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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 6 UBIQUITINATION OF MHC CLASS I
HEAVY CHAINS IS ESSENTIAL FOR
DISLOCATION BY HUMAN
CYTOMEGALOVIRUS-ENCODED US2
BUT NOT BY US11**

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Submitted

Abstract

The HCMV-encoded glycoproteins US2 and US11 target newly synthesized MHC class I heavy chains for degradation by mediating their dislocation from the ER back into the cytosol, where they are degraded by proteasomes. A functional ubiquitin system is required for US2- and US11-dependent dislocation of the class I heavy chains. It has been assumed that the class I heavy chain itself is ubiquitinated during the dislocation reaction. To test this hypothesis, all lysines within the class I heavy chain were substituted. The lysine-less class I molecules could no longer be dislocated by US2, despite the fact that the interaction between the two proteins was maintained. Interestingly, US11 was still capable of dislocating the lysine-less heavy chains into the cytosol. Ubiquitination does not necessarily require lysine residues, but can also occur at a protein's N-terminus. To investigate the potential role of N-terminal ubiquitination in heavy chain dislocation, a lysine-less ubiquitin moiety was fused to the N-terminus of the class I molecule. This lysine-less fusion protein was still dislocated in the presence of US11. Ubiquitination could not be detected *in vitro*, either for the lysine-less heavy chains, or for the lysine-less ubiquitin-heavy chain fusion protein.

Our data show that, even though dislocation of the lysine-less class I heavy chains requires a functional ubiquitin system, the heavy chain itself does not serve as the ubiquitin acceptor. This finding sheds new light on the role of the ubiquitin system in the dislocation process.

Introduction

A key step in the mammalian defense against viral infections is the presentation of virus-encoded peptides by MHC class I molecules to cytotoxic T-cells. The peptides presented by the class I molecules are frequently derived from newly synthesized proteins, degraded by proteasomes^{1,2}. In case of a virus infection, a proportion of the peptides will be of viral origin. This system allows efficient detection and elimination of the infected cells by cytotoxic T cells. HCMV encodes two glycoproteins, US2 and US11, which cause rapid degradation of newly synthesized MHC class I heavy chains by mediating their retrograde transport or 'dislocation' from the ER into the cytosol, where they are degraded by proteasomes^{3,4}. In this way, HCMV eludes the surveying cytotoxic T-cells.

The disposal of ER proteins via dislocation into the cytosol, followed by proteasomal degradation, is a common feature of all eukaryotic cells. The

US2- and US11-mediated degradation of MHC class I molecules has been instrumental in elucidating several mechanistic aspects of this pathway³⁻¹⁶. A wide variety of ER-luminal proteins are degraded through dislocation to the cytosol. They include mutated and/or misfolded proteins such as CFTR Δ F508 and CPY*, oxidized proteins, and non-complexed, redundant subunits of multimeric complexes like TCR α chains (reviewed in¹⁷⁻¹⁹, MHC class I heavy chains²⁰, and MHC class II α and β chains^{21,22}). In addition, the expression of various proteins is regulated through dislocation and proteasomal degradation, e.g. HMG-CoA reductase (HMGR) and apolipoprotein B100 (reviewed in¹⁷).

How exactly ER proteins are exported to the cytosol is still poorly understood. It has been suggested that misfolded proteins are transported to the cytosol via the same channel through which they entered the ER, the Sec61 translocation complex^{3,23-29}. Other potential constituents of a 'dislocon' are Derlin-1-3 and multi-membrane spanning E3 ligases such as HRD1, GP78, and TEB4^{16,30-33}. In the cytosol, glycoproteins are stripped of their N-linked glycans by an N-glycanase³⁴⁻³⁷ and subsequently degraded by the proteasome in a ubiquitin-dependent manner.

A functional ubiquitin system is not only essential for the final proteasomal degradation, it has also been shown to be indispensable for the initial dislocation of ER proteins^{7,29,38-46}. Dislocation of CPY* and HMGR, for example, depends on the ubiquitin ligase Hrd1p^{29,41-43}. The mammalian ortholog of Hrd1p, HRD1, facilitates the dislocation of CD3 δ and TCR α ⁴⁵. Furthermore, the US11-dependent dislocation of class I heavy chains^{7,44}, the dislocation of TCR α ^{38,40}, as well as the dislocation of the μ s chain of IgM⁴⁶ and IR₃₂₂³⁹ all depend on a functional ubiquitin system.

Initially, lysine residues within the dislocation substrate were considered to be the main target for ubiquitination, but it is becoming increasingly clear that this is not necessarily true in all cases. Replacement of all the lysines within the TCR α chain, or substitution of the lysines within the cytoplasmic tail of the MHC class I heavy chain by arginines does not affect the dislocation of these proteins^{13,38,47}. The lysine residues within the cytoplasmic tail of MHC class I are also dispensable for the ubiquitin-dependent dislocation by the gamma herpesvirus 68-encoded Mk3 protein, a viral E3 ligase⁴⁸. These findings are surprising, since the lysines within the cytoplasmic tail of these proteins would appear to be the most obvious targets for the cytosolic ubiquitin system. In other cases, however, cytoplasmic lysines are essential ubiquitination targets, as is illustrated by the HIV Vpu-induced dislocation of CD4, which depends on lysine residues within the cytosolic domain of CD4⁴⁹. The dislocation of the soluble ER-luminal proteins CPY*

and RI₃₃₂ also depends on the ubiquitinating system^{39,50,51}. In those cases, luminal residues are only accessible to the ubiquitin ligation machinery after (partial) dislocation, implying that the initial steps of the retrotranslocation reaction do not require ubiquitination of the substrate *per se*.

To acquire further insight into the role of the ubiquitin system in the retrograde transport of ER (glyco-)proteins, we utilized the extremely efficient dislocation of MHC class I heavy chains in the context of HCMV US2 and US11. In this model, the dislocation and the subsequent degradation of the heavy chains can be evaluated as two separate events, both temporally and spatially. We investigated whether the actual retrotranslocation requires the conjugation of ubiquitin to the target protein itself. To this end, a series of class I heavy chain mutants was constructed in which all lysine residues were substituted. To evaluate the possible contribution of the substrate's N-terminus, a lysine-less, non-cleavable ubiquitin moiety was fused to the N-terminus of the class I heavy chains. While the lysine-less class I molecules were no longer dislocated by US2, retrograde movement took place as normal in the presence of US11. Dislocation of the mutant heavy chains nevertheless required a functional ubiquitin system. While these results confirm the essential contribution of the ubiquitin system to glycoprotein dislocation, they indicate at the same time that retrotranslocation of the substrate can take place in the absence of ubiquitination of the substrate itself.

Results

US2-mediated dislocation of MHC class I heavy chains is ubiquitin-dependent

Previous studies have shown that the dislocation of MHC class I heavy chains by US11 requires a functional ubiquitin system⁴⁴. It has become clear that US2 and US11-dependent dislocation of class I heavy chains differ in several ways. For example, US2 and US11 differ in their preference for HLA class I haplotypes and cytoplasmic tail composition of the heavy chains⁵²⁻⁵⁴. The fact that US11-mediated dislocation of class I heavy chains requires Derlin-1, but US2-mediated dislocation does not, indicates that the mechanism of the dislocation reaction is also different^{14,15}. To investigate whether, like US11, US2-mediated dislocation requires a functional ubiquitin system, MHC class I heavy chains were co-expressed with US2 in TS20 cells, which have a temperature-sensitive mutation within the ubiquitin activating enzyme E1⁵⁵. Degradation of the class I heavy chains was compared at the permissive (33°C) and non-permissive temperature (40°C), in the presence of proteasome inhibitor. At the permissive temperature, dislocation of the class I heavy chains proceeded as normal (Fig. 1A, lanes 2-4). Removal of the single

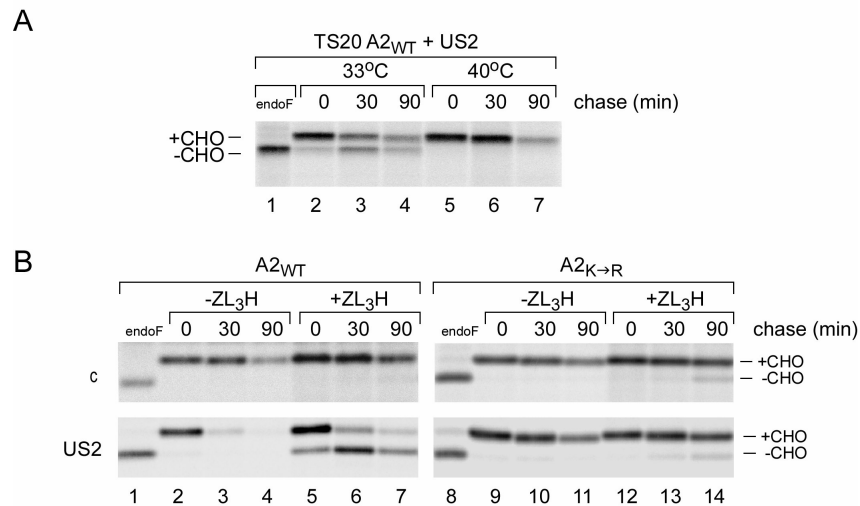


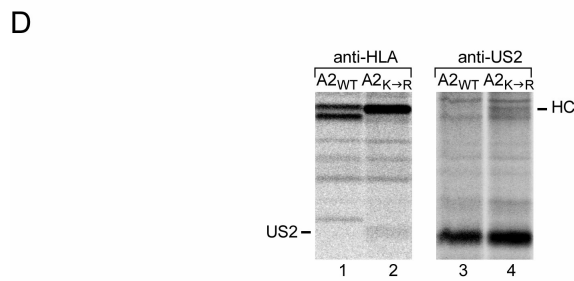
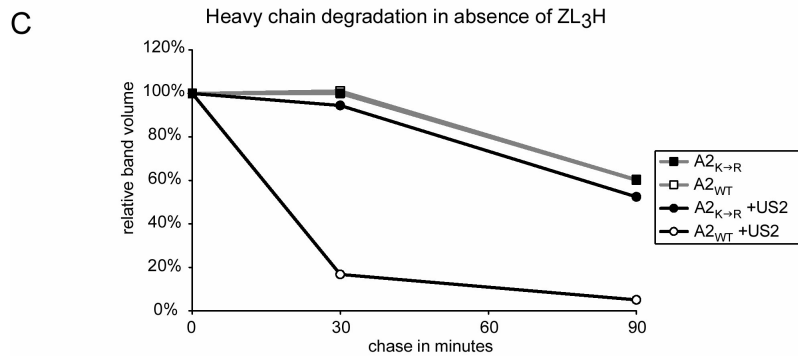
Figure 1. Dislocation of MHC class I molecules by US2 is dependent on the ubiquitin system and requires lysine residues within the class I heavy chains.

(A) First, TS20 cells expressing wild type HLA-A2 ($A2_{WT}$) and US2 were incubated at either 33°C or 40°C for 2 h. Next, the cells were starved, pulse-labeled with ^{35}S Met/Cys for 10 min, and chased for 30 and 90 min. The latter steps were all performed in the presence of proteasome inhibitor (ZL_3H), at the indicated temperatures. HLA-A2 molecules were immunoprecipitated with the MR24 anti-heavy chain antibody and separated on SDS-polyacrylamide gel (10 %). The presence or absence of carbohydrate side chains is indicated as +CHO or -CHO, respectively. The $t=0$ sample was treated with N-glycosidase F as described (EndoF; displayed in the first lane). (B) TS20 cells expressing either $A2_{WT}$ or $A2_{K\rightarrow R}$ were transduced with control (c) or US2-expressing retroviruses. Cells were pulse-labeled for 10 min and chased for 30 and 90 min at 33°C, with or without the proteasome inhibitor ZL_3H . MHC class I heavy chains were immunoprecipitated using the MR24 antiserum. Samples were displayed on SDS/PAGE (10 %). (C) Graph depicting the quantified results of the pulse-chase analysis shown in **A** (results obtained in the absence of proteasome inhibitor). (D) $A2_{WT}$ and $A2_{K\rightarrow R}$ -expressing TS20-US2 cells were metabolically labeled for 60 min with ^{35}S Met/Cys in presence of the proteasome inhibitor ZL_3H . The cells were lysed in the presence of digitonin as described, and subjected to immunoprecipitations with either the MR24 anti-heavy chain antibody or an antibody directed against US2.

N-linked glycan from the class I heavy chains by cytosolic N-glycanase resulted in the emergence of a deglycosylated degradation intermediate (lanes 2-4). The latter migrates at the same level as class I heavy chains, obtained at the end of the pulse, treated with N-glycanase *in vitro* (lane 1). At the non-permissive temperature, the breakdown intermediate was not observed, indicating that dislocation of the class I heavy chains was inhibited (Fig. 1A, lanes 5-7). These results show that a functional ubiquitin system is essential for the dislocation of MHC class I heavy chains in the presence of US2.

Lysine-less MHC class I heavy chains are not dislocated by US2

MHC class I heavy chains carry a number of lysine residues that may



serve as acceptor sites for ubiquitin in the course of the dislocation and degradation process. To investigate whether these lysines are required for the ubiquitin-dependent dislocation by US2, we constructed a recombinant HLA A*0201 molecule, A2_{K→R}, in which all 13 lysines were substituted for arginines. TS20 cells were transduced with retroviruses expressing the A2_{K→R} or A2_{WT} heavy chains. In addition, the cells were transduced with a retrovirus expressing US2, or with a control virus. The stability of the wild type and recombinant class I heavy chains was evaluated in pulse-chase experiments (Fig. 1B). The A2_{WT} heavy chains were relatively stable in the absence of US2 (Fig. 1A, upper-left panel, lanes 2-4). When US2 was expressed in these cells, the A2_{WT} heavy chains were degraded rapidly (Fig. 1B, lower-left panel, lanes 2-4). In the presence of the proteasome inhibitor ZL₃H, a deglycosylated degradation intermediate appeared, migrating at the level of endoglycosidase F-treated heavy chains (compare lanes 1 and 5-7).

Like the A2_{WT} heavy chains, the A2_{K→R} molecules were stable in cells not expressing US2 (Fig. 1B, upper-right panel). Contrary to the A2_{WT} molecules, however, the A2_{K→R} heavy chains were not degraded in the presence of US2 (Fig. 1B, lower panels, compare lanes 2-4 and 9-11). A

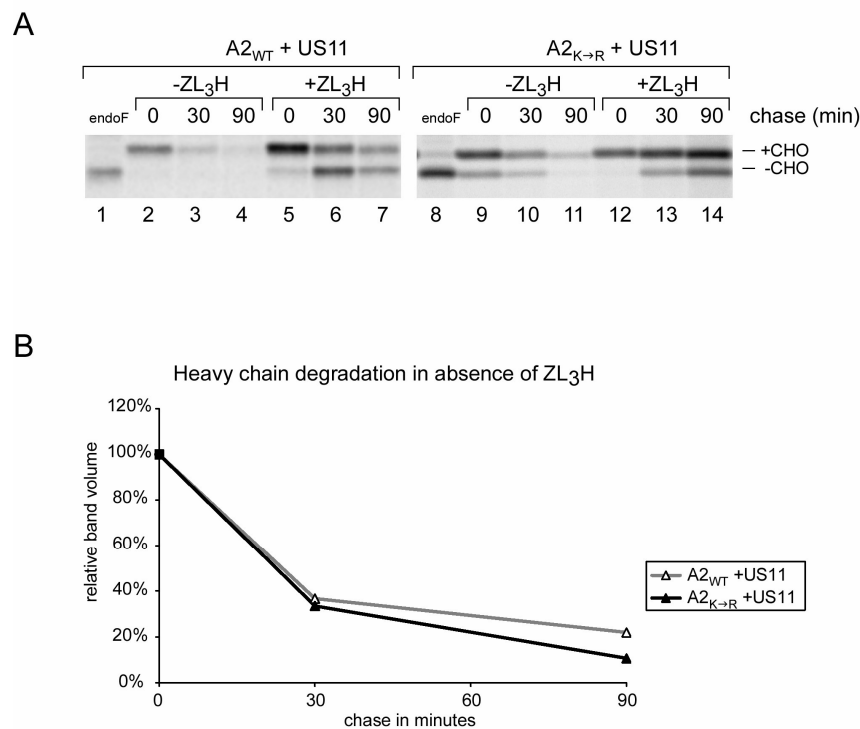


Figure 2. US11-mediated dislocation of MHC class I molecules does not depend on lysine residues within the class I heavy chains.

(A) US11-positive TS20 cells expressing either A2_{WT} or A2_{K→R} were pulsed and chased as described in Fig. 1. MHC class I heavy chains were immunoprecipitated using the MR24 antiserum. Samples were analyzed on SDS/PAGE (10 %). (B) Quantification of the pulse-chase experiments performed in the absence of proteasome inhibitor (Fig. A, lanes 2-4 and 9-11; glycosylated heavy chains only).

quantification of the degradation of full-length heavy chains in the absence of proteasome inhibitor is shown in Fig. 1C. These results indicate that the lysine residues within MHC class I heavy chains are essential for the US2-mediated dislocation of these chains.

US2 can interact with lysine-less MHC class I heavy chains

The stabilization of the A2_{K→R} molecules in the presence of US2 might be the result of a lack of interaction of the lysine-less heavy chains with US2. Although the lysines had been replaced with arginines, which have very similar characteristics as far as side chain composition, structure and polarity are concerned, it cannot be excluded that these alterations affected the interaction with US2. Therefore, we investigated whether US2 was still capable of interacting with the lysine-less class I heavy chains in co-

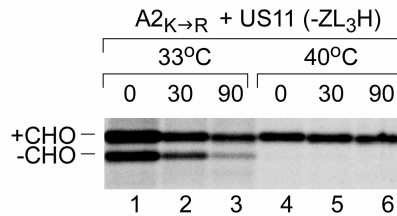


Figure 3. US11-mediated dislocation of A2_{K→R} is ubiquitin-dependent. TS20 cells expressing A2_{K→R} and US11 were pre-incubated at either 33°C or 40°C for 2 h. Subsequently, the cells were starved, pulse-labeled with ³⁵S Met/Cys for 10 min, and chased for 30 and 90 min at the temperatures indicated. The experiment was performed in absence of proteasome inhibitor. MHC class I heavy chains were immunoprecipitated and displayed on SDS/PAGE (10 %).

immunoprecipitation experiments (Fig. 1D). Immunoprecipitation of the A2_{K→R} molecules resulted in co-precipitation of US2 (lane 2). Conversely, the anti-US2 antibody was able to co-precipitate A2_{K→R} heavy chains (lane 4). More US2/heavy chain material could be co-precipitated in the A2_{K→R} expressing cell line than in the A2_{WT} cells. The latter was likely due to the fact that a substantial amount of A2_{WT} heavy chains had been dislocated and, hence, occurred as free molecules in the cytosol. In conclusion, these data show that the lysine-less MHC class I heavy chains were not insensitive to US2-mediated degradation as a result of their inability to interact with the viral protein.

MHC class I heavy chains lacking lysine residues are dislocated by US11 in a ubiquitin-dependent manner

Next, we investigated whether the lysine-less class I heavy chains could be dislocated in the presence of US11. For this purpose, US11 was co-expressed with either A2_{WT} or A2_{K→R} heavy chains in TS20 cells. Wild-type heavy chains were rapidly degraded in the US11-expressing cells (Fig. 2A, lanes 2-4). Interestingly, unlike US2, US11 was capable of inducing dislocation of the A2_{K→R} molecules (Fig. 2A, lanes 9-11; Fig. 2B). Deglycosylated heavy chains emerged in the US11-expressing cells in the absence of ZL₃H (lanes 8-11), indicating that proteasomal degradation of the dislocated A2_{K→R} molecules was inhibited. Note that in the presence of proteasome inhibitor, the dislocation became the rate-limiting step, resulting in delayed appearance of the deglycosylated degradation intermediate (compare conversion rates of glycosylated (upper band) to deglycosylated (lower band) heavy chains in lanes 9-11 and 12-14).

These results clearly show that, in contrast to US2, US11 can dislocate heavy chains that lack lysine residues. To investigate whether the dislocation of the lysine-less heavy chains requires a functional ubiquitin system

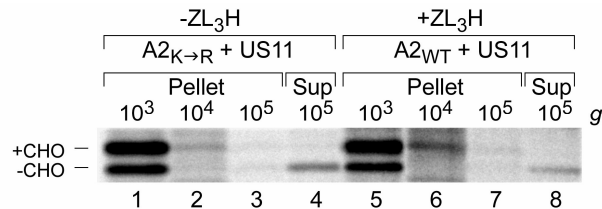


Figure 4. A soluble cytosolic A2_{K→R} degradation intermediate appears in US11-positive cells. US11-positive TS20 cells expressing either A2_{WT} or A2_{K→R}, were labeled with ³⁵S Met/Cys for 30 minutes and subjected to subcellular fractionation using differential centrifugation as described. Pellet fractions were solubilized in NP40 lysis mix. MHC class I heavy chains were immunoprecipitated from the pellet fractions and the 10⁵ g supernatant (Sup) and displayed on SDS/PAGE (10%). The experiments with the A2_{WT}-expressing cells, but not the A2_{K→R}-expressing cells, were performed in the presence of ZL₃H.

nonetheless, we compared the degradation of the lysine-less class I molecules in the TS20 cells at 33°C and 40°C (Fig. 3). At 33°C, the A2_{K→R} molecules were dislocated, and, again, deglycosylated heavy chains emerged in the absence of proteasome inhibitor (lanes 1-3). At 40°C, the dislocation of lysine-less class I molecules was inhibited completely. These observations indicate that a functional ubiquitin system is essential for US11-dependent dislocation of lysine-less MHC class I heavy chains.

In the absence of proteasome inhibitor, a soluble degradation intermediate of A2_{K→R} can be observed in the cytosol of US11-expressing cells

The AAA ATPase p97-Ufd-Npl4 complex is required for membrane extraction of (partly) dislocated ER proteins that are still associated with the ER membrane. This final step might require ubiquitination of the substrate ^{8,9,39,46}. Deglycosylated lysine-less degradation intermediates of MHC class I molecules are detectable in the US11 cells (Fig. 2A and 3), but it is unknown if these are still associated with the ER membrane. To investigate whether these deglycosylated heavy chains had been fully released into the cytosol, a cell fractionation experiment was performed (Fig. 4). Note that the experiment with the A2_{K→R}-expressing cells was performed in the absence of proteasome inhibitor. Deglycosylated A2_{K→R} and A2_{WT} accumulated in the 10⁵g supernatants representing the cytosolic fractions (Fig. 4, lanes 4 and 8), confirming that lysine-less class I heavy chains are indeed extracted from the ER membrane.

A2_{K→R} forms a complex with US11 and Derlin-1

Besides being dependent on the cytosolic p97 complex, US11-mediated dislocation of MHC class I heavy chains also requires the ER-resident membrane protein Derlin-1 ^{14,15}. US2-mediated dislocation, on the other

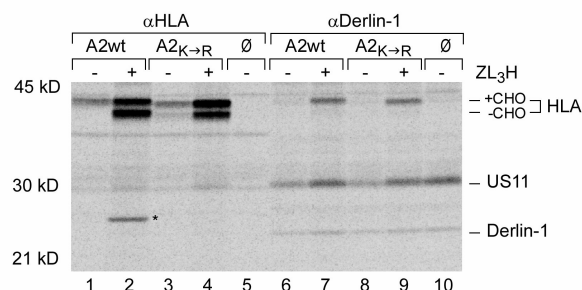


Figure 5. Derlin-1 interacts with A2_{K→R}.

A2_{WT}, A2_{K→R}-expressing, or HLA-negative TS20-US11 cells were pulse-labeled for 15 minutes with ³⁵S Met/Cys and chased for 30 minutes in the presence or absence of ZL₃H. Cells were lysed in digitonin-containing lysis buffer and subjected to immunoprecipitation with either the MR24 anti-heavy chain antibody or anti Derlin-1 antibody. * unidentified 25kD protein.

hand, does not depend on Derlin-1, although degradation of US2 itself does¹⁵. Derlin-1 is the human homologue of the yeast Der1p, which is required for the degradation of ER proteins in yeast, including misfolded CPY*⁵⁶. To evaluate whether A2_{K→R} can also form a complex with Derlin-1, we investigated whether we could co-isolate A2_{WT} and A2_{K→R} with Derlin-1 from digitonin lysates prepared from metabolically labeled cells. Fig. 5 shows that A2_{WT} as well as A2_{K→R} molecules co-precipitated with Derlin-1 (lanes 7 and 9, respectively). Despite the presence of both intact and deglycosylated MHC class I heavy chains in the lysates (lanes 2 and 4), Derlin-1 selectively interacted with the glycosylated form of the class I molecules. Interestingly, US11 co-precipitated with Derlin-1 even in the absence of class I heavy chains, suggesting that US11 links Derlin-1 to the class I heavy chains.

In conclusion, these results indicate that lysine-less MHC class I heavy chains also associate with Derlin-1 in US11-expressing cells.

US11-mediated dislocation of MHC class I does not require N-terminal ubiquitination

Theoretically, the lysine-less class I heavy chain could still be ubiquitinated at its N-terminus. Therefore, the role of N-terminal ubiquitination was investigated in more detail using various ubiquitin-heavy chain fusion proteins. Either wild type ubiquitin or a lysine-less ubiquitin moiety was fused to the N-terminus of the class I heavy chains. Previously, it has been shown that US11-mediated dislocation of class I molecules requires polyubiquitination¹³. Attachment of lysine-less ubiquitin to the N-terminus of the class I heavy chain will prevent the formation of multi-ubiquitin chains at this site. The C-terminal glycine 76 of ubiquitin was replaced by a valine, creating a version that is no longer sensitive to deubiquitinating enzymes⁵⁷⁻⁶⁰.

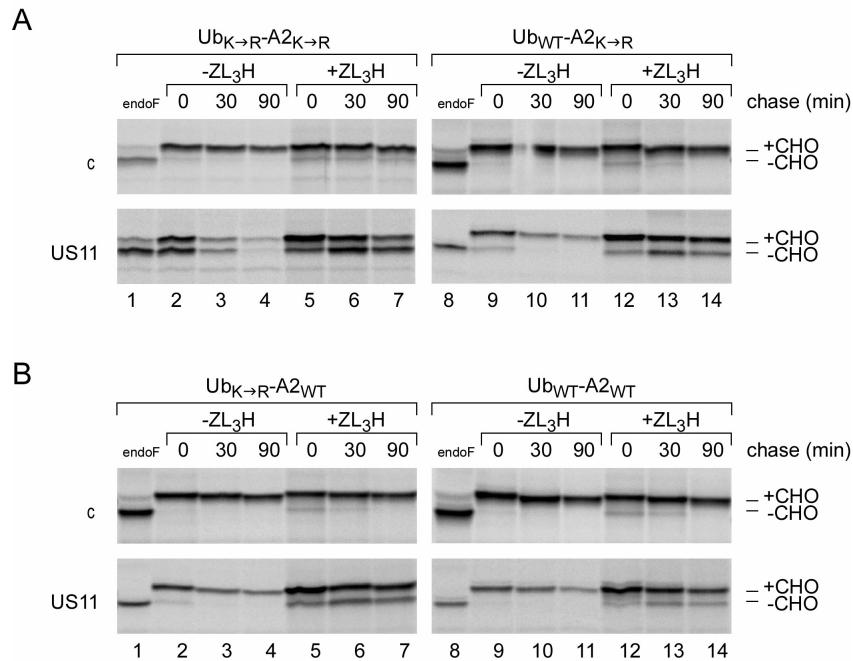
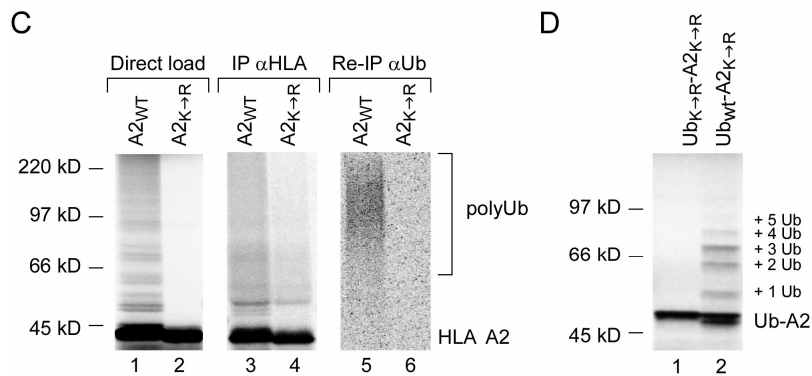


Figure 6. Dislocation of MHC class I-ubiquitin fusion proteins by US11.

(A) TS20 and TS20-US11 cells expressing Ub_{K→R}-A2_{K→R} or Ub_{WT}-A2_{K→R} fusion proteins were pulse-labeled for 10 min with ³⁵S Met/Cys and chased for 30 and 90 min at 33°C, with or without the proteasome inhibitor ZL₃H. HLA-A2 molecules with (+CHO) or without (-CHO) carbohydrate side chain were immunoprecipitated with the MR24 anti-heavy chain serum and analyzed on an SDS/polyacrylamide gel (10 %). The t=0 samples were treated with N-glycanase F (F). (A) Experiment as in (A) using cells expressing Ub_{K→R}-A2_{WT} or Ub_{WT}-A2_{WT} fusion proteins. (C) A2_{WT} and A2_{K→R} constructs were translated *in vitro* in the presence of ³⁵S-methionine in the absence of microsomes. One third of the translation product was loaded on gel directly (left panel). Two third of the translation product were subjected to immunoprecipitation with anti heavy chain antibody (MR24). One half of the immunoprecipitate was loaded on gel (middle panel), and the other half was subjected to a subsequent re-immunoprecipitation with the monoclonal antibody P4D1, recognizing polyubiquitin chains (right panel). (D) Ub_{K→R}-A2_{K→R} and Ub_{WT}-A2_{K→R} constructs were translated *in vitro* and loaded onto a 10 % SDS-polyacrylamide gel.

Four fusion proteins were constructed, consisting of ubiquitin (Ub) or lysine-less ubiquitin (Ub_{K→R}) fused with its C-terminus to the N-terminus of either wild type or lysine-less HLA-A2. The fusion proteins were stably expressed in TS20 cells using recombinant retroviruses.

In pulse chase experiments performed with US11-negative control cells, the Ub_{K→R}-A2_{K→R} fusion protein remained stable throughout the chase (Fig. 6A, upper-left panel, lanes 2-4). In US11-expressing cells, Ub_{K→R}-A2_{K→R} was dislocated and degraded (Fig. 6A, lower-left panel, lanes 2-4). This indicates that polyubiquitination at the N-terminus of the class I molecules is



not essential for US11-mediated dislocation. Dislocation of Ub_{K→R}-A2_{K→R} resulted in the immediate emergence of the deglycosylated breakdown intermediate, despite the absence of proteasome inhibitor in the experiment (Fig. 6A, lanes 2-4). Apparently, like A2_{K→R}, Ub_{K→R}-A2_{K→R} can be dislocated into the cytosol, but is degraded less efficiently, resulting in a temporary accumulation of deglycosylated heavy chains.

Ub_{WT}-A2_{K→R}, Ub_{K→R}-A2_{WT} and Ub_{WT}-A2_{WT} were all dislocated and degraded in the presence of US11 (Fig. 6A, lanes 9-11; Fig. 6B). For the Ub_{WT}-A2_{K→R} fusion protein, very little deglycosylated class I was observed (Fig. 6A, lanes 9-11). This indicates that the N-terminal ubiquitin successfully created a ubiquitin fusion degradation (UFD) substrate^{60,61}. In the absence of US11, all fusion proteins were stable throughout the chase, showing that ubiquitin fusion by itself did not induce dislocation of the class I heavy chains (Fig. 6A and B, upper panels).

The data presented so far are based on the assumption that A2_{K→R} and Ub_{K→R}-A2_{K→R} cannot serve as ubiquitination substrates. To test this premise, ubiquitin conjugation to A2_{K→R} and Ub_{K→R}-A2_{K→R} was evaluated *in vitro*. Translation of the MHC class I heavy chain constructs in the absence of microsomal membranes renders their hydrophobic transmembrane domains accessible to cytosolic chaperones and quality control mechanisms^{18,19} thereby promoting their degradation through the ubiquitin-proteasome pathway. Fig. 6C, left panel, shows a direct load of translated wild type and lysine-less heavy chains. The wild-type construct produces an additional smear of high molecular weight material. Immunoprecipitation with an anti-MHC class I heavy chain antibody revealed that this smear contained class I molecules (middle panel). Subsequent re-immunoprecipitation with a poly-ubiquitin-specific antibody identified this material as ubiquitin-conjugated

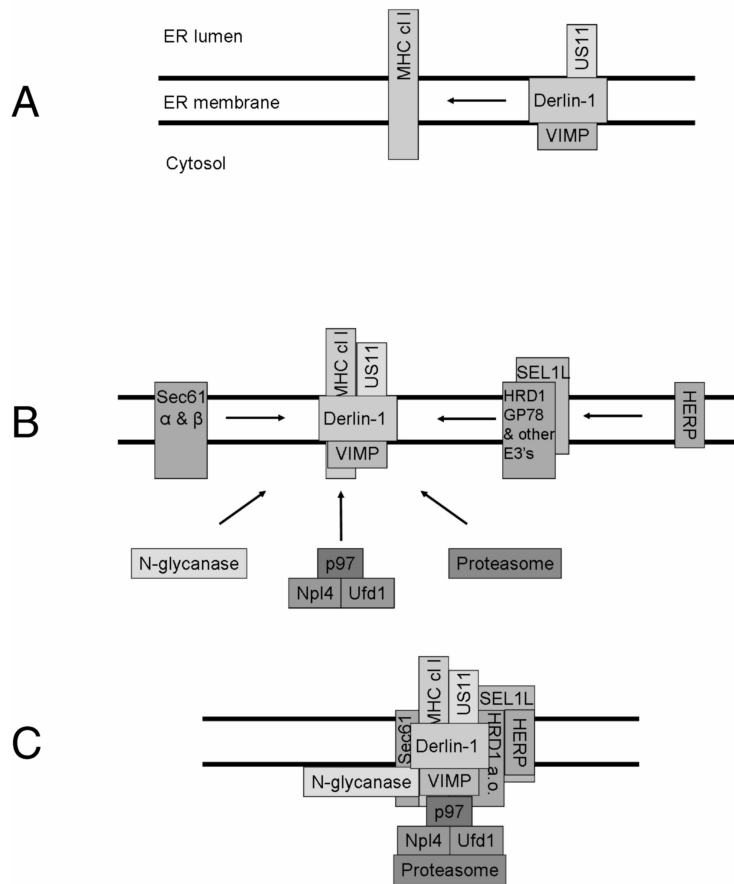


Figure 7. Formation of the dislocation complex mediating retrograde transport of MHC class I heavy chains in the context of HCMV US11.

class I heavy chains (right panel). Ubiquitinated heavy chains were not detectable for $A2_{K \rightarrow R}$ (Fig. 6C, lanes 2, 4 and 6).

Ubiquitination of $Ub_{K \rightarrow R}-A2_{K \rightarrow R}$ and $Ub_{WT}-A2_{K \rightarrow R}$ was evaluated in the experiment presented in Fig. 6D. Whereas translation of $Ub_{K \rightarrow R}-A2_{K \rightarrow R}$ did not yield ubiquitinated material, $Ub_{WT}-A2_{K \rightarrow R}$ resulted in a ladder of bands corresponding to heavy chains carrying one or more ubiquitin moieties. These experiments show that neither the lysine-less MHC class I heavy chains, nor the $Ub_{K \rightarrow R}-A2_{K \rightarrow R}$ fusion protein serve as ubiquitination substrates *in vitro*.

Altogether, these results indicate that dislocation of MHC class I heavy chains in the context of US11 does not require ubiquitination of the heavy chains themselves. However, ubiquitination promotes the degradation of dislocated heavy chains by proteasomes. Fusion of ubiquitin to the class I molecules does not accelerate their dislocation, supporting the conclusion that N-terminal ubiquitination does not play a role in US11-initiated extraction of heavy chains from the ER membrane. The finding that ubiquitinated A2_{K→R} cannot be detected *in vitro* suggests that the N-terminus is not a favorable ubiquitination site.

Discussion

In this study, we used the HCMV US2- and US11-mediated dislocation of MHC class I heavy chains as a model to investigate the role of the ubiquitin system in the dislocation and degradation of ER proteins. We show that the lysines within the class I molecule are essential for dislocation in the context of US2. In contrast, in US11-dependent dislocation, ubiquitination of the lysines or the N-terminus of the class I heavy chain does not play a role. US2 and US11, however, both require a functional E1 enzyme to effect dislocation of MHC class I molecules.

Although substitution of lysine residues within the MHC class I molecules does not prohibit their dislocation by US11, it does affect the efficiency of degradation once the heavy chains have reached the cytosol. As a result, deglycosylated heavy chains become visible in the cytosol in the absence of proteasome inhibitor (Figures 2A, 3 and 6). The breakdown intermediates appear early after synthesis. Inhibition of the catalytic activity of the proteasome results in a different pattern, with the deglycosylated heavy chains emerging at later time points during the chase (Fig. 2A). This difference in kinetics may be explained by the fact that the substitution of lysines does not block heavy chain dislocation, but mainly affects the degradation of dislocated molecules by the proteasome. In contrast, inhibition of the proteolytic activity of the proteasome seems to also cause a retardation of heavy chain dislocation, resulting in the delayed appearance of the heavy chains in the cytosol and accumulation at later time points. Thus, whereas heavy chain degradation is the rate-limiting step in case of lysine-less substrates, dislocation appears to be the rate-limiting step when the catalytic activity of the proteasome is blocked. It is interesting to note that proteasome inhibitors completely inhibit the retrograde movement of other ER dislocation substrates, including tyrosinase⁶², ribophorin 332³⁹, parathyroid hormone related protein⁶³, TCR α ⁶⁴, CD3 δ ^{65,66}, and MHC

class II α and β chains^{21,22}. For those substrates, dislocation and proteasomal degradation may be more closely linked than for US11 mediated degradation of class I heavy chains.

Contrary to US11, US2 requires lysines within the MHC class I heavy chain for its dislocation into the cytosol. The stabilization of the A2_{K→R} molecules in the presence of US2 might be the result of a lack of interaction of the lysine-less heavy chains with US2. According to crystal structure data, two lysine residues occur within the US2 interaction region of the class I heavy chain. One of these, K₁₇₆, is directly in contact with US2⁶⁷. In the A2 mutants used in our study, however, the lysines were replaced with arginines, which have very similar characteristics as far as side chain composition, structure and polarity are concerned. Our data indicate that US2 can indeed still interact with the A2_{K→R} heavy chains. In addition, it has been shown that substitution of the lysine residues at position 127 and 144 of HLA-A2 does not affect the ability of US2 to retain this MHC class I mutant⁶⁸. The question remains: how do lysines within the class I molecules contribute to US2-mediated dislocation? Previously, it has been shown that lysines within the cytoplasmic tail of class I heavy chains, and even the entire cytoplasmic tail, are dispensable for dislocation by US2^{47,52,53}. Combined, these findings suggest that ER-located lysine residues of the heavy chains play a role in dislocation by US2. Theoretically, US2 could induce a partial dislocation or 'prolapse' of part of the heavy chain into the cytosol, resulting in cytosolic deposition of luminal lysine residues. Although this possibility cannot be excluded, this scenario is unlikely. Alternatively, ubiquitin could serve as a ratcheting molecule during the extraction of the class I heavy chains. Previous studies have indicated that a ratcheting function of polyubiquitin alone is insufficient for dislocation of class I heavy chains by US11¹³. Whether this is also the case for US2-mediated retro-translocation of heavy chains remains to be established.

It has become clear that the dislocation of glycoproteins from the ER involves a wide array of proteins. The precise composition of the 'dislocation complex' is unclear as yet and could, in fact, be flexible, depending on which substrate has to be relocated to the cytosol. In addition to Derlin-1, VIMP, and p97, E3-ligases may also form a part of the dislocation complex^{16,30,31}. In the case of MHC class I dislocation by US11, the reaction commences with the interaction of the viral glycoprotein with the newly synthesized class I heavy chain (Fig. 7). US11 links the class I heavy chain to the complex of Derlin-1 and the small membrane spanning molecule VIMP^{14,15}. Interestingly, US11 also occurs in a complex with Derlin-1 in the absence of class I heavy chains (Fig. 5, lane 10), indicating that it is indeed US11 that targets Derlin-1 to the heavy chains. The next step might be the recruitment of translocon

subunits ^{3,23-29} and ER-resident ubiquitin ligase-complexes such as HERP-HRD1-SEL1L and GP78 ^{16,30,31}. The transmembrane regions of the multi-membrane spanning E3 enzymes may form a conduit around the class I heavy chain, possibly together with Derlin-1 and other membrane proteins, as has been hypothesized for the twin-arginine translocons occurring in peroxisomes and plant thylakoid membranes ^{69,70}. The advantage of forming a 'dislocon' after the substrate has been identified is that it will be adapted to the nature of the substrate ^{11,12}. Such an *ad hoc* arrangement would also allow the inclusion of transmembrane regions of substrates such as CFTR Δ 508 and apolipoprotein B100 (reviewed in ¹⁷). Finally, it is possible that the p97-Ufd1-Npl4 complex and the proteasome are attracted to, or even stabilize the dislocation complex ^{8,9}. Using the ATPase activity of p97 and/or the proteasome, the dislocation substrate may be extracted from the ER membrane. In the course of this process, the heavy chain is unfolded and deglycosylated by N-glycanase. The latter has been found to bind to Derlin-1 ^{37,71}. The fact that the dislocation reaction is dependent on a functional ubiquitin system, combined with the observation that US11-mediated dislocation of the MHC class I molecule does not involve ubiquitination of the class I heavy chain itself, suggests that the dislocation reaction requires ubiquitination of adaptor proteins that recruit other components into the dislocation complex. Thus, the ubiquitinated proteins may serve as a recognition signal for the p97-Ufd1-Npl4 complex and, possibly, for other components implicated in the retrograde movement of substrates into the cytosol.

Materials & Methods

Plasmids

Constructs expressing lysine-less MHC class I molecules were prepared by PCR using primers containing lysine to arginine codon mutations (see below); HLA A2*0201 was used as a template ⁴⁴. The following primers were used:

GAGTGGGCCCTCACTCTCCGTGTCTCC (antisense) and GGAGACACGGAGAGTGAGGGCCCACTC (sense) (bp 258-284), CCTCTCTCAGGGCGATGTAATCCCTGCCGTCG (antisense) and CGGCAGGGATTACATCGCCCTGAGAGAGG (sense) (bp 426-457), CGCCTCCACCTGTGCCTGGTGGTC (antisense) and CAGACCAC CAGGCACAGGTGGGAGG (sense) (bp 493-519), TGC GTTCTGGG GCGTCCGTGCGCTGCAGCGTCTCCCTCCCGTTC (antisense) and GAACGGGAGGGAGACGCTGCAGCGCACGGACGCCCCCAGAAC GCA (sense) (bp 591-635), ACCACAGCCGCCACCTCTGGAAGG

(antisense) and GAACCTTCCAGAGGTGGGCGGCTG (sense) (bp 788-815), GGGTGAGGGGCCTGGGCAAACC (antisense) and AGGGTTTGCCAGGCCCTCAC (sense) (bp 863-886), CTCCTCTTCTATCTGAGCTCCTCCTCCTCCA (antisense) and GGAGGAGGAGCTCAGATAGAAGAGGAGGGAGCT (sense) (bp 994-130), and flanking sequences AGACCTCGAGCAGCTGTCTCACACTCTACAAGC (antisense) and TAATACGACTCACTATAGGGAGA (sense).

The PCR products were fused in three additional rounds of PCR. The resulting lysine-less MHC class I constructs were cloned into the pCR2 vector using the TOPO technology (Invitrogen) and subsequently subcloned into the pLZRS-EGFP vector (<http://www.stanford.edu/group/nolan/>). Ubiquitin-HLA fusion constructs were generated using a fusion PCR. Ubiquitin with the glycine at position 76 mutated to valine, and its lysine-less version⁶⁰ (kindly provided by Dr. Nico Dantuma, Stockholm), were fused to wild-type or lysine-less HLA-A2. US2⁵³ was subcloned into the pLZRS vector expressing an IRES element and truncated nerve growth factor receptor (Δ NGFR), using GATEWAY technology (Invitrogen).

Antibodies

MR24, a polyclonal antiserum directed against the luminal α 3 domain of HLA-A2⁴⁴, and the polyclonal anti-US11 and anti-HRD1 rabbit sera have been described previously^{44,45}. Ubiquitin was detected using monoclonal antibody P4D1 (Santa Cruz). Anti-US2 rabbit serum⁷² was kindly donated by Dr. T. Jones (Pearl River, NY). Anti-NGFR antibody was provided by Dr. M. Heemskerk (Leiden). Rabbit serum against Derlin-1¹⁴ was kindly donated by Dr. T. Rapoport (Boston) and Dr. Y. Ye (Washington D.C.). The monoclonal antibody 20.4, directed against the nerve growth factor receptor (NGFR), was obtained from ATCC (#HB-8737).

Cells

The hamster cell lines E36 and E36ts20 (the latter referred to as TS20)⁵⁵ were maintained at 33°C in minimum essential medium (MEM α ; Gibco BRL) supplemented with 10% (v/v) fetal calf serum (Greiner, Alphen a/d Rijn, The Netherlands), penicillin (100 units/ml) and streptomycin (100 μ g/ml; Gibco BRL).

Retroviral transduction and FACS sorting

Replication-deficient recombinant retroviruses were produced using the pLZRS-EGFP or Δ NGFR vectors and the Phoenix-A producer cells as described (<http://www.stanford.edu/group/nolan/>). Stable cell lines were generated by transduction of wild-type or US11-expressing TS20 cells⁴⁴ with

recombinant retrovirus encoding wild-type or mutant MHC class I molecules as well as EGFP. US2-expressing cells were generated by transduction of TS20 cells with retrovirus expressing US2 and Δ NGFR. A retrovirus encoding only EGFP served as a control. EGFP- and Δ NGFR-positive cells were sorted using a FACS Vantage cell sorter (Becton Dickinson). Δ NGFR-positive cells were stained with anti-NGFR antibody 20.4 (ATCC #HB-8737) and goat anti-mouse-TexasRed conjugate (Jackson, WA, U.S.A.).

Pulse-chase analysis, immunoprecipitation and SDS/PAGE

For metabolic labeling/pulse chase experiments, cells were starved in RPMI 1640 medium (Bio Whittaker) without methionine and cysteine at either 33°C or 40°C for approximately 1 h. For experiments performed at the non-permissive temperature, TS20 cells were pre-incubated at 40°C for 2-4 h, prior to the pulse-chase experiment. Starvation and pulse-chase incubations were all performed at the same temperature. Where indicated, proteasome inhibitor ZL₃H (Peptide Institute, Osaka, Japan) was used, added during starvation at a final concentration of 20 μ M. The cells were metabolically labeled with 250 μ Ci of ³⁵S-labelled Redivue Promix (a mixture of L-[³⁵S]methionine and L-[³⁵S] cysteine) (Amersham) per 10⁷ cells in starvation medium. For chase samples, radioactive medium was replaced with RPMI 1640 medium supplemented with 1 mM methionine and 0.1 mM cysteine (Sigma).

The cells were lysed in Nonidet P-40 (NP40)-containing lysis buffer as described ⁴⁴. Immunoprecipitations were performed at 4°C on cleared lysates for 2-4 h using specific antiserum and Protein A Sepharose beads (Amersham, Roosendaal, The Netherlands). The beads were washed with NET buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5 % (v/v) NP-40) supplemented with 0.1 % SDS. The digitonin lysis buffer contained 1% (w/v) digitonin, 50 mM Tris/HCl (pH 7.5), 5 mM MgCl₂, 150 mM NaCl, 1 mM leupeptin, and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF; Roche, Woerden, The Netherlands). The washed beads were boiled in Laemmly sample buffer (40 mM Tris/HCl, pH 8.0, 4 mM EDTA, 8 % (w/v) SDS, 40 % (v/v) glycerol, 10 % (v/v) β -mercaptoethanol, and 0.1 % Bromophenol Blue) for 5 to 10 min. For re-immunoprecipitations, Laemmly samples containing 10 mM DTT were diluted with equal amounts of 10 mM iodo acetic acid (IAA) and subsequently diluted with NP40 lysis mix to a final volume of 1 ml. The samples were subjected to a sequential round of immunoprecipitation with the desired antibody. Samples were loaded onto SDS-polyacrylamide gels and exposed to a storage PhosphorImaging screen, which was scanned in a Personal Molecular Imager FX and analyzed with Quantity One software (Bio-Rad, Veenendaal, The Netherlands).

Subcellular fractionation

Fractionation of cells was performed essentially as described⁴. In brief, about 10^7 cells were starved and labeled for 30 min with ^{35}S -Redivue Promix as described above. Cells were washed and resuspended in 1 ml of homogenization buffer (0.25 M sucrose, 10 mM triethanolamine, 10 mM potassium acetate, 1 mM EDTA, pH 7.6, supplemented with protease inhibitors leupeptin (0.1 mM) and AEBSF (10 mM)). Cells were placed on ice and homogenized using a Dounce homogenizer with a tight-fitting pestle (50 strokes). The homogenate was spun at $1,000g$ at 4°C in an Eppendorf centrifuge for 10 min. The pellet was saved and the supernatant was spun at $10,000g$ at 4°C for 30 min. The pellet was again saved and the supernatant spun at $100,000g$ at 4°C for 1 h. The latter two centrifugations were performed in a TLA 120.2 fixed-angle rotor, operated in a Beckman Optima2 TLX ultracentrifuge. All pellets were resuspended in NP-40 lysis buffer. HLA-A2 and US11 were immunoprecipitated simultaneously from the solubilized pellets and the $100,000g$ supernatant, and separated by SDS-PAGE.

In vitro transcription and translation and in vitro ubiquitination assays

HLA-A0201 in pCDNA3 or TOPO plasmids (Invitrogen) were linearized with XhoI and HindIII, respectively, and used for *in vitro* transcription with T7 polymerase (Promega). Transcripts were translated in the presence of L- ^{35}S methionine in rabbit reticulocyte lysate without microsomes. Reaction mixtures of 25 μl were incubated at 30°C for 45 minutes. 16 μl samples were denatured by adding 34 μl PBS containing 1 % SDS and 10 mM DTT and boiling at 95°C for 5 min. After the addition of 1 ml of non-denaturing buffer (1% Triton X-100, 50 mM Tris/HCl pH 7.5, 300 mM NaCl, 5mM EDTA, 0,02% sodium azide) and 10 mM IAA, immunoprecipitations were performed as described above.

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