4], a process in which the HCMV genes *US2* and *US11* play a key role[4,5]. Such a multiplicity of mechanisms to cripple MHC class I expression suggests that cytolytic T cells are pivotal in the host defence against HCMV.

MHC class I molecules consist of two subunits, a heavy chain and $\beta_2$-microglobulin, whose association is required for peptide binding in the ER and for efficient transport of the complex out of the ER[6]. The heavy chain is targeted to the endoplasmic reticulum (ER) membrane by an amino-terminal, cleaved signal sequence, contains a glycosylated lumenal domain, and has a carboxy-terminal membrane anchor (type I membrane protein). The light chain $\beta_2$-microglobulin is a secreted protein with a cleavable signal sequence. Both proteins are translocated across the ER membrane during their synthesis by an apparatus whose major constituent is the Sec61 complex. This membrane protein complex consists of three subunits, $\alpha$, $\beta$, $\gamma$, and probably forms a protein-conducting channel[7].

In cells expressing the HCMV *US11* gene product, a type I membrane protein, the heavy-chain molecules are inserted into the ER membrane and are glycosylated cotranslationally, but shortly thereafter they are rapidly transported back into the cytosol, where they are deglycosylated by an *N*-glycanase and degraded by the proteasome[5]. How dislocation of the polypeptide chains occurs is unknown. However, a similar process may also occur in normal cells and may correspond to the process referred to as ER degradation[8,9]. Several membrane proteins that misfold in the ER are degraded following the attachment of ubiquitin[10–13]. Involvement of the ubiquitin pathway[14], and inhibition of breakdown by agents that target the proteasome[15] imply that there is a cytosolic component to such degradation. Again, the mechanism by which these proteins are extracted from the membrane and transferred to the proteasome is unknown.

We have now found a second HCMV gene product, encoded by *US2*, that also triggers dislocation of newly synthesized class I heavy chain molecules into the cytosol for degradation by the proteasome. Characterization of the fate of MHC class I molecules in cells expressing the US2 gene product illuminates the underlying molecular mechanisms. US2 binds to newly synthesized class I molecules and escorts them into the cytosolic compartment where the heavy chains, and possibly US2, are deglycosylated by a *N*-glycanase. The deglycosylated breakdown intermediate is associated with the Sec61 complex, suggesting that retrograde transport occurs through the same protein-conducting channel that allowed the original membrane insertion of the heavy chain. The deglycosylated intermediate is also associated with the

proteasome, in further support of the idea that this cytosolic organelle is the site of its subsequent degradation. When misfolding of class I molecules is induced in cells that do not express US2, the proportion of heavy chains found in association with the Sec61 complex increases dramatically immediately before their destruction. We therefore propose that degradation of misfolded proteins in the ER may generally occur by the retrograde transport of polypeptides through the protein-conducting channel, followed by their degradation in the cytosol by the proteasome.

## Breakdown of MHC class I molecules

In U373 cells transfected with *US2* (US2$^+$ cells), MHC class I molecules are markedly less stable than in control cells. In a pulse–chase experiment, most of the newly synthesized class I heavy chains (HC) are degraded within 30 min, regardless of whether they are unassembled—as detected by immunoprecipitation with the antibody RaHC—or properly folded and associated with $\beta_2$-microglobulin as detected by the conformation-sensitive monoclonal antibody W6/32 (ref. 16) (Fig. 1*a*, *b*). In the presence of the proteasome inhibitors lactacystin[17], carboxybenzyl-leucyl-leucyl-leucinal ($ZL_3H$)[5] or carboxybenzyl-leucyl-leucyl-leucyl-vinylsulphone ($ZL_3VS$; M. B., J. McMaster and H. L. P., unpublished observations), degradation is inhibited, but not completely blocked, and a 40K breakdown intermediate is observed (Fig. 1*a*). The intermediate, which comigrates with class I HC from tunicamycin-treated cells, is the product of an *N*-glycanase-catalysed reaction, as demonstrated by analysis of the intermediate by isoelectric focusing (data not shown, and ref. 5). Other intermediates, such as ubiquitin-conjugated species, could not be detected.

We next did experiments to confirm that even properly folded MHC class I molecules can be degraded in a US2-dependent fashion. The assembly of class I molecules requires an oxidizing environment in the ER[18]. In cells that do not express US2, free heavy chains are converted into properly folded class I molecules, the W6/32 reactive complex[19,20] (Fig. 2*a*). In the presence of the reducing agent dithiothreitol (DTT), there is a stark reduction in the quantity of properly folded, W6/32 reactive class I molecules. Instead, we found free heavy chains that tend to form aggregates. After 40 min of chase, these chains abruptly disappeared. Removal of DTT allows efficient refolding[21] of MHC class I molecules (Fig. 2). When cells are first incubated with DTT and the reducing agent is then removed, free class I HC disappear, and efficient refolding of MHC class I molecules occurs.

In US2$^+$ cells, upon DTT removal, the decrease of free heavy chains coincides with a transient increase of W6/32 reactive, folded heavy chains (Fig. 2*b*). Therefore, these ER-resident glycosylated free heavy chains are first folded and subsequently degraded in a US2-dependent manner.

To link a precursor–product relationship of the MHC class I HC precursor and the deglycosylated intermediate with intracellular location we did a pulse-chase experiment in the presence of the proteasome inhibitor $ZL_3H$ in combination with subcellular fractionation (Fig. 2*c*). The 1000*g* pellet, comprising debris, cell remnants and nuclei, together with trapped cytosol, reveals the presence of both precursor and product. When the postnuclear supernatant fraction of the 1000*g* sedimentation is further analysed, most of the deglycosylated class I breakdown intermediate is cytosolic (100,000*g* supernatant), in agreement with the suggestion that it would normally be broken down by the proteasome (see below). Some of the deglycosylated breakdown intermediate is present in the 100,000*g* pellet, where it reaches a maximum at 10 min of chase, after which it remains constant. This may represent material associated with a larger cytosolic complex such as the 26S proteasome. In the cytosol, some glycosylated precursor is present at equal intensities at the 1- and 5-min chase points but not later, suggesting that at least some of the deglycosylation reaction may also occur to class I HC released in the cytosol. In the 10,000*g* pellet which contains microsomes, the intact heavy chain essentially showed the same time course as the total cellular population. Taken together, these results demonstrate that US2 triggers the dislocation of glycosylated class I HC into the cytosol where they are deglycosylated by a *N*-glycanase and subsequently degraded by the proteasome.

## ATP-dependent dislocation

As a first step towards the clarification of the mechanism of dislocation, we tested whether the process is ATP dependent. US2$^+$ cells were depleted of ATP by addition of 2-deoxyglucose and antimycin A[22] immediately after a 1-min pulse-labelling period. Conversion of the class I HC precursor to the deglycosylated product is significantly inhibited (Fig. 3*a*). The fraction of class I HC that fails to undergo conversion to the deglycosylated product is accounted for by an increase in folded, W6/32 reactive class I molecules, indicating again that degradation and folding are competing processes.

The ATP dependence of dislocation was confirmed in an experiment in which ATP depletion was imposed before, at the onset of, or at later times in the chase period (Fig. 3*b*). Within 1 min of ATP depletion, protein synthesis is reduced by more than 90%. ATP depletion produced a marked reduction in the relative amount of dislocated class I HC. This was again accompanied by an increase in the relative amount of W6/32 reactive class I molecules. Protein folding in the ER can therefore continue, even at intracellular ATP levels where protein synthesis has essentially stopped. We conclude that the dislocation reaction requires ATP, like other transport processes across membranes.

## US2 association

Next, we addressed the role of US2. The US2 protein is probably a membrane protein containing a C-terminal transmembrane segment, which contains two of the three possible *N*-linked glycan attachment sites, whereas the third site is located N-terminal of the transmembrane segment. The *US2* gene product occurs in at

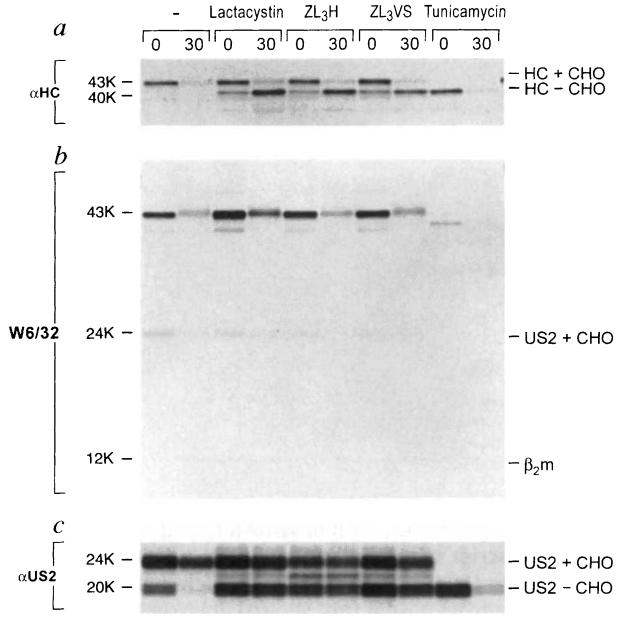


FIG. 1 US2 targets MHC class I molecules for proteasomal destruction. *a*, In US2$^+$ cells, MHC class I heavy chains (43K, HC + CHO) are rapidly destroyed and give rise to a deglycosylated breakdown intermediate (40K, HC – CHO) in the presence of proteasome inhibitors. CHO designates the *N*-linked oligosaccharide. *b*, Properly folded MHC class I molecules (W6/32 reactive material) form a complex with US2 (24K) and are targeted for destruction in US2$^+$ cells. The deglycosylated class I heavy chain cannot be detected in the W6/32 immunoprecipitates. The positions of migration of $\beta_2$m (12K) is indicated. *c*, US2 occurs in a glycosylated (24K, US2 + CHO) and a non-glycosylated form (21K, US2 – CHO). The non-glycosylated form of US2 is rapidly destroyed in a manner sensitive to proteasome inhibitors.

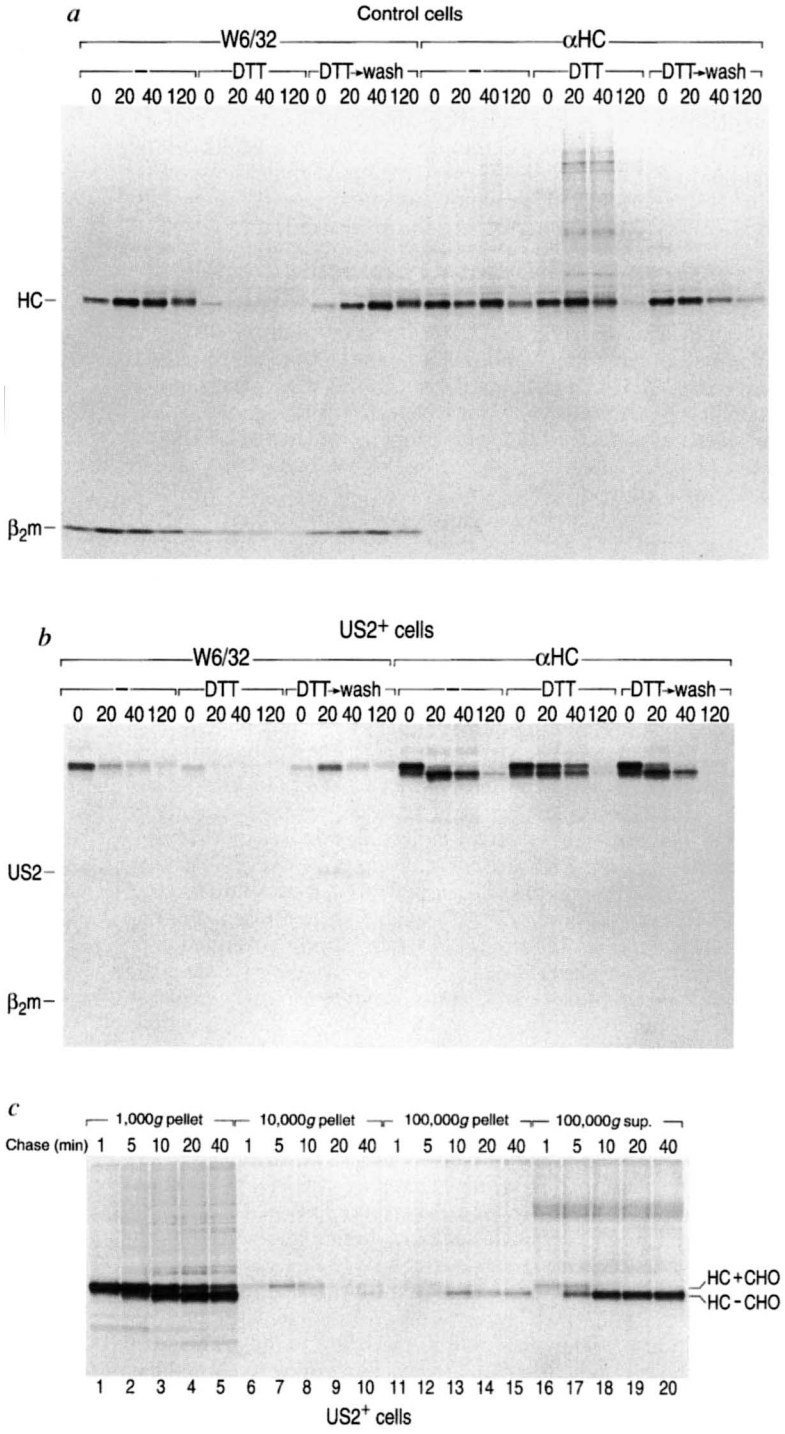


FIG. 2 Folded class I molecules are degraded in US2$^+$ cells and the deglycosylated intermediate is found in the cytosol. *a*, Class I molecules were recovered with either rabbit anti-heavy chain serum (αHC) or W6/32 from control cells, cells treated with dithiothreitol (DTT), and cells treated with DTT followed by DTT wash-out, as indicated. All pulse-chase experiments were done in the presence of $ZL_3H$. *b*, As *a*, but for US2$^+$ cells. Note the refolding of class I HC, as is evident from acquisition of W6/32 reactivity, upon removal of DTT. Note also the presence of glycosylated US2 in association with the refolded molecules. *c*, Deglycosylated breakdown intermediate (HC – CHO) occurs in the cytosol (100,000*g* supernatant), as assessed by a pulse-chase experiment combined with subcellular fractionation. Note that some of the deglycosylated breakdown intermediate can be sedimented after 1 h at 100,000*g* at later chase times. This fraction probably corresponds to proteasome-associated class I HC. CHO designates the *N*-linked oligosaccharide.

least two principal forms, both in virus-infected cells (T.R.J. and L. Sun, manuscript submitted) and in US2$^+$ cells (Figs 1*c*, 4*a*, *b*). These differ by the presence of a single high-mannose *N*-linked glycan, as seen from partial (not shown) and complete digestions of immunoprecipitated US2 with endoglycosidaseH (EndoH) (Fig. 4*a*), and from labelling experiments in the presence of tunicamycin (Fig. 1*c*). The occurrence of *N*-linked glycosylation indicates initial exposure of US2 to the lumen of the ER, and failure to acquire EndoH resistance suggests that it is an ER-resident protein, consistent with its activity on newly synthesized class I molecules. In a subcellular fractionation experiment, glycosylated US2 was indeed mostly found in a particulate fraction, whereas non-glycosylated US2 was located in the cytosol (data not shown). In pulse-labelling experiments even as short as 45 s, both forms are present (Fig. 4*b*, right). The non-glycosylated form is readily degraded, but the glycosylated form is more stable (Fig. 1*c*). Degradation of the non-glycosylated form can be largely prevented by the proteasome inhibitors tested (Fig. 1*c*). Poor solubility in urea-containing isoelectric focusing (IEF) sample buffer precludes analysis by IEF of US2. Hence we cannot be certain that non-glycosylated cytosolic US2 originates from the action of an *N*-glycanase, but otherwise its fate is strikingly similar to that of the deglycosylated class I HC intermediate.

To test whether US2 acts directly on MHC class I HC, co-immunoprecipitation experiments were carried out. Lysates of labelled US2$^+$ cells were produced in digitonin, a relatively mild detergent, and immunoprecipitated with the antibodies indicated (Fig. 4*a*). After dissociation of immune complexes in SDS and dilution into NP-40-containing buffer, the proteins were reprecipitated with anti-US2 antibodies. Association of US2 with class I

molecules was observed. The glycosylated form of US2 was found predominantly in a complex with the folded, W6/32 reactive class I molecules (Figs 1, 2*b*), whereas non-glycosylated US2 was recovered mainly from anti-heavy-chain precipitates, which contain mostly class I breakdown intermediate. A similar reimmunoprecipitation in a pulse–chase experiment (Fig. 4*b*, right) confirms the US2–class I association. Because the amount of US2-complexed HC breakdown intermediate reaches a plateau at a time where the total amount of HC is already in steep decline we suggest that US2 escorts class I molecules into the cytosol. As expected, given its lectin-like nature[23], calnexin exclusively interacts with the glycosylated form of US2 (Fig. 4*a*). Combined, our results demonstrate a direct interaction of US2 and class I molecules, both of which are delivered from the ER to the cytosol where they are destroyed. We have not detected similar complexes for US11 and class I HC (not shown).

## Association with the proteasome

Next, we tested whether a physical complex between class I heavy chains and the proteasome could be detected in inhibitor treated cells. Proteasome antibodies precipitate a number of polypeptides, including the characteristic α- and β-subunits of relative molecular mass ~30K (Fig. 4*b*; confirmed by site-specific labelling of β-subunits with a radioactive peptide-vinylsulphone (M.B. and H.L.P., manuscript in preparation)). Reimmunoprecipitation with anti-heavy chain antibodies reveals the presence of the breakdown intermediate, but not of the glycosylated precursor, with maximum recovery at 7 min of chase (Fig. 4*b*). At this time, US2$^+$ cells contain both the precursor and the deglycosylated product, but only the latter associates with the proteasome. This association reaches a maximum before the total deglycosylated population peaks (20 min). Complexes between MHC class I heavy chains and the proteasome can also be detected in US11-expressing cells but not in control cells, even though the latter always contain some free class I HC (Fig. 4*c*). Taken together, these results support the notion that the proteasome is the site of further degradation of the deglycosylated heavy chains.

## Association with the Sec61 complex

Given the rapidity of the dislocation and breakdown reaction, newly synthesized class I heavy chains are unlikely to have strayed far from their point of insertion, the Sec61 complex. We therefore explored the possible involvement of the translocation complex in the dislocation reaction by co-immunoprecipitation. Digitonin extracts were prepared from US2$^+$ cells treated with the proteasome inhibitor $ZL_3H$ and immunoprecipitated with Sec61β antibodies. These conditions leave the Sec61 complex intact[24]. The immune complexes were dissociated in SDS and reimmunoprecipitated with RaHC. Only the deglycosylated class I heavy chains were found to be associated with Sec61 in US2$^+$ cells (Fig. 5*a*). A similar complex of the deglycosylated heavy chain and the Sec61 complex can also be recovered with antibodies against the γ-subunit of the Sec61 complex (our unpublished observations). In the absence of $ZL_3H$, no complexes of class I HC and Sec61 were detectable (not shown). The amount of class I breakdown intermediate that is associated with Sec61 increases with time and reaches its maximum when the breakdown intermediate is actually destroyed. Because only deglycosylated class I HC are complexed with Sec61, the reportedly cytosolic *N*-glycanase[25] must be able to act on the class I heavy chain while it is tethered to the Sec61 complex (Fig. 6). US2, but not transferrin receptor and calnexin (not shown), is also recovered in a complex with Sec61 (Fig. 5*a*). Interestingly, it is predominantly the non-glycosylated form of US2 that is coprecipitated, consistent with the suggestion that US2 escorts class I heavy chains on their way to destruction.

## A complex of Sec61 and class I heavy chains

Class I heavy chains are also degraded in normal cells if they fail to assemble properly[20,26], a process that probably occurs in the cytosol because it can be partially inhibited by proteasome inhibitors (data not shown). Degradation of class I HC is greatly stimulated if their misfolding is induced by DTT (Fig. 2). To test the possible involvement of the Sec61 complex in the degradative pathway, co-immunoprecipitation experiments were done (Fig. 5). In the absence of DTT, association of class I molecules with Sec61 was only seen at late times of the chase period. In the presence of DTT, the association occurred earlier and was more pronounced. These data therefore suggest that even in normal cells, unfolded class I heavy chains interact with the Sec61 complex before being degraded in the cytosol. We detected only the glycosylated heavy chain in a complex with Sec61. Although the formation of a complex of class I heavy chains with Sec61 precedes their proteolytic destruction and suggests that Sec61 is involved, the absence of a deglycosylated class I HC in association with Sec61 indicates that the rate-limiting steps for degradation of class I HC in control and in US2$^+$ cells must differ.

## Discussion

HCMV uses a remarkable mechanism to downregulate the expression of the MHC class I molecules in infected cells. Two viral gene products, US11[5] and—as shown here—US2, induce newly synthesized MHC class I molecules to be transported back

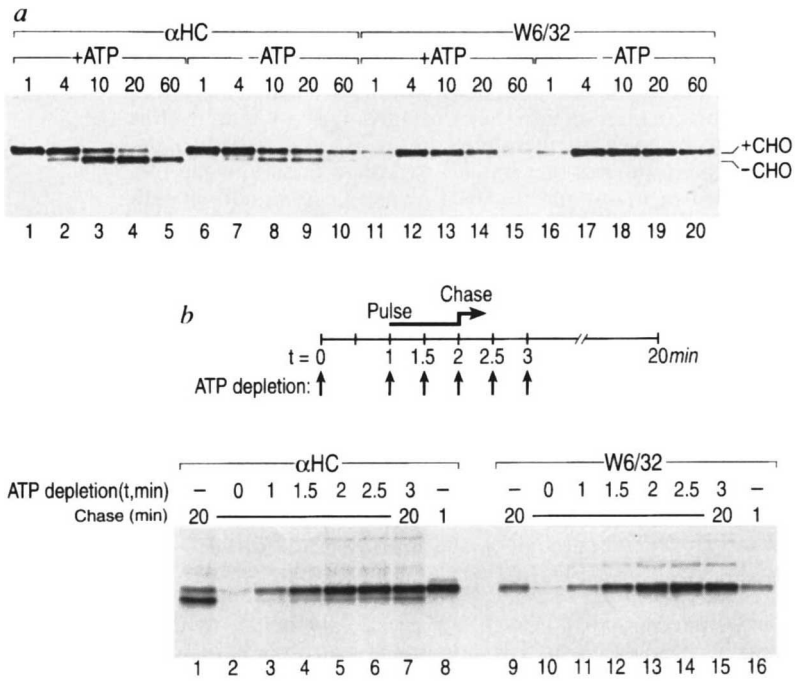


FIG. 3 The dislocation of MHC class I heavy chains is ATP-dependent. *a*, US2$^+$ cells treated with $ZL_3H$ were pulse-chased in the presence (−ATP) and absence (+ATP) of 2-deoxyglucose and antimycin A. Folded class I molecules were recovered with W6/32, and free heavy chains with anti-HC. Immunoprecipitates were resolved by SDS–PAGE. In the absence of ATP, the decreased conversion of the glycosylated precursor (+CHO) to the deglycosylated product (−CHO) is compensated by an increase in properly folded (W6/32-reactive) class I molecules. *b*, Pulse–chase experiment on US2$^+$ cells in the presence of $ZL_3H$, in which all samples were labelled and chased for the same times, with the ATP-depletion mixture added at the indicated times (see scheme). Addition of ATP-depletion mix at the onset of labelling completely suppresses the conversion of glycosylated heavy chain to the deglycosylated intermediate.

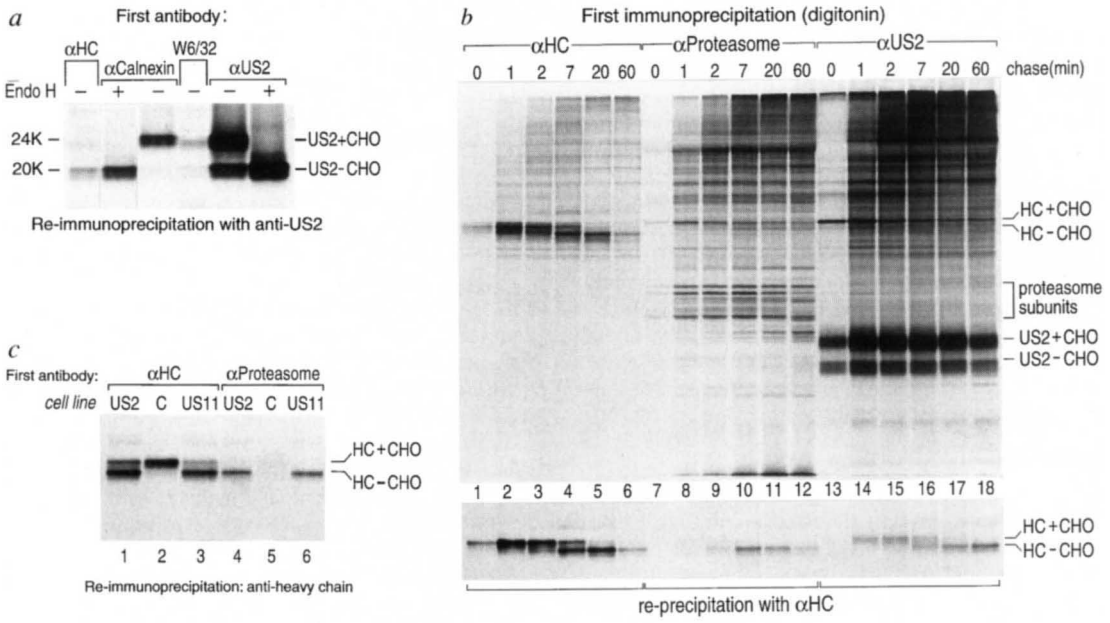


FIG. 4 US2 associates with MHC class I heavy chains and the breakdown intermediate occurs as a soluble intermediate complexed with the proteasome. *a*, Glycosylated (US2 + CHO) US2 associates with calnexin and properly folded class I molecules (W6/32), whereas non-glycosylated US2 (US2 – CHO) is found in a complex with the class I heavy chain breakdown intermediate. Glycosylation status was assessed by digestion with EndoH, as indicated. *b*, Digitonin lysates of $ZL_3H$ treated $US2^+$ cells were immunoprecipitated by the antibodies indicated and the precipitates were reimmunoprecipitated with rabbit anti-heavy chain serum (bottom panel). The positions of migration of the heavy chains (HC) and US2 molecules (US2) with or without their *N*-linked glycan (±CHO) are indicated. *c*, Deglycosylated intermediate (HC – CHO) is found in association with the proteasome in digitonin extracts of $ZL_3H$ treated $US2^+$ and $US11^+$ cells, but not control cells, even though class I heavy chains are present in all cell lines.

from the ER into the cytosol. Following deglycosylation they are degraded by the proteasome. Using US2-expressing cells, we have been able to elucidate the mechanism of this process.

US2 interacts directly with newly synthesized class I molecules and escorts them back into the cytosol in a reaction that involves the Sec61 complex and therefore appears to be the reversal of the translocation process that initially deposited them into the ER. Misfolded or unassembled class I heavy chains in normal cells seem to be routed in a similar pathway for their destruction in the cytosol.

The Sec61 complex was thought to have a role only in the translocation of proteins from the cytosol into the ER. It is an essential translocation component that can associate with either ribosomes or the Sec62/63 complex to perform co- and post-translational transport, respectively[7,27–29]. The Sec61 complex forms a protein-conducting channel while the driving force for transport is provided by other, interacting components. There is no reason to assume that this channel must allow transport in only one direction, and one could therefore envisage that the channel may indeed by used for retrograde transport if associated with appropriate additional components. Retrograde transport *in vitro*, presumably through the Sec61 channel, has been reported[30–32] but its physiological significance is unclear. Our conjecture that regrograde transport through the Sec61 channel is used for cytosolic degradation of ER proteins is based on the specific association of class I HC breakdown intermediates with the Sec61 complex at times when degradation proceeds at maximum rate. To our knowledge, our data provide the first indication that polypeptide chains can be caught in transit through the channel.

Reversal of the translocation process would be easier to explain if the newly synthesized polypeptide chain had never left the Sec61 channel. Indeed, this may be true for many class I HC in US2- or US11-expressing cells, because their degradation occurs so soon after membrane insertion (Fig. 6*a*). However, in US2-expressing cells, dislocation into the cytosol is possible even for folded class I molecules, which therefore have probably left the translocation site. We postulate that membrane proteins can be brought back

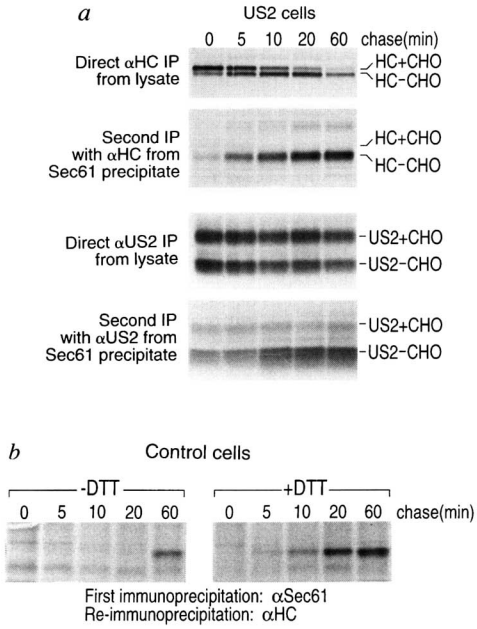


FIG. 5 Class I molecules destined for degradation associate with Sec61p. *a*, Sec61 was immunoprecipitated from digitonin lysates of $ZL_3H$-treated $US2^+$ cells and the precipitates were reimmunoprecipitated with either rabbit anti-HC serum (αHC) and or rabbit anti-US2 serum (αUS2). Sec61-depleted lysates were also immunoprecipitated with αHC or αUS2. These samples are designated as direct immunoprecipitates. The positions of migration of the heavy chains (HC) and US2 molecules (US2) with or without their *N*-linked glycan (±CHO) are indicated. Exposure time of the autoradiogram for the reprecipitation experiments was 14 days; for direct precipitations, it was 2 days. *b*, Control cells were chased in the absence or presence of DTT, as indicated. Sec61 was immunoprecipitated as for *a*. These immunoprecipitates were reimmunoprecipitated with αHC. Protein misfolding promoted by DTT results in an increased representation of glycosylated class I HC in the Sec61 complex.

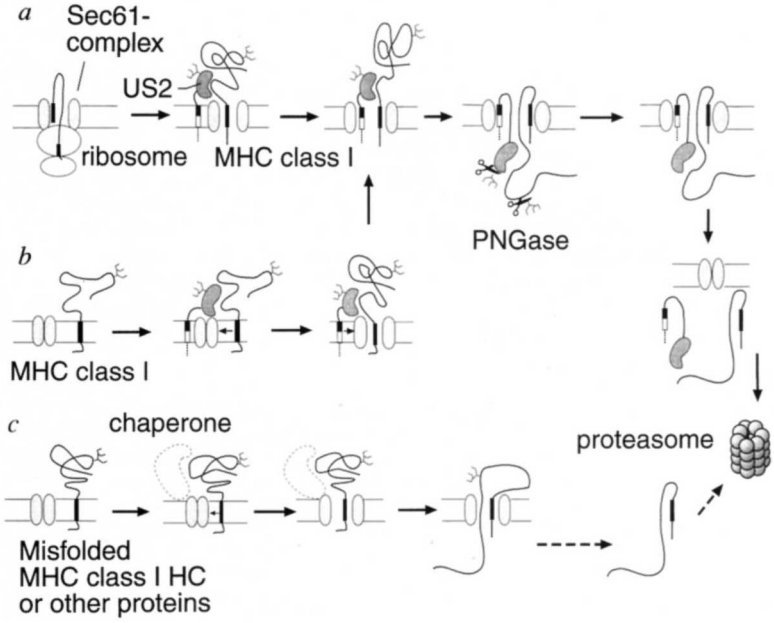


FIG. 6 Model for Sec61-dependent dislocation. *a*, A nascent class I heavy chain inserts into the ER in a signal sequence-dependent fashion. US2 interacts with the class I heavy chains before their lateral escape from the Sec61 complex into the ER membrane. The lumenal, glycosylated domains of both class I HC and US2 are subject to dislocation, a reaction that culminates in the action of *N*-glycanase and proteasomal proteolysis. *b*, An ER-resident, properly folded class I HC can be recruited by US2 to re-enter the Sec61 complex. US2 itself re-enters the Sec61 complex and both polypeptides are dislocated. *c*, In normal cells, any misfolded or incompletely assembled protein could be recruited by the action of an ER-resident chaperone to re-engage the Sec61 complex for dislocation and cytosolic destruction. Whereas in *a* and *b*, proteolysis may become rate-limiting owing to the high rate of dislocation, in *c* dislocation is the rate-limiting step.

into the translocation channel, perhaps by reversal of the lateral gating mechanism that led to their release into the phospholipid bilayer in the first place. Because glycosylated US2 associates with glycosylated class I molecules, we believe that it is this association that triggers the re-entry of the heavy-chain molecules into the translocation channel (see model in Fig. 6*b*).

The next step in the US2-dependent breakdown of the heavy chains is deglycosylation by a cytosolic *N*-glycanase[5,25]. The resulting class I HC intermediate is associated with non-glycosylated US2, which suggests that they are dislocated in a complex. The association of both deglycosylated class I HC and US2 with the Sec61 complex strengthens this proposal. We envisage that the lumenal domains of class I HC and US2 are threaded into the channel so that they achieve loop structures similar to those formed during their insertion into the ER (Fig. 6). The occurrence of the deglycosylated class I HC in association with Sec61 implies that the lumenal domain of the class I HC must be accessible to the cytosolic *N*-glycanase while still tethered to Sec61.

The final step in the degradation pathway is the proteasome. Inhibition of degradation by proteasome inhibitors is required to visualize the class I HC intermediate, and for the detection of a complex between the proteasome and the intermediate in US2-expressing cells. A complex between the proteasome and a substrate is without precedent and may be detectable in US2$^+$ cells only because of the unusually high rate of dislocation of MHC molecules. We have been unable to detect ubiquitin-conjugated class I HC in the course of pulse-chase experiments, perhaps because such intermediates are too short-lived, but the ability of the 26S proteasome to attack non-ubiquitinylated proteins is on record[33–35].

Most of the substrates of the proteasome are soluble, deglycosylated class I HC, but those still located in the translocation channel may also be attacked by a population of proteasomes that is membrane-bound[36]. Indeed, in mouse epithelial cells engaged in the breakdown of misfolded human class I molecules, the ubiquitin-activating enzyme E1 and ubiquitin itself were found associated with an expanded compartment to which the misfolded class I heavy chains were targeted[37]. Although ubiquitinylation may be involved only in US2-independent degradation, membrane association of the proteasome may be more generally involved in disposal of dislocated proteins.

Whether the degradation pathway of MHC class I molecules in normal cells is identical to that in US2$^+$ cells is not entirely clear, because proteasome inhibitors did not block degradation completely and no deglycosylated breakdown intermediate could be detected as yet. The latter may simply be too short-lived. If the rate-limiting step in the overall pathway is the feeding of a polypeptide into the translocation site for dislocation, it may be difficult to detect breakdown intermediates. For those proteins where ER breakdown has been invoked, their half-lives are on the 15–180-min timescale[9] without the occurrence of obvious breakdown intermediates. Only if the dislocation step is greatly accelerated, as in the case of US2- or US11-expressing cells, and if proteasome function is inhibited, may it be possible to catch intermediates.

Regardless of whether the later steps are the same in US2$^+$ and control cells, our results suggest that dislocation occurs by the same mechanism. MHC class I molecules that are induced to misfold in the ER are associated with the Sec61 complex at the time point of their maximum degradation. Misfolded heavy chains may associate with ER-resident chaperones[38] that, analogously to US2 and US11, bring the polypeptides back into the translocation channel for dislocation into the cytosol and subsequent degradation (Fig. 6). The postulated chaperones need not be homologous with US2 or US11 as these are also not related to each other[39], and still induce the same process. As far as we have been able to determine, the effects of US2 and US11 are specific for MHC class I molecules[5], whereas the putative chaperones would have broader substrate specificity. Because the structures of US2 and US11 are quite different[39], they may either target unique determinants of the translocation/dislocation machinery, as suggested by their differential attack on murine class I HC, or they may interact with different regions of class I HC (R. P. Machold and H.L.P., unpublished observations), while otherwise exploiting a similar mechanism of dislocation.

We propose that the pathway outlined in Fig. 6*c* is generally used to purge the ER of misfolded or unassembled proteins. If some proteins can be dislocated back into the cytosol, there must be a mechanism that distinguishes them from those that stay in the ER or are transported further along the secretory pathway. With few exceptions, type I and type II membrane proteins occur in homo-oligomeric or hetero-oligomeric forms early in their biosynthesis. The original concept of architectural editing encompassed a check on proper oligomeric state as one of the criteria for quality control[40]. Failure to oligomerize would leave exposed those areas of the protein involved in intersubunit contacts, which could now be used to allow an interaction, either directly, or via the above-mentioned chaperones, with the Sec61 complex (Fig. 6*c*). Such a model bears obvious similarities with cytosolic proteolysis, where recognition of unfolded proteins by chaperones

precedes degradation by cytosolic proteases such as the proteasome[15,41].

ER degradation is a long known phenomenon but was hitherto assumed to occur in an ATP-dependent manner[42] within the membrane or in a lumenal compartment of the ER or of an ER derivative. ATP may be required both for the dislocation step that precedes cytosolic destruction and for proteasomal proteolysis[15,43]. It becomes increasingly clear that many soluble and membrane-bound proteins in yeast[44] and in mammals[45–47] are degraded in the cytosol. Our results suggest that the Sec61 channel may provide the general pathway by which retrograde transport to the cytosol occurs. □

## Methods

**Antibodies and inhibitors.** The following antibodies were used: anti-proteasome (Organonteknika, Oss, The Netherlands); anti-Sec61β[24]; monoclonal antibody W6/32[16]; rabbit anti-heavy chain serum (RaHC)[3]; and rabbit anti-US2 serum (T.R.J. and L. Sun, manuscript submitted). The proteasome inhibitor lactacystin[17] was obtained from E. J. Corey. Carboxybenzyl-leucyl-leucyl-leucinal[48] ($ZL_3H$) was synthesized as described[5]. The synthesis of carboxybenzyl-leucyl-leucyl-leucyl vinylsulfone ($ZL_3VS$), a compound that covalently and specifically modifies the proteasomal β-subunits will be reported elsewhere. Antimycin A, 2-deoxyglucose, and tunicamycin were from Sigma; digintonin was from Calbiochem.

**Pulse–chase experiments.** U373-MG astrocytoma cells used as control cells, US2 transfectants ($US2^+$) and US11 transfectants ($US11^+$)[4,49] were maintained and used for pulse–chase experiments essentially as described[5]. Procedures for preparation of cell lysates using NP-40 lysis mix and immunoprecipitation have been described[5,20].

**Gel electrophoresis.** SDS–polyacrylamide gel electrophoresis (SDS–PAGE), one-dimensional isolectric focusing (IEF) and fluorography were performed as described[50].

**Subcellular fractionation of a pulse-chase experiment of $US2^+$ cells.** A pulse–chase experiment was performed at 37 °C on $US2^+$ cells labelled in the presence of $ZL_3H$ (20 μM). The cells were pulsed with 400 μCi[$^{35}$S]methionine for 1 min and chased for 0, 1, 5, 10, 20 and 40 min. The subcellular fractionation and subsequent immunoprecipitation were done as described[5].

**Pulse–chase experiment including DTT during the chase.** A pulse–chase experiment was done at 37 °C with control and $US2^+$ cells in the presence of $ZL_3H$ (20 μM). The cells were pulsed with 200 μCi of [$^{35}$S]methionine for 10 min and chased for 0, 20, 40 and 120 min. Dithiothreitol (DTT) was added at a final concentration of 2 mM at 7 min into the pulse. Where indicated, DTT was washed out by the addition of 50 ml of culture medium. The cells were lysed in NP-40 lysis mix and class I molecules were immunoprecipitated with RaHC or W6/32. The proteins were separated by SDS–PAGE.

**ATP depletion of $US2^+$ cells.** A pulse-chase experiment was done at 37 °C with $US2^+$ cells in the presence of $ZL_3H$ (20 μM). The cells were pulsed with 50 μCi [$^{35}$]methionine for 1 min. Excess non-radioactive methionine was added simultaneously with ATP-depletion mix[22], comprising 2-deoxyglucose and antimycin A to final concentrations of 22.5 mM and 13.5 μM, respectively. Cells were chased for 0, 1, 4, 10, 20 and 60 min. Alternatively, ATP-depletion mix was added before, during or after a 1-min pulse, followed by a chase, so that all samples were chased for 20 min (Fig. 3*b*). Class I molecules were recovered with either RaHC or W6/32. Proteins were separated by SDS–PAGE.

**Re-immunoprecipitation of class I heavy chains from immune complexes containing proteasomes or US2.** $US2^+$ cells were pulsed for 45 s with 200 μCi of [S]methionine and chased for 0, 1, 2, 7, 20 and 60 min in the presence of $ZL_3H$ (20 μM) at 37 °C. Cells were lysed in a digitonin lysis mix (1% digitonin in 2.5 mM HEPES, pH7.6, 10 mM $CaCl_2$). MHC class I molecules were immunoprecipitated from SDS-dissociated immune complexes prepared in a first round of immunoprecipitation with RaHC, anti-proteasome, or anti-US2 antibodies. Proteins were separated by SDS–PAGE.

**Re-immunoprecipitating class I heavy chains and US2 from Sec61 immune complexes.** A pulse–chase experiment was done at 37 °C with control and $US2^+$ cells in the presence of $ZL_3H$ (20 μM). Cells were pulsed with 100 μCi of [$^{35}$S]methionine for 10 min and chased for 0, 5, 10, 20 and 60 min. To one set of samples, a final concentration of 2 mM DTT was added 7 min into the pulse. Cells were lysed with a digitonin lysis mix and anti-Sec61β was used to immunoprecipitate the Sec61 complex from the cell lysates. The precipitated complexes were dissociated with SDS and suspended in NP-40 lysis mix. Sequential immunoprecipitations with RaHC and anti-US2 were carried out. Heavy chains and US2 molecules were also recovered from Sec61-depleted cell lysates with RaHC or anti-US2. Proteins were separated by SDS–PAGE.

ACKNOWLEDGEMENTS. We thank E. J. Corey for making available lactacystin and K. B. Hendil for anti-proteasome antibody MCP20. We thank ARRCO (Boston) for photographic and art work. This work was supported by the NIH and by Boehringer Ingelheim. E. J. H. J. Wiertz was supported by a Talent Stipendium from the Netherlands Organization for Scientific Research and an EMBO long-term fellowship.

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Received 5 September; accepted 5 November 1996.

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