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# The role of the ubiquitin system in human cytomegalovirus-mediated degradation of MHC class I heavy chains

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# CHAPTER 8 MHC CLASS I HEAVY CHAINS ARE DISLOCATED FROM THE ENDOPLASMIC RETICULUM TO THE CYTOSOL IN A VECTORIAL FASHION, COMMENCING AT THE C-TERMINUS

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*Submitted*

## Abstract

ER-resident proteins that fail to pass ER quality control are destined for degradation by proteasomes in the cytosol. This process requires the retrograde movement or 'dislocation' of proteins from the ER into the cytosol. The dislocation pathway is also used by the human cytomegalovirus (HCMV), which targets the antigen-presenting MHC class I molecules for proteasomal degradation immediately after their synthesis and translocation into the ER. The mechanism of protein transportation from the ER back into the cytosol is poorly understood. In this study, we explored the dislocation reaction in more detail, using the HCMV-mediated degradation of MHC class I heavy chains as a model. Antibody epitopes present within the α1, α2 and α3 domains of the class I molecule were used to monitor the order in which the class I heavy chain domains migrate back into the cytosol. We show that during dislocation of the heavy chains, ER-luminal epitopes close to the transmembrane segment of MHC class I molecules, i.e. within the α3 domain, appear in the cytosol earlier than epitopes located towards the N-terminus (α1 domain). These results indicate that dislocation of a type I membrane protein takes place in a vectorial fashion, from the C-terminus to the N-terminus.

## Introduction

The human cytomegalovirus has adopted the cellular ER quality control system as part of its immune evasion strategy. The HCMV glycoproteins US2 and US11 facilitate the rapid retro-translocation or 'dislocation' of newly synthesized MHC class I molecules from the ER into the cytosol where they are degraded by proteasomes [1,2]. The dislocation pathway is generally used by mammalian cells to degrade misfolded ER proteins [3,4]. The US11-dependent degradation of MHC class I heavy chains serves as a paradigm of protein dislocation and has been instrumental in characterizing several features of this process, including the dependence on a functional ubiquitin system [5,6], the p97 ATPase complex [7-10], the multi-spanning membrane protein Derlin-1 [11,12], and the proteasome [1]. Furthermore, US11-mediated degradation is dependent on luminal ATP [13].

The ubiquitin system fulfills several crucial functions in the process that leads to the degradation of ER proteins. It is required for the initial extraction of MHC class I heavy chains from the ER as well as their ultimate proteasomal degradation [5,6]. Recently, we have found that the lysine residues and the N-terminus of the class I heavy chains are both dispensable as

ubiquitin acceptor sites during the dislocation reaction (Hassink et al., submitted for publication). This finding suggests that an adaptor protein is ubiquitinated, thereby serving as a recognition signal for the recruitment of other components into the dislocation complex. One of these components may be the p97-Npl4-Ufd1 ATPase complex that has been implicated as playing an important role in the extraction process [7-10]. The p97-Npl4-Ufd1 complex has at least three different poly-ubiquitin binding sites, but whether these are absolutely necessary for heavy chain dislocation is unclear at the moment [8,14]. It has been suggested that p97 might drive the release of substrates from the cytosolic side of the membrane into the cytosol [8,9,14]. P97 ATPase activity is required for this process, at least in the presence of proteasome inhibitors. In the absence of proteasome inhibitors, however, a mutant p97 without ATPase activity still can facilitate the degradation of heavy chains, suggesting an additional involvement of the proteasome in the release of proteins from the membrane[14]. Since the proteasome has ATPase activity in its 19S cap [15], this might provide an alternative source of energy for the release in the absence of p97 ATPase activity.

The dislocation channel is ill defined. Co-precipitation of Sec61β chains with dislocated MHC class I molecules in the presence of US2, and studies with Sec61α mutants in yeast, suggest that the Sec61 complex forms part of a dislocation channel [2,16-23]. The Translocon Associated Membrane protein (TRAM) may also form part of this channel, facilitating re-entrance of multispanning membrane proteins into the 'dislocon' [24]. The translocon has a diameter of only 40-60 Å [25], which corresponds to the predicted size of several dislocation substrates. For example, MHC class I molecules with an N-terminally fused GFP, which has a cylinder-shaped structure with a length of 42 Å and a diameter of 24 Å [26], can be detected fluorescently active in cytosol in the presence of US2, suggesting that it is dislocated in a folded state [27]. Furthermore, fusion of a dihydrofolate reductase (DHFR) domain to the N-terminus of MHC class I does not inhibit dislocation in the presence of metotrexate [28], which induces rigid folding in the DHFR domain resulting in a structure with an estimated diameter of at least 35 Å. Substrates with a size exceeding the diameter of the dislocation channel may have to be unfolded by ER-resident chaperones prior to their dislocation. Alternatively, additional components may be recruited into the dislocation conduit, such as the ubiquitin ligases HRD1, gp78 and TEB4, all multispanning membrane proteins occurring in the ER (reviewed in[29-31]). In addition, Derlin-1 has been proposed as a possible constituent of the dislocon. This multi-spanning ER membrane protein is essential for US11-dependent dislocation of MHC class I heavy chains[11,12]. Derlin-1 can be detected in a complex with US11, p97, HERP and the ER-associated ubiquitin ligase HRD1 [11,12,32-34].

In this paper we explored the dislocation behavior of MHC class I molecules in the presence of US11, using two novel strategies. First, HA-tagged MHC class I molecules were expressed simultaneously with US11 in hybridoma cells that produce antibodies directed against the HA-tag. Contrary to our expectations, the formation of antibody-heavy chain complexes in the ER did not stall heavy chain dislocation. At the same time, the co-expression of HA-tagged class I heavy chains and the HA-specific antibodies resulted in increased degradation of the IgG heavy chains. The experiments suggest that the ER can facilitate the dislocation of large oligomerized complexes. In the second strategy, US11-mediated dislocation of MHC class I heavy chains was monitored in permeabilized cells, using antibodies that recognize epitopes within the α1, α2 and α3 domains of the class I heavy chains. The α-3 domain of the heavy chain appeared in the cytosol earlier than the α-1 domain, indicating that the class I heavy chains were dislocated starting at their cytosolic tail.

## Results

### ER luminal anti-HA antibodies interact with MHC class I heavy chains containing an ER-luminal HA tag

To evaluate the topology of the retrograde movement of MHC class I heavy chains, it would be advantageous if the heavy chains could be halted on their way to the cytosol. We investigated if the dislocating heavy chains could be stopped in the dislocation channel by the interaction with antibodies directed against determinants present within the class I heavy chains. To this purpose we made use of the 12CA5 hybridoma, producing an antibody directed against the epitope YPYDVPDYA of the haemaglutinin (HA) protein of influenza virus [35]. MHC class I heavy chains carrying the HA-tag were expressed in the 12CA5 cells (Figure 1A). The epitope tag was placed in the ER luminal domain between the transmembrane region and the α3 domain (HLA-275-HA), between the α2 and α3 domains (HLA M-HA), or near the N-terminus (HLA N-HA) (Figure 1B). It has been shown by Pelkmans et al. that antibodies can be employed to study the folding of haemaglutinin complexes within the ER [36]. Furthermore, Jakes et al. have shown that antibody-antigen interactions are strong enough to prevent rearrangement of hydrophilic protein segments from the *cis*- to the *trans*-side of artificial lipid membranes [37]. Likewise, the binding of antibodies to MHC class I heavy chains within the ER may cause the formation of a protein complex that is no longer dislocated to the cytosol.

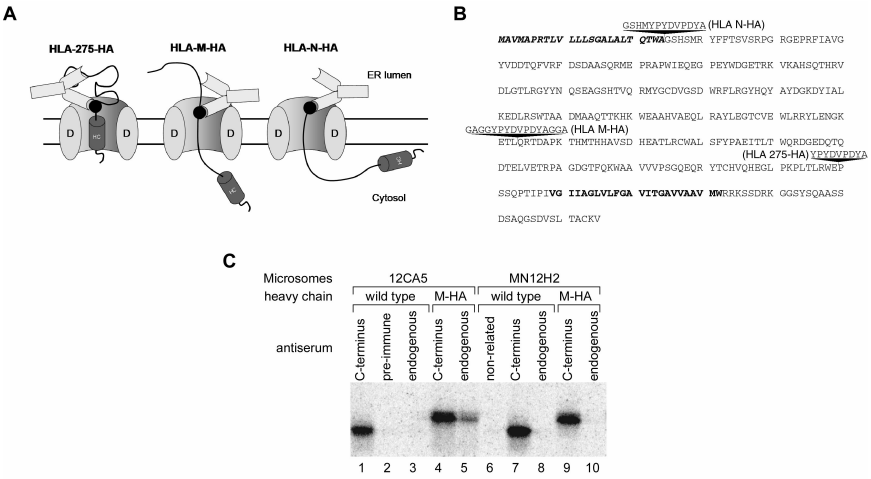


**Figure 1. Binding of anti-HA antibodies to HA-tagged MHC class I heavy chains in the ER of 12CA5 hybridoma cells.**
(A) Model of dislocating MHC class I heavy chains carrying HA-tags at different positions (closed circles). Binding of 12CA5-produced anti-HA antibodies is indicated. (D), Dislocation channel. (B) Amino acid sequence of the HLA A*0201 heavy chain with locations of the inserted HA epitope indicated. Three constructs were generated: HLA N-HA, HLA M-HA and HLA 275-HA. The signal sequence is shown in italic bold face, and the transmembrane region in bold face. (C) The HLA wt protein or the HLA M-HA construct were translated *in vitro* in the presence of microsomes derived from the hybridoma cell lines 12CA5 and MN12H2. Microsomes were lysed and radio-labeled class I heavy chains were immunoprecipitated using antibodies endogenously present in the microsomes, or through exogenously added antibodies directed against the MHC class I C-terminus.

To confirm that endogenous antibodies can indeed bind to the epitope-tagged MHC class I heavy chains within the lumen of the ER, non-tagged or HA-tagged MHC class I heavy chains were translated *in vitro* in the presence of microsomes from 12CA5 cells. MN12H2 hybridoma cells were used as a control. The MN12H2 cells express an antibody that recognizes the epitope TKDTNNNL of the *Neisseria meningitidis* type 1 outer membrane protein PorA [38]. After translation, the microsomes were lysed and BSA-coated protein-A and -G beads were added to the lysates to precipitate the endogenous antibodies. Alternatively, an antibody directed against the C-terminus of the class I heavy chains was added to the lysates. The results are shown in Figure 1C. The non-tagged, wild-type MHC class I heavy chains could be precipitated using the exogenously added C-terminus-specific antiserum, but not with the pre-immune serum or the anti-HA antibodies endogenously present in the 12CA5 cells (lanes 1-3). The HA-tagged class I heavy chains, on the other hand, could be precipitated with the anti-HA antibodies present within the 12CA5 microsomes (lane 5). As expected, the

mobility of the HA-tagged class I heavy chains is retarded slightly in the SDS-PAGE. As a control, parallel experiments were performed with microsomes derived from the MN12H2 hybridoma. Whereas wild-type and HA-tagged class I heavy chains could be retrieved efficiently with the exogenously added antibody directed against the C-terminus of the MHC class I heavy chains (lanes 7 and 9), the endogenous MN12H2 antibody could not precipitate the class I heavy chains (lanes 8 and 10). These data indicate that microsomes derived from 12CA5 hybridoma cells contain functional antibodies that are able to interact with newly synthesized, HA-tagged MHC class I heavy chains.

**Interaction of endogenous antibodies with epitope-tagged MHC class I heavy chains in the ER does not prohibit their dislocation**

Next, attempts were undertaken to prepare 12CA5 hybridoma cells stably expressing wild-type or HA-tagged MHC class I heavy chains. Of the 12 clones obtained by limited dilution of cells transfected with HA-tagged MHC class I heavy chains, only 4 showed expression of the HA-tagged class I heavy chains as examined by western blotting. This ratio was 5 out of 6 for cells transfected with the wild-type MHC class I heavy chains. Surprisingly, although all the clones obtained produced antibodies, only the antibodies derived from cells transfected with the wild-type heavy chains were able to recognize HA-tagged MHC class I heavy chains. Apparently, the immunoglobulins from the clones expressing the HA-tagged MHC class I heavy chains had an altered antigen recognition motif that could no longer recognize the HA-epitope. This suggests that the HA-antibodies did indeed interact with the HA-tagged MHC class I molecules but that this interaction was toxic for the hybridoma cells.

One of the reasons why we did not obtain any clones that were capable of simultaneously producing antibodies and tagged proteins might be that the antibody-protein complexes are halted in the ER and cannot be released into the cytosol. This would suggest that complexes have to be dissociated before dislocation can commence. To investigate this, we transiently transfected the constructs encoding HLA wt, HLA 275-HA, HLA M-HA, or HLA N-HA in 12CA5 hybridoma cells. To induce dislocation of the MHC class I heavy chains, these same constructs were also transfected into 12CA5 cells expressing US11. Pulse-chase experiments were performed to evaluate the stability of the class I heavy chains. Cells were collected after a pulse of 10 min (time point 0) and after 30 or 120 min of chase. The wild-type class I heavy chains were immunoprecipitated from cell lysates using the MHC class I-specific antibody MR24. MHC class I heavy chains carying an HA-tag were isolated using beads only, via the antibodies produced by the hybridoma cells. The results shown in Fig. 2A indicate that all the MHC class I variants were

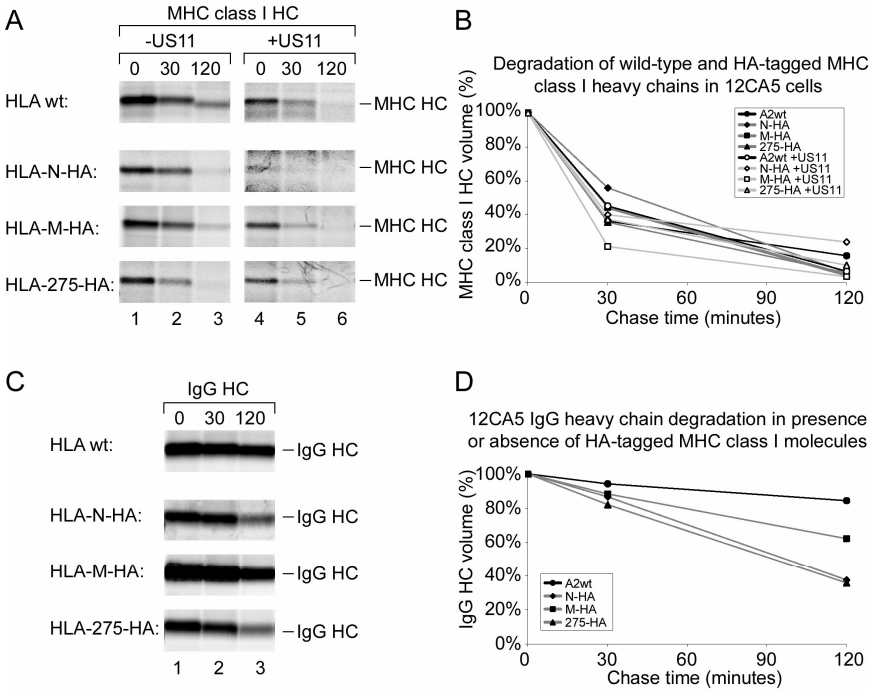


**Figure 2. MHC class I and IgG heavy chain degradation in 12CA5 wild-type and 12CA5-US11 cells transiently expressing wild-type or HA-tagged MHC class I heavy chains.**
(A) 12CA5 cells transiently expressing wild-type or HA-tagged MHC class I heavy chains were pulsed for 10 minutes and chased for the times indicated. The cells were lysed in NP40 lysis mix and subjected to immunoprecipitations with MR24 anti-heavy chain serum (HLA wild type-expressing cells) or with ProteinG-Sepharose beads alone (HLA HA-tag transfectants). (B) Quantification of the data shown in **A**. The degradation of the MHC class I heavy chains was expressed relative to the amount present at time point 0. (C) IgG heavy chains precipitated from the 12CA5 hybridoma cells transfected with wild type or HA-tagged HLA molecules. The anti-HA IgG heavy chains were isolated from the samples shown in **A,** left column, using Protein-G Sepharose beads. (D) Quantification of the data shown in **C.** The degradation of the anti-HA IgG heavy chains was expressed relative to the amount present at time point 0.

dislocated, regardless of the presence of HA-specific antibodies, even in the absence of US11 (compare lanes 1-3 and 4-6). Quantification of the pulse-chase data showed that the HA-tagged MHC class I heavy chains were degraded with the same kinetics as wild-type MHC class I molecules (Figure 2B). This suggests that MHC class I heavy chains with ER luminal HA-tags are dislocated despite the presence within the ER of antibodies that recognize these tags.

If tagged MHC class I heavy chains are dislocated while they are associated with IgG antibodies, these antibodies should also end up in the cytosol and be degraded. Analyses of IgG heavy chain stability revealed that

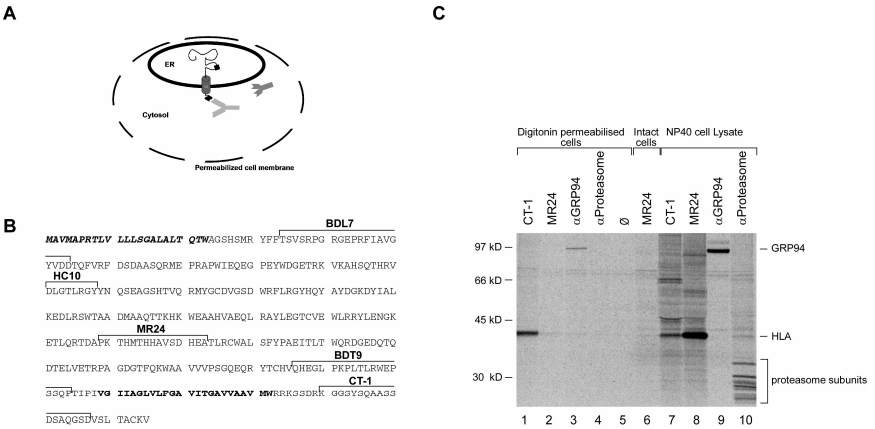


**Figure 3. Detection of cytosol-exposed epitopes of ER-resident proteins in permeabilized cells.** (A) U373 cells were incubated with digitonin at 4°C to permeabilize the cell membrane but not the ER membrane. Antibodies can enter the cell, but not the ER, and bind to epitopes exposed to the cytosol. (B) The epitopes recognized by the different anti-HLA antibodies are indicated in the amino acid sequence of the HLA A*0201 heavy chain. The signal sequence is shown in italic bold face; the transmembrae region in bold face. (C) U373 cells were labeled with $^{35}$S-methionine/cysteine for 10 minutes, permeabilized using digitonin (lanes 1-5), and incubated with antibodies directed against the cytoplasmic tail (CT-1) or the ER luminal/extracellular domain of MHC class I heavy chains (MR24). As a control, anti-GRP94 and anti-proteasome antibodies (mixture of MCP20 and MCP21) were used. Incubation of non-permeabilized cells with the MR24 antiserum is shown in lane 6. An aliquot of the cells used in lanes 1-5 was lysed using NP40 lysis-mix and subjected to immunoprecipitation with the antibodies indicated (lanes 7-10). The immunoprecipitates were dissolved in SDS/β-mercaptoethanol sample buffer and separated on SDS-PAGE.

degradation of IgG heavy chains was increased in the presence of HA-tagged MHC class I heavy chains (Fig. 2C and D). This suggests that IgG antibodies of cells that express HA-tagged MHC class I heavy chains do indeed interact with these class I heavy chains. The 12CA5 IgG and the HA-tagged MHC class I heavy chains may be dislocated in a complex.

### Immunological detection of cytosol-exposed ER luminal epitopes in digitonin permeabilized cells

A permeabilized cell system was used as an alternative approach to investigate the orientation of dislocating MHC class I heavy chains. During the migration of the heavy chains into the cytosol, epitopes within the class I molecules will become available in the cytosol in a particular order. This can be monitored using antibodies that recognize defined epitopes within the class I heavy chains. In addition, the single N-linked glycan can serve as a beacon: as soon as it is exposed to the cytosol, it will be removed by N-glycanase.

The experiment has been depicted schematically in Fig. 3A. After metabolic labeling, the cells were permeabilized with digitonin at 4 °C for a

short period of time. In this way, the ER membrane stays intact [39]. Subsequently, the cells were incubated with antibodies directed against distinct epitopes within the MHC class I heavy chains. The positions of the epitopes is indicated in Fig. 3B. The excess antibodies were washed out, together with the fully dislocated MHC class I heavy chains. As a result, the residual antibodies could now bind to either cell surface-expressed MHC class I molecules or cytosol-exposed domains of class I heavy chains associated with intracellular organelles. Due to the short labeling time (10 min), however, only the ER-resident class I molecules were anticipated to yield a signal.

First, the integrity of the ER membrane was evaluated in digitonin-permeabilized cells (Fig. 3C). An antibody specific for the C-terminus of the MHC class I heavy chains was capable of precipitating the heavy chains, indicating that the antibodies were able to penetrate permeabilized cells (Fig. 3C, lane 1). Antibodies that recognize a luminal/extracellular epitope within the MHC class I heavy chains, could only marginally detect heavy chains in permeabilized cells, indicating that cell membrane-associated class I molecules had not acquired radioactivity within the short labeling period of 10 minutes (lane 2, MR24). The MR24 antiserum did precipitate the class I heavy chains extremely well from an NP40 lysate (compare lanes 2 and 8). The product precipitated was endoglycosidase-H sensitive, which is indicative of ER localization (data not shown). Antibodies directed against the ER-luminal GRP94 also bound only marginally to labeled GRP94 in digitonin-permeabilized cells (Fig. 3C, compare lanes 3 and 9). These results indicated that the ER membranes remained intact in the permeabilized cells. The fact that proteasomes could not be detected in permeabilized cells but could be precipitated from an NP40 lysate (compare lanes 4 and 10) indicated that most of the cytosol was indeed washed out of the cells during the permeabilization procedure. In conclusion, the permeabilized cell system allows the selective detection of cytosol-exposed epitopes within ER membrane proteins.

**The α3 domains of the MHC class I heavy chains appear in the cytosol prior to the α1 domains during US11-mediated dislocation**

Permeabilized US11-expressing cells were used to evaluate the accessibility to antibodies of ER-luminal domains of the class I heavy chains in the course of their dislocation to the cytosol. The positions of the epitopes recognized by the antibodies used have been indicated in Fig. 3B. In NP40 lysates, equivalent amounts of the intact, glycosylated (+CHO) and deglycosylated (-CHO) MHC class I heavy chains were retrieved using all four antibodies (Fig. 4A, upper panel). In the digitonin-permeabilized cells, the antibodies against the membrane-proximal epitope BDT9 and the α3-specific

MR24 precipitated the glycosylated MHC class I heavy chain, in addition to the deglycosylated form (Fig. 4A, middle panel, lanes 1 and 2). The antibodies directed against epitopes located towards the N-terminus of the class I heavy chains (HC10 and BDL7) predominantly recognized deglycosylated class I molecules (Fig. 4A, middle panel, lanes 3 and 4). In the US11-expressing cells some deglycosylated class I heavy chains were apparently attached to the outside of the ER membranes. In cells not expressing US11, hardly any class I heavy chains could be precipitated using the same antibodies, indicating that the integrity of the ER membrane was maintained (Figure 4A, lower panel). To evaluate whether equivalent numbers of cells were used, NP40-lysates and digitonin-permeabilized cells were incubated with antibodies against the transferrin receptor (TfR). Comparable amounts of TfR were detected in each of the samples (Fig. 4B). In conclusion, these data show that glycosylated class I heavy chains are selectively isolated from the ER of permeabilized cells using antibodies directed against epitopes located within the α3 domain of the class I heavy chains. The fact that the N-linked glycans are still present on these heavy chains indicates that they are still located in the ER lumen. In contrast, the antibodies directed against epitopes located within the α1 domain of the heavy chains failed to retrieve the glycolysated forms of the class I molecules, suggesting that dislocation of the heavy chains commences at their C-terminus.

The finding that deglycosylated class I heavy chains could be retrieved from permeabilized cells suggested that the heavy chains were still attached to the ER membrane. To investigate whether these deglycosylated species were indeed located on the exterior of the ER membrane, permeabilized cells were subjected to proteinase K digestion. The antibody CT-1, directed against the cytoplasmic tail of the heavy chains, precipitated the heavy chains from untreated, but not from proteinase K-treated cells, indicating that the cytoplasmic tails were removed from the glycosylated MHC class I molecules (Figure 4C, compare lanes 1 and 3). With the BDT9 antibody, directed against the ER luminal domain of the class I heavy chains, MHC class I heavy chains were detected in untreated and proteinase-K digested samples (lanes 5 and 7). Precipitation with the BDT9 antibody also yielded deglycosylated class I heavy chains (lane 5). Immunoprecipitations with this antibody on proteinase K-treated permeabilized cells yielded tail-less, glycosylated class I heavy chains (lane 7), but not tail-less deglycosylated heavy chains (as present in endoglycosidase-F treated samples, lane 8), indicating that the deglycosylated MHC class I molecules are *de facto* outside the ER.

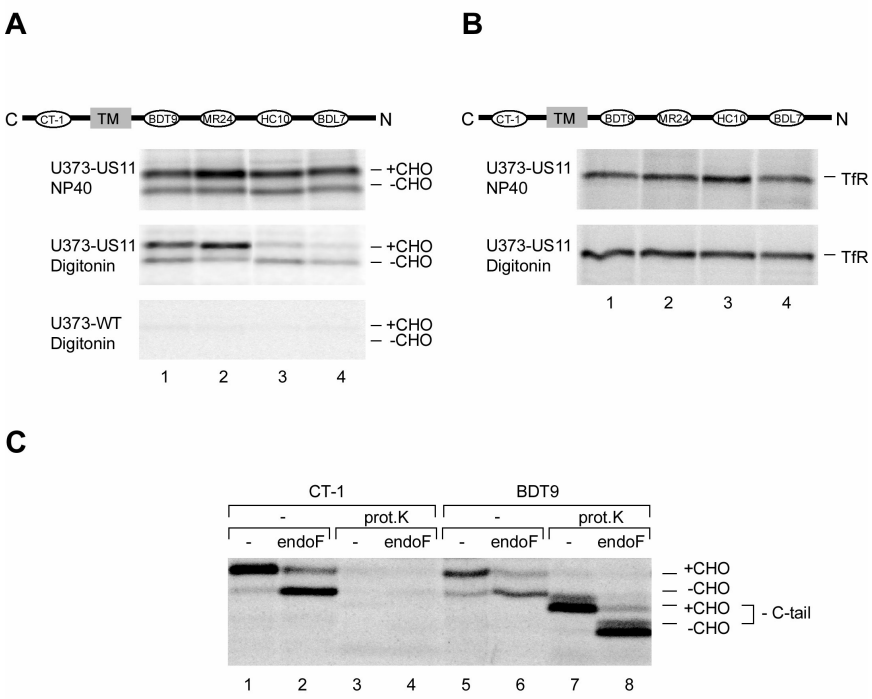


**Figure 4. Detection of cytosol-exposed epitopes of ER-resident MHC class I heavy chains during dislocation in U373-US11 cells.**
(A) U373-US11 and U373 wild-type cells were labeled with $^{35}$S-methionine/cysteine mix for 10 minutes in the presence of proteasome inhibitor ($ZL_3H$). One aliquot of the cells was lysed in the presence of NP40. Another aliquot was permeabilized using digitonin. MHC class I heavy chains were immunoprecipitated from the NP40 lysates using the antibodies indicated in the schematic representation of the MHC class I heavy chain. The digitonin-permeabilized cells were washed extensively in permeabilization buffer and incubated with the antibodies indicated. After this incubation, the digitonin-permeabilized cells were washed again thoroughly. The cells were then lysed in NP40 lysis mix and incubated with Protein-A and -G Sepharose beads. The immunoprecipitates were dissolved in SDS/βME sample buffer and separated on SDS-PAGE. (B) In parallel with the experiment shown in Fig. A, the NP40 lysates and the digitonin-permeabilized cells were incubated with antibodies directed against the cytoplasmic tail of the transferrin receptor (TfR). Immunoprecipitations were performed as in **A**. (C) U373-US11 cells were digitonin-permeabilized and either mock-treated or incubated with proteinase K (prot. K) for 15 minutes. To inactivate the proteinase K, the cells were lysed in NP40 lysis-mix, supplemented with 1% SDS, at 95°C for 5 minutes. The samples were diluted 5 times in standard NP40 lysis mix and incubated with the antibodies indicated. An aliquot of each sample was digested with endoglycosydase F (endoF). The proteins were displayed using SDS-PAGE and BioRad PhosporImaging equipment.

## Discussion

Several studies suggest that US11-dependent dislocation occurs from the N-terminus to the C-terminus, based on the premise that ubiquitination of

the ER-luminal domains of the heavy chain is required for its dislocation. The lysines within the cytosolic tail are not required for heavy chain dislocation [6,12]. Consequently, it is assumed that lysines in the luminal domain of the heavy chain are involved. Extraction via the N-terminus is, however, more complicated than C-terminal extraction, as the N-terminus has to be re-inserted into the ER membrane. The molecule will have to bend and probably even unfold to accomplish this. We have recently demonstrated that none of the lysines or the N-terminus of the heavy chains have to be ubiquitinated in order to accomplish US11-dependent dislocation (Hassink et al., submitted for publication). We favor the hypothesis that extraction of the heavy chains starts from the cytosolic C-terminus. In the present study we show that epitopes close to the transmembrane domains of the dislocating heavy chain become exposed to the cytosol when the heavy chain is still associated with the ER membrane and has not been deglycosylated by the cytosolic N-glycanase. Epitopes located further towards the N-terminus are only available after the heavy chain has been released into the cytosol and has lost its single N-linked glycan. These results indicate that extraction of MHC class I heavy chains from the ER membrane most likely starts at the C-terminus.

Antibodies added to digitonin-permeabilized cells interacted with deglycosylated heavy chains, suggesting that a proportion of the dislocating heavy chains remained attached to the ER membrane after partial dislocation. By adding proteinase K to the permeabilized cells, we were able to show that the deglycosylated heavy chains were completely protease-sensitive. This suggests that, in the course of the dislocation reaction, deglycosylated heavy chains are situated outside the ER, but remain associated with the ER membrane. Attachment of dislocating heavy chains to the outside of the ER membrane has also been observed in the context of US2 [40]. In addition, dislocating IgM heavy chains [8] and ribophorin I [41] have been found in association with the ER. Observations by Lilley et al. and ourselves [11](Hassink et al., manuscript submitted for publication) suggest that either US11 or Derlin-1 bind to class I heavy chains. As US11 and Derlin-1 are membrane proteins and do not dislocate in a complex with MHC class I heavy chains, we suggest that deglycosylated heavy chains remain ER membrane-associated by interacting with either US11/Derlin-1 or VIMP. Alternatively, deglycosylated MHC class I heavy chains may associate with molecules that form part of the dislocation channel. The finding in our experiments that the heavy chains remain attached to the ER membrane may be related to the fact that the P97 complex and the proteasomes in the permeabilized cells have been depleted. These complexes have been implicated in the extraction of proteins from the ER membrane [7,8,10].

MHC class I heavy chains may be dislocated while folded, as suggested

by experiments with N-terminally fused GFP and DHFR [27,28]. Our data indicate that hybridoma cells, producing antibodies against HA-tagged MHC class I heavy chains, were still able to facilitate the dislocation of these heavy chains, despite the possibility that antibody-antigen complexes can be formed within the ER lumen. Our observations that ER-resident antibodies can bind to HA-tagged MHC class I heavy chains *in vitro* and our inability to obtain stable transfectants of 12CA5 hybridoma cells expressing HA-tagged MHC class I heavy chains, strongly suggest that antibody-heavy chain complexes are indeed formed. In addition, we observed an increase in IgG degradation in cells expressing epitope-tagged MHC class I molecules as compared to cells expressing wild-type heavy chains. This suggests that a fraction of IgG heavy chains is degraded when HA-tagged substrates are expressed. Possibly, the MHC class I molecules are dislocated into the cytosol while associated to IgG antibodies.

As folded MHC class I molecules have a minimum diameter of 50 Å and an Ig light chain-heavy chain heterodimer already has a diameter of 50 Å, a complex of IgG and MHC class I molecules must be larger than 60 Å. This would rule out the possibility of the Sec61 subunits acting as a dislocon for MHC class I molecules by themselves [25]. A flexible composition of the dislocon would be most suitable for transporting large folded substrates from the ER lumen to the cytosol [42-44]. Therefore, the hypothesis of a dislocon that assembles around a substrate may be viable [45,46].

For signal gated twin arginine translocons, used by peroxisomes and plant thylakoid membranes, it has been suggested that they assemble at the site of translocation around the folded substrate, thereby maintaining the permeability barrier [45,46]. Likewise, it is imaginable that the transmembrane regions of Sec61α and β, Derlin-1, and HRD1 found associated to MHC class I heavy chains in U373-US2 and US11 cells form a conduit around the class I heavy chain[11,12](Hassink et al., manuscript submitted for publication). The advantage of forming a dislocon around the substrate to be extracted from the ER is that it will be adapted to the dimensions of the substrate. This can explain the observed dislocation of large, folded proteins such as GFP- and DHFR-MHC class I heavy chain fusion proteins [27,28]. *Ad hoc* formation of dislocation channels may also allow easy embrace of misfolded proteins carrying multiple transmembrane domains, for instance CFTR mutants [47,48] and apolipoprotein B100 [49,50].

Looking at other translocation mechanisms, it is obvious that there is a lot of variation in the mechanisms used by the different organelles within cells to translocate proteins from one side of the membrane to the other (reviewed in [45]). Whereas the signal- gated Sec61 channel uses vectorial transport of newly synthesized amino acid chains, mitochondria first have to unfold

proteins destined for transport over the membrane [51]. Proteins transported to the nucleus and some of the proteins targeted to peroxisomes are translocated while folded or oligomerized [45,46]. With respect to protein dislocation from the ER, a universal dislocon would either have to be of high plasticity or very flexible regarding its composition to accommodate a range of differently shaped and sized substrates.

## Materials & Methods

#### Reagents and cells

Peptide-N-glycosidase H and F were obtained from Roche Diagnostics (Mannheim, Germany). Proteinase K was procured from Life Technologies. Digitonin was obtained from Calbiochem and Protein A- and G-Sepharose were purchased from Amersham Biosciences. The proteasome inhibitor carboxybenzyl-leucyl-leucyl-leucinal ($ZL_3H$) was obtained from the Peptide Institute (Osaka, Japan) and used at a final concentration of 20 μM. U373 wild type and U373 US11 cells [1,2] and the MN12H2 hybridoma cells have been described [38].

Polyclonal antisera against the C-terminus (CT-1), the membrane proximal region (BDT9), the α3 domain (MR24), and the N-terminus (BDL7) of human leucocyte antigen subtype A2 (HLA-A2) were produced in rabbits as described [5], using the synthetic peptides KGGSYSQAASSDSAQGSD, QHEGLPKPLTLRWEPSSQP, PKTHMTHHAVSDHEA, and TSVSRPGRGEPRFIAVGYVDDT, respectively. The HC10 antibody has been described elsewhere and recognizes the amino acid sequence DLGTLRGY, located at the end of the α1 domain [52]. The antibody H68.4 recognizing the transferrin receptor was purchased from Zymed Laboratories (San Francisco). The antibody directed against GRP94 (C-19) was obtained from Santa Cruz. The anti-proteasome antibodies MCP20 and MCP21 were kindly donated by Dr. K. Hendil (Copenhagen, Denmark).

#### DNA constructs

Plasmids encoding wild-type MHC class I heavy chains (HLA wt) and MHC class I heavy chains with an HA-tag near the N-terminus (HLA N-HA), between the α2 and α3 domains (HLA M-HA), between the transmembrane and the α3 domain (HLA 275-HA) or at the extreme C-terminus (HLA C-HA) (See Figure 3B for AA sequences) were constructed using as a template cDNA of HLA A *0201 in pCDNA3.1 (pLUMC9901), described elsewhere [5]. HLA N-HA and HLA 275-HA were constructed by fusion PCR. For HLA M-HA and HLA C-HA, SfiI-EcoRI-SfiI cassettes were inserted, after which the HA-tags were inserted via ligation using pre-annealed oligonucleotides

with 3' overhangs encoding the HA-tag.

#### Epitope availability assay and proteinase K digestions

For the epitope availability assay, $5x10^6$ U373 cells were labeled for 10 minutes with 125 μCi of $^{35}$S-labelled Redivue Promix (a mixture of l-[$^{35}$S]methionine and l-[$^{35}$S] cysteine; Amersham) in the presence of proteasome inhibitor. Cells were washed in PBS at 4°C, centrifuged for 1 min at 2000*g* in an Eppendorf centrifuge, and resuspended in 200 μl permeabilization buffer (25 mM HEPES pH7.2, 115 mM KAc, 5 mM NaAc, 2.5 mM $MgCl_2$, 0.5 mM EGTA) supplemented with 400 μg/ml digitonin. The cells were incubated for 15 min at 4 °C. Subsequently, the cells were washed twice, spun at 2000 *g* for 1 min, and resuspended in 200 μl permeabilization buffer. Digitonin-permeabilized cells were then incubated with antibodies for 2 hrs in permeabilization buffer, after which time excess of antibodies was removed by washing twice. The final centrifugation step was performed at 14,000 g for 15 minutes. Next, the pellets were resuspended in NP40 lysis mix and postnuclear supernatants were prepared [5]. Lysates were incubated with BSA-coated protein A and G beads, or BSA coated beads and anti-Transferrin receptor antibody (TfR) when indicated. Heavy chains were precipitated from untreated NP40-lysed cells using the same amount of antibody per sample as was used for the digitonin permeabilized cells.

For proteinase K digestions, $10x10^6$ cells were permeabilized using digitonin as described above, but instead of the two hour antibody incubation, the cells were incubated with 100 μg/ml proteinase K (LifeTechnologies) in 400 μl permeabilization buffer for 15 min. After washing, the cells were lysed for 20 min in 400 μl NP40 lysis-mix also containing 1% SDS. The samples were boiled for 10 minutes at 95 °C while vortexing regularly during the boiling. To shear the DNA and prepare the samples for immunoprecipitation they were passed 5 times through a 25G needle and diluted 5 times in NP40 lysis-mix.

#### Preparation of permeabilized cells for in vitro translations

The protocol for the preparation of permeabilized cells for *in vitro* translations was adapted from Wilson et al. 1995 [53]. $8x10^6$ 12CA5 or MN12H2 cells were permeabilized by incubation in 4 ml KMH buffer (110 mM KAc, 2 mM MgAc and 20 HEPES-KOH pH 7.2), supplemented with 20-40 μg/ml digitonin, on ice for 5 min. The digitonin was then diluted by adding 10 ml KMH. The cells were subsequently centrifuged for 3 minutes at 1000 *g* at 4°C and resuspended gently in 5 ml Hepes-KAc buffer (50 mM KAc, 90 mM Hepes-KOH pH 7.2) and incubated on ice for 10 minutes. Next, the cells were centrifuged again, resuspended in 1 ml KMH, and

transferred to a 1,5 ml Eppendorf tube. Cells were then centrifuged for 5-10 seconds at 13,000 *g*. The cells were subsequently resuspended in 100 µl KMH and incubated with 1 µl of 0.1 M $CaCl_2$ and 1 µl micrococcal nuclease at room temperature for 12 min, after which 1 µl 0.4 M EGTA was added. The cells were centrifuged for 5-10 seconds at 13,000 *g*. Finally, the digitonin-permeabilized cells were resuspended in KMH at $10^5$ cells/3-5 µl.

#### In vitro translation assay

For *in vitro* transcription and translation reactions, HLA wt or HLA M-HA plasmids were linearized with XhoI, and used for *in vitro* transcription with T7 polymerase (Promega). 1 µl of capped mRNA transcripts were translated in the presence of 1 µl 15 mCi/ml Redivue L-[35S]methionine (Amersham), 17.5 µl rabbit reticulocyte lysate, 0.5 µl 19 amino acid mix (minus methionine), and 4 µl of digitonin-permeabilized 12CA5 or MN12H2 cells (prepared as described above). The reaction mixtures were incubated at 30°C for 30 minutes. Microsomes and supernatant were separated by centrifugation at 14,000 *g* at 4°C for 15 minutes. The pellets were washed twice with KMH buffer and 1mM $CaCl_2$ prior to lysis in NP40 lysis mix.

#### Transfections

Hybridoma cells were transiently transfected using electroporation [54]. The cells were washed in a buffer containing 2 mM HEPES pH 7.6, 15 mM $K_2HPO_4/KH_2PO_4$ pH 7.2, and 1mM $MgCl_2$, supplemented with 250 mM mannitol. Five µg of purified plasmid DNA of the appropriate constructs was added to a pellet of $2x10^6$ cells. The cells were resuspended in 350 µl of buffer containing175 mM mannitol at 37 degrees for 3 min. The cells were transferred to a 2 mm electroporation cuvette (Biorad) and electroporated using a Genepulser II with RF module (Biorad) at 190 V, 100% modulation, amplitude of 140 V, 40 kHz, 1.5 msec burst duration, for 15 times. The cells were allowed to recover for 10 minutes before being transferred to normal growth medium. The cells were expanded by growing on a selection medium containing G418.

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