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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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### **Citation**

Hassink, G. C. (2006, May 22). *The role of the ubiquitin system in human cytomegalovirus-mediated degradation of MHC class I heavy chains*. Retrieved from <https://hdl.handle.net/1887/4414>

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**CHAPTER 3 UBIQUITINATION IS ESSENTIAL FOR  
HUMAN CYTOMEGALOVIRUS US11-  
MEDIATED DISLOCATION OF MHC  
CLASS I MOLECULES FROM THE  
ENDOPLASMIC RETICULUM TO THE  
CYTOSOL**

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*Biochemical Journal* 358 (2001) pp369-77



## Abstract

Human cytomegalovirus encodes two glycoproteins, US2 and US11, which cause rapid degradation of MHC class I molecules, thus preventing recognition of virus-infected cells by the immune system. This degradation process involves retrograde transport or 'dislocation' of MHC class I molecules from the endoplasmic reticulum (ER) to the cytosol, where they are deglycosylated by an N-glycanase and degraded by the proteasome. At present it is unknown whether ubiquitination is required for US2- and US11-mediated dislocation and degradation of MHC class I molecules. Here, we show that in E36ts20 hamster cells, which contain a temperature-sensitive mutation in the E1 ubiquitin-activating enzyme, US11-mediated degradation of MHC class I molecules is strongly impaired at the non-permissive temperature, indicating the necessity for ubiquitination in this process. We next addressed the question of whether ubiquitination is a condition for the retrograde movement of MHC class I molecules from the ER to the cytosol, or whether ubiquitination is merely required for recognition of dislocated MHC class I molecules by the proteasome. In the absence of a functional ubiquitin system, complexes of US11 and MHC class I molecules accumulate in the ER. In this state the membrane topology of MHC class I molecules does not significantly change, as judged from proteinase K digestions. Thus the results indicate that a functional ubiquitin system is essential for dislocation of MHC class I molecules from the ER to the cytosol.

## Introduction

The proteasome mediates degradation not only of cytosolic and nuclear proteins <sup>1,2</sup>, but also of proteins that reside in the endoplasmic reticulum (ER) <sup>3,4</sup>. This discovery implied that ER proteins destined for degradation must be transported, or 'dislocated', back to the cytosol for them to access the degradation machinery. Dislocation and subsequent degradation by the proteasome has been observed for an increasing number of ER proteins in several different organisms (reviewed in <sup>2-5</sup>). Of particular interest is the degradation of MHC class I molecules, which are neither misfolded, nor destined for degradation by cellular signals like most known substrates, but which are targeted for degradation by either of two human cytomegalovirus (HCMV)-encoded glycoproteins, US2 or US11 <sup>6,7</sup>.

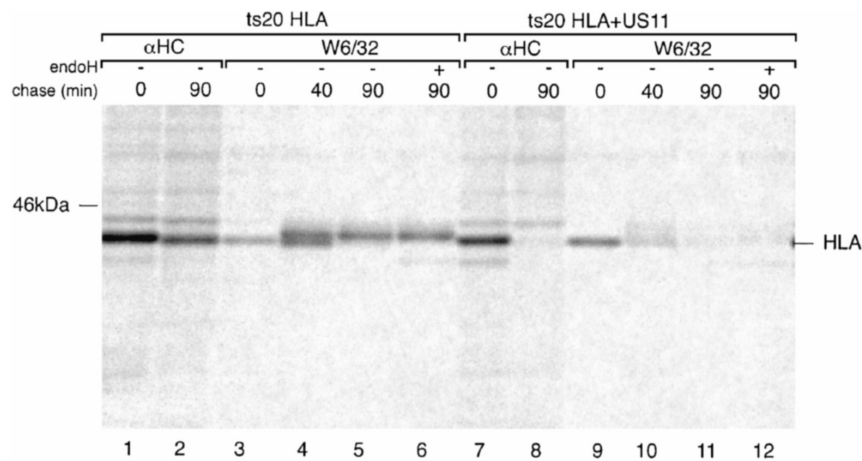
Proteasomes were implicated in this degradation process based on the

use of proteasome inhibitors, which caused accumulation of deglycosylated MHC class I breakdown intermediates in the cytosol <sup>6,7</sup>. Such cytosolic deglycosylated intermediates were also found in the course of degradation of T-cell receptor (TCR)α chains transiently expressed in non-T-cells, using proteasome inhibitors <sup>8,9</sup>. Based on co-precipitation of deglycosylated MHC class I heavy-chain intermediates with sec61b <sup>7</sup>, the translocon is probably involved in both anterograde and retrograde transport of proteins across the ER membrane. Experiments involving sec61p mutants in yeast support this notion <sup>10-12</sup>.

Covalent attachment of ubiquitin chains to lysine residues is the main mode of targeting proteins to proteasomes. Ubiquitinated proteins are recognized by subunits of the 19 S cap of the 26 S proteasome <sup>2,4</sup>. Involvement of ubiquitination in degradation of ER proteins by the proteasome would therefore be anticipated, and indeed ubiquitination was shown to be essential for degradation of several ER substrates <sup>13-18</sup>. Some substrates, however, are degraded by the proteasome in a ubiquitin-independent manner <sup>19-21</sup>.

At present, it is unknown whether ubiquitination is required for US2- and US11-mediated dislocation and degradation of MHC class I molecules. Shamu and co-workers <sup>22</sup> showed that a proportion of the MHC class I heavy chains are ubiquitinated in US11-expressing cells, but their results failed to show whether ubiquitination is a condition for dislocation or whether it is merely a consequence of dislocation to the cytosol.

The attachment of multiple ubiquitin molecules to proteins involves the action of three enzymes, the ubiquitin-activating enzyme, designated E1, a ubiquitin-conjugating/carrier enzyme or E2, and a ubiquitin ligase or E3 <sup>2</sup>. We used the E36ts20 hamster cell line, which contains a temperature-sensitive mutation in the E1 ubiquitin-activating enzyme, to monitor the effects of a disrupted ubiquitin system on US11-mediated degradation of MHC class I molecules. The results indicate that the early step of dislocation of MHC class I molecules from the ER to the cytosol is blocked when the ubiquitin system is not functional. This suggests dual ubiquitin dependence, since it is anticipated that ubiquitination is also needed for recognition of the degradation substrate by the proteasome at a later stage of the degradation pathway.



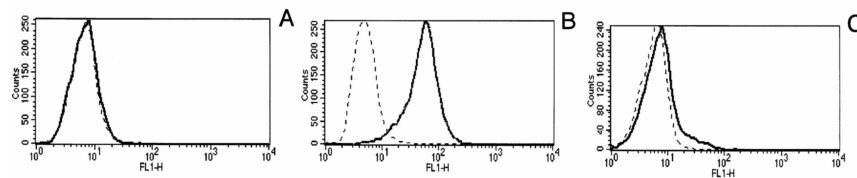
**Figure 1. US11 mediated degradation of HLA-A2 in ts20 hamster cells.** ts20 cells stably transfected with HLA-A2 alone (ts20 HLA), or both HLA-A2 and US11 (ts20 HLA+US11) were pulse-labelled with [<sup>35</sup>S]methionine/ [<sup>35</sup>S]cysteine as described in Materials and Methods for 8 min and chased for the periods indicated at 33°C. Lysates were split and subjected to immunoprecipitation with polyclonal antiserum against the unfolded MHC class I heavy chain (αHC), or conformation dependent antiserum (W6/32), which recognizes the complex of MHC class I heavy chain with β2m and peptide. Samples in lanes 6 and 12 were treated with endoglycosidase H (endoH). The immunoprecipitates were separated on an SDS/polyacrylamide (10%) gel. The experiment was repeated twice with very similar results, the data shown here are from one of these experiments.

## Results

### Reconstitution of US11-mediated breakdown of HLA-A2 in hamster cells

The hamster cell line ts20, containing a temperature-sensitive mutation in the E1 ubiquitin-activating enzyme, and the parental cell line E36<sup>23</sup> were stably transfected with human HLA-A2, HCMV US11 or both genes together (see the Experimental section).

HLA-A2 transfected into ts20 cells is stable at the permissive temperature of 33°C (Figure 1), and matures from free heavy chains detected by rabbit anti-heavy-chain serum (Figure 1, lanes 1 and 2) to mature, fully assembled molecules as detected by the conformation-dependent antibody W6/32 (Figure 1, lanes 3–5). For HLA-A2, W6/32 reactivity not only requires association with β2m but also binding of antigenic peptide<sup>25</sup>. W6/32-reactive MHC class I molecules obtained after 90min of chase are endoglycanase H (endoH)-resistant (Figure 1, lane 6), indicating their transport to the Golgi apparatus. Cell-surface expression of transfected HLA-A2 molecules was analysed using flow cytometry (Figure 2). Figure 2(B) shows that ts20 cells



**Figure 2. HLA-A2 surface expression is down-regulated in ts20 cells expressing US11.**

A FACS experiment was performed to assess the cell-surface expression of HLA-A2 in different cell lines. At least 105 cells were sorted per measurement. The x axes represent fluorescence intensity (arbitrary units) and y axes represent cell counts. Cells grown at 33°C were incubated with W6/32 antiserum and subsequently with goat anti-mouse antiserum coupled with FITC (black lines). As a control, cells were incubated with goat anti-mouse-FITC alone (dotted lines). (A) Wild-type ts20 cells; (B) ts20 cells transfected with HLA-A2; (C) ts20 cells transfected with HLA-A2 and US11.

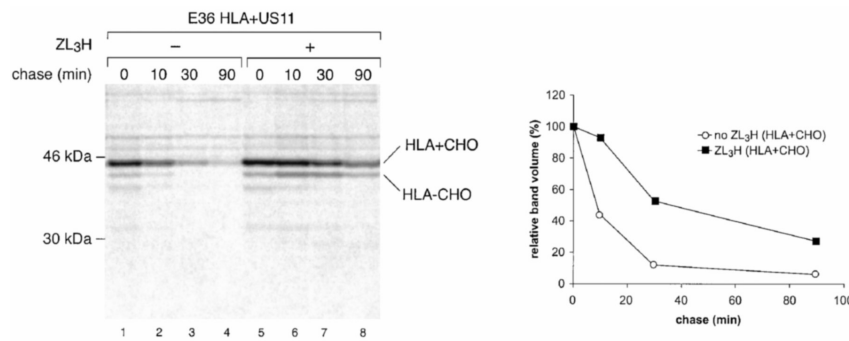
transfected with HLA-A2 express these molecules on their surface. Taken together the data indicate that HLA-A2 is stably expressed in hamster cells, associates with hamster b2m, acquires antigenic peptides and is transported to the cell surface at 33°C.

When US11 was co-transfected together with HLA-A2 into ts20 cells, the MHC class I molecules were degraded at the permissive temperature of 33°C, as assessed by immunoprecipitations with either polyclonal rabbit anti-(MHC class I heavy chain) serum (Figure 1, lanes 7 and 8) or the conformation-dependent antibody W6/32 (Figure 1, lanes 9–12). The amount of surface-expressed HLA-A2 was dramatically lower in the cells that express HLA-A2 and US11 (Figure 2C), which is attributed to efficient degradation of HLA-A2 at the permissive temperature.

Very similar results were obtained with E36 cells transfected with HLA-A2 and/or US11 (see below). Pulse-chase analysis of ts20 cells transfected with US11 alone showed that endogenous hamster MHC class I molecules were not degraded (results not shown).

#### **Inhibition of the proteasome interferes with dislocation and degradation of HLA-A2**

Treatment of E36 HLA+US11 cells (Figure 3) and ts20 HLA+US11 cells (results not shown) with the proteasome inhibitor ZL3H delays US11-mediated degradation of MHC class I molecules and causes a deglycosylated intermediate to accumulate in the cytosol. In the experiment shown in Figure 3 an antiserum against the cytoplasmic tail of HLA-A2 was used for immunoprecipitation, which recognizes both folded and unfolded molecules.

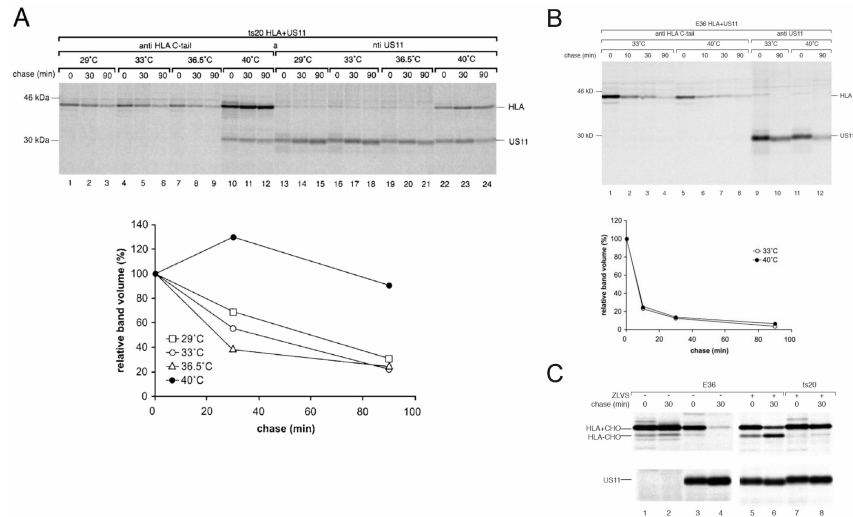


**Figure 3. Effect of proteasome inhibitor on US11-mediated degradation of HLA-A2 in E36 cells.** E36 HLA+US11 cells were pulse-labelled (10min) with [35S]methionine/[35S]cysteine as described in the Experimental section and chased for 0, 10, 30 and 90min at 33°C, with or without the proteasome inhibitor ZL3H. HLA-A2 molecules with (HLA+CHO) or without (HLA-CHO) carbohydrate were immunoprecipitated with antiserum against the cytoplasmic tail of MHC class I molecules and separated on an SDS/polyacrylamide gel (10% gel). In the graph, relative volumes of bands representing HLA-A2 molecules with carbohydrate (HLA+CHO) were plotted (measured in arbitrary units, counts/mm<sup>2</sup>). The experiment was performed twice with similar results; this figure represents one such experiment.

In the graph on Figure 3 the relative amounts of glycosylated HLA molecules only are represented. The disappearance of glycosylated HLA molecules is a measure of the dislocation of HLA molecules to the cytosol, where they are degraded (in the absence of ZL3H) or accumulate as deglycosylated breakdown intermediates (in the presence of ZL3H). The graph indicates that inhibition of the proteolytic activity of the proteasome also inhibits the dislocation process.

At later time points in the chase (>30min) the deglycosylated HLA molecules also begin to disappear (Figure 3, lane 8). It is not very likely that this disappearance is related to the concentrations of proteasome inhibitor being too low. Degradation of the deglycosylated breakdown intermediate has also been observed in other cell lines, at higher concentrations of proteasome inhibitor<sup>6,7,30</sup>. Rather, at later time points cytosolic deglycosylated HLA molecules may be degraded by other proteases, which may form a by-pass for the inhibited proteasome.





**Figure 4. Ubiquitination is required for US11-mediated degradation of MHC class I heavy chains.** (A) ts20 HLA+US11 cells were pre-incubated at the indicated temperatures for 2h and subsequently starved, pulse-labelled for 8min with [35S]methionine/[35S]cysteine as described in the Experimental section, and chased for 0, 30 and 90min at the same temperature, as shown. Immunoprecipitations were performed sequentially with antiserum against the cytoplasmic tail of MHC class I molecules (lanes 1–12) and anti US11 serum (lanes 13–24), respectively. Samples were separated by SDS/PAGE (10% gel) and a PhosphorImage was generated. The panel shows one experiment that was performed five times in a comparable fashion, all with similar results. (B) Pulse-chase analysis of E36 HLA+US11 cells (10min pulse labelling, chase times as indicated) at 33°C and 40°C using antiserum against the cytoplasmic tail of MHC class I molecules (lanes 1–8) and anti US11 serum (lanes 9–12). Samples were separated by SDS/PAGE (10% gel) and a PhosphorImage was generated. The experiment was performed twice, with similar results; this panel represents one such experiment. For (A) and (B), in the graphs relative volumes of HLA-A2 bands, from lanes (A) 1–12 and (B) 1–8, were plotted (measured in arbitrary units, counts/mm<sup>2</sup>). (C) E36 and ts20 cells were infected with vaccinia viruses expressing HLA-A2 (lanes 1 and 2), or simultaneously with vaccinia viruses expressing HLA-A2 and US11 (lanes 3–8) as indicated in the Experimental section. After 4h of incubation the cells were pulsed for 10min and chased for 30min. All incubations were performed at 40°C. Proteasome inhibitor ZLVS was included where indicated. HLA-A2 and US11 were immunoprecipitated sequentially, the precipitates were analysed by SDS/PAGE and PhosphorImages were generated as described in the Experimental section. Bands representing HLA-A2 molecules with (HLA+CHO) or without (HLA-CHO) carbohydrate are indicated.

**A defective ubiquitination system results in stabilization of HLA-A2 molecules and causes HLA-A2-US11 complexes to accumulate**

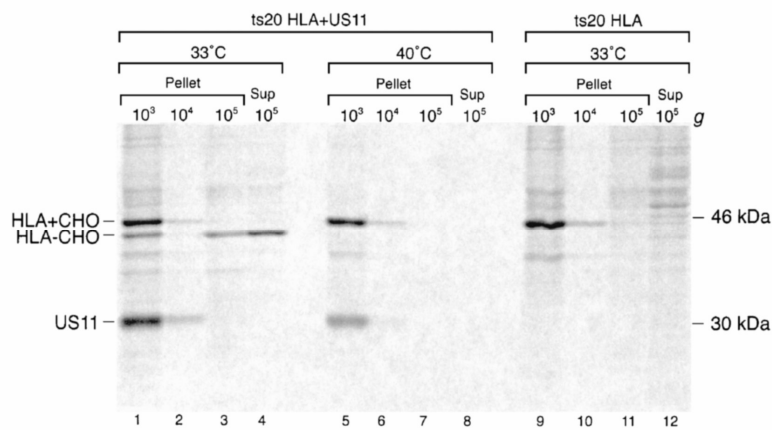
US11-mediated degradation of HLA-A2 was observed in ts20 cells at any temperature below the non-permissive temperature of 40°C (Figure 4A, lanes 1–9). When the experiment was performed at 40°C, the temperature at which the ubiquitin-activating enzyme E1 is inactive, HLA-A2 molecules were stabilized (Figure 4A, lanes 10–12). This result indicates that the ubiquitin system plays an essential role in US11-dependent degradation of HLA-A2. A

considerable amount of US11 co-precipitated with stabilized HLA-A2 at 40°C (Figure 4A, lanes 10–12), while at the same time HLA-A2 co-precipitated with US11 (Figure 4A, lanes 22–24). The identity of the co-precipitating molecules was confirmed in re-immunoprecipitation experiments (results not shown). Thus whereas binding of US11 to HLA-A2 at the permissive temperature is quickly lost due to the degradation of HLA-A2, the interaction of US11 and HLA-A2 is stabilized when ubiquitination is prohibited. In the course of the chase the mobility of US11 and HLA-A2 altered slightly, probably owing to a post-translational modification, which was not pursued further at this stage.

In the E36 parental cells US11-mediated degradation of HLA-A2 was as efficient at 33°C as it was at 40°C (Figure 4B). The stabilization of HLA-A2 in ts20 cells observed at the non-permissive temperature is therefore solely due to paralysis of the ubiquitin system. In E36 cells not expressing US11, HLA-A2 was stable (Figure 4C, lanes 1 and 2). The MHC class I breakdown intermediate was observed in E36 cells expressing HLA-A2 and US11, but not in ts20 cells expressing HLA-A2 and US11, incubated at 40°C in the presence of proteasome inhibitor ZLVS (Figure 4C, lanes 5–8). Note that in the experiment shown in Figure 4(C) HLA-A2 and US11 were expressed using recombinant vaccinia viruses. During later stages of vaccinia virus infection host protein synthesis is shut off, and expression of viral (trans-)genes is generally high. To avoid artefacts due to loss of stoichiometry, cells were monitored for expression of HLA and US11 only 4h after infection. The ratio between US11 and HLA was influenced by using a multiplicity of infection of 1.5 for vvUS11 and of 1 for vVA2, to ensure sufficient US11 to degrade HLA-A2 at the permissive temperature. Degradation and stabilization of MHC class I molecules occurred in a similar fashion in stably transfected and vaccinia virus-infected cells.

#### **HLA-A2–US11 complexes accumulate in the membrane fraction at 40°C**

Is ubiquitination required only for targeting of dislocated MHC class I molecules to proteasomes, or is ubiquitination also a prerequisite for the actual dislocation of these molecules? To distinguish between these possibilities, a cell-fractionation experiment was carried out (Figure 5). ts20 cells expressing HLA-A2 and US11 or HLA-A2 only were metabolically labelled for 30min in the presence of a proteasome inhibitor. At 33°C in the double transfectant, US11 and all of the glycosylated MHC class I resided exclusively in the membrane fraction (Figure 5, 1000g and 10000g pellets, lanes 1 and 2). In contrast, the deglycosylated intermediate was found predominantly in the cytosolic fraction (Figure 5, 100000g supernatant, lane 4). This pattern reflects dislocation to the cytosol and deglycosylation of MHC class I heavy chains, similar to what was shown earlier for endogenous MHC

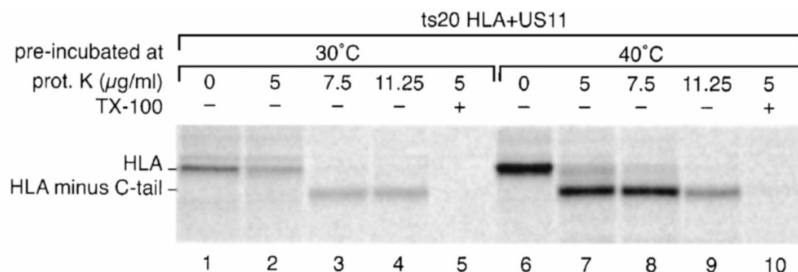


**Figure 5. In the absence of ubiquitination HLA-A2 accumulates in the membrane fraction.**

ts20 HLA+US11 and ts20 HLA cells were labelled with  $[^{35}\text{S}]\text{methionine}/[^{35}\text{S}]\text{cysteine}$  as described in the Experimental section for 30min in the presence of ZL3H either at 33°C or 40°C, as indicated. The cells were homogenized with a Dounce homogenizer and subjected to repeated centrifugations at the indicated g forces (see also the Experimental section). HLA-A2 and US11 were immunoprecipitated simultaneously from each fraction using antisera against the cytoplasmic tails of MHC class I and US11. Samples were separated by SDS/PAGE (10% gel). Sup, supernatant. Bands representing HLA-A2 molecules with (HLA+CHO) or without (HLA-CHO) carbohydrate are indicated.

class I molecules in US11-transfected U373 cells <sup>6</sup>.

When a similar experiment was performed on cells that were pre-incubated at 40°C, a deglycosylated intermediate was not detected. Instead, all of the HLA-A2 material was found in the membrane fraction along with US11 (Figure 5, lanes 5–8). EndoH digestion experiments showed that the HLA-A2 population rescued at 40°C is endoH-sensitive (results not shown). Together, these data suggest that, when the ubiquitin system is disrupted, HLA-A2 molecules are not dislocated to the cytosol, but remain associated with a pre-Golgi compartment, most likely the ER. HLA-A2 from cells that do not express US11 resided in the membrane fractions (Figure 5, lanes 9–12), as expected, since these molecules are stable during the 30min labelling period.



**Figure 6. HLA-A2 molecules that accumulate at 40°C do not have an altered membrane insertion.** ts20 HLA+US11 cells were pre-incubated at the temperatures indicated and labelled with [<sup>35</sup>S]methionine/[<sup>35</sup>S]cysteine for 10min at the same temperature. The cells were semi-permeabilized and digested with proteinase K (prot. K) at 0°C, as described in the Experimental section, at concentrations indicated. Immunoprecipitations were performed using antibodies against the ER luminal domain of HLA-A2 heavy chains (monoclonal antibody HCA2). Samples in lanes 5 and 10 were treated with 1% (v/v) Triton X-100 (TX-100) prior to proteinase K digestion. Samples were separated by SDS/PAGE (10% gel). The experiment was repeated twice with similar results; the data shown in this figure are from one such experiment.

#### **At 40°C, membrane insertion of MHC class I molecules is not altered**

We next asked the question: does the membrane topology of HLA-A2 molecules change while they accumulate in the ER in the absence of ubiquitination? Previous experiments have indicated that lysines in the cytosolic tail of HLA-A2 heavy chain can be substituted without affecting dislocation and degradation<sup>22</sup>. The cytosolic tail is therefore not likely to be the primary target for ubiquitination. Assuming that dislocation involves ubiquitination of MHC class I molecules themselves, this would then require ER luminal domains to become exposed to the cytosolic ubiquitin system. Such conformational changes would take place before attachment of ubiquitin, they may be independent of the ubiquitin machinery, and they may possibly be induced by the binding of US11 to the MHC class I molecules. Proteinase K digestion of MHC class I molecules in semi-permeabilized ts20 HLA+US11 cells, kept at 30°C, results in products that lack their cytosolic C-terminal tail (Figure 6, lanes 1–4). At 40°C, MHC class I molecules accumulate in the ER membrane, as was shown above. Figure 6 (lanes 6–9) shows that this state does not involve dramatic changes in the membrane insertion of MHC class I molecules, such as partial dislocation to the cytosol, since proteinase K digestion again resulted in removal of the cytosolic tail only.

## Discussion

The results described here indicate that dislocation of the MHC class I molecules across the ER membrane is fully dependent on a functional ubiquitin system. Obviously, not only dislocation requires ubiquitin, but subsequent proteasomal degradation is likely to require ubiquitination as well, since proteins are usually targeted to the proteasome via the attachment of at least four ubiquitin molecules<sup>1,2,31</sup>. It is not clear whether a single ubiquitination event accounts for both aspects of the degradation process, or whether several distinct ubiquitination events would be required.

The degradation process mediated by US2 and US11 now evidently includes interaction of the MHC class I molecule with the translocon channel (shown for US2-mediated degradation<sup>7</sup>), involvement of the ubiquitin system (<sup>22</sup>and this study), dislocation to the cytosol and de-glycosylation<sup>6</sup> and degradation by the proteasome<sup>6,7</sup>.

### **Effects of disruption of the ubiquitin system**

Our experiments indicate that complexes of US11 and HLA-A2 are retained in the ER membrane when ubiquitin conjugation is blocked. Similar ER retention was observed for proteins that are degraded via endogenously initiated, quality-control associated, dislocation. For example, the TCR $\alpha$  chain<sup>17</sup> and RI322, a truncated ER luminal form of ribophorin A<sup>32</sup>, are also not dislocated when ubiquitination is prevented. Mutation of the E2 ubiquitin carrier enzymes involved in the degradation pathway of a soluble misfolded yeast protein CPY\* (mutated carboxypeptidase yscY) similarly caused the accumulation of the substrate in the ER<sup>13</sup>. Together, these results indicate that the ubiquitin system plays an important role in both endogenously and exogenously triggered dislocation of proteins across the ER membrane.

### **Possible mechanisms of ubiquitin-dependent dislocation of MHC class I molecules**

Although the cytoplasmic tail of the MHC class I heavy chain is obviously the part most accessible to the ubiquitination machinery, it is unlikely to be the primary target for ubiquitination, as removal of the lysine residues from the cytoplasmic tail of HLA-A2 neither prohibits its dislocation and degradation, nor its ubiquitination<sup>22</sup>. Interestingly, removal of all lysines from the  $\alpha$  subunit of the TCR affects neither its dislocation nor its degradation, whereas a functional ubiquitination system is still required for dislocation of the mutant  $\alpha$  chain<sup>9,17</sup>. These results can be explained by assuming that an interacting protein is ubiquitinated, not the degradation

substrate itself, leading to ubiquitin-mediated dislocation of the substrate in trans. In the case of  $\alpha$ 1-antitrypsin Z it was shown that the chaperone calnexin is ubiquitinated in trans, leading to degradation of  $\alpha$ 1-antitrypsin Z. In the course of the process calnexin is released from the substrate and is neither dislocated nor degraded itself<sup>33</sup>.

Since for MHC class I molecules not all of the lysines were removed, it is still a possibility that ubiquitination within ER luminal domains of the MHC class I heavy chains mediates dislocation. Apart from lysine residues within a protein, the N-terminus can serve as a target for ubiquitination. Exclusive N-terminal ubiquitination was found for the short-lived cytosolic protein MyoD and the Epstein–Barr virus membrane protein LMP1 (latent membrane protein 1)<sup>34,35</sup>. Thus if MHC class I molecules would be ubiquitinated while still in the ER membrane, this would either involve luminal lysine residues, or the N-terminus, which then would have to be exposed to the cytosol prior to attachment of ubiquitin. Proteinase K digestion experiments (Figure 6) suggest that in the absence of a functional ubiquitin system the membrane topology of HLA-A2 does not change drastically. Assuming that MHC class I molecules are ubiquitinated themselves prior to dislocation, the conformational change may involve only minor exposure of HLA-A2 luminal sequences to the cytosol, which cannot be observed after proteinase K digestion. Alternatively, the topological change may not take place at the non-permissive temperature, because this by itself already requires the action of the ubiquitination machinery. Binding of MHC class I molecules to US11, which occurs without ubiquitination, apparently does not induce obvious topological changes of the MHC class I molecules.

The molecular basis of the dislocation reaction still remains obscure. It has been proposed that cytosolic chaperones such as the heat-shock proteins Hsp70 and Hsp90 facilitate the actual dislocation<sup>36,37</sup>. The important role of ubiquitin in the dislocation process supports the suggestion that the proteasome provides the pulling force that extracts proteins from the ER membrane<sup>38,39</sup>.

#### **The roles of US2 and US11**

Inhibition of ubiquitination did not only cause a dramatic stabilization of HLA-A2, but also revealed strong binding of US11 to HLA-A2 (Figure 4A). This obviously confirms that the binding of US11 to MHC class I molecules precedes ubiquitination and that MHC class I heavy chain is not released from US11 when ubiquitination is prevented. Since US11 is not degraded and has never been found in the cytosol along with MHC class I molecules (ref<sup>6</sup> and Figure 5), the release from US11 must take place before MHC class I heavy chain is dislocated to the cytosol.

Although rather extensive research has been done on the US2- and US11-mediated degradation of MHC class I molecules, the exact functions of US2 and US11 in the dislocation process have remained elusive. Binding of US2 and US11 to MHC class I could alter the conformation of the latter such that it is recognized by the ER quality-control machinery and is degraded via the constitutive dislocation pathway. US2 and US11 could also mimic components of the quality-control machinery, which normally target misfolded cellular proteins for destruction. Although to date no sequence similarities have been found between US11 or US2 and any cellular protein, one could speculate that US2 and US11 themselves function as E3 enzymes, which specifically catalyse ubiquitination of MHC class I molecules, thus inducing their dislocation and degradation.

## Materials & Methods

### Materials

The hamster cell lines E36 and E36ts20 (the latter referred to as ts20 throughout the rest of the article) <sup>23</sup> were maintained at 33°C under an air/CO<sub>2</sub> (19:1) atmosphere in minimum essential medium (MEMa; Gibco BRL) supplemented with 10% (v/v) foetal calf serum (Greiner), penicillin (100units/ml) and streptomycin (100µg/ml; Gibco BRL).

A polyclonal antiserum against the cytoplasmic tail of human leucocyte antigen subtype A2 (HLA-A2) was produced in rabbits using the synthetic peptide KGGSYSQAASSDSAQGS. Polyclonal antiserum against US11 was raised in rabbits using the synthetic peptide LSLTLFDEPPPLVETEPL, derived from the cytoplasmic tail of US11. Polyclonal rabbit serum specific for unfolded MHC class I heavy chains has been described <sup>24</sup>, as well as W6/32 monoclonal antiserum, specific for assembled MHC class I heavy chain–β<sub>2</sub>-microglobulin (β<sub>2</sub>m) complexes <sup>25</sup> and monoclonal antiserum HCA2 against the luminal domain of HLA-A2 heavy chains <sup>26</sup>.

The proteasome inhibitors carboxybenzyl-leucyl-leucyl-leucinal (ZL3H) and carboxybenzyl-leucyl-leucyl-leucyl vinylsulphone (ZLVS) were from the Peptide Institute (Osaka, Japan) and used at a final concentration of 20µM. A pCDNA3 derivative, encoding HLA-A0201 under control of the CMV promoter, was constructed as follows. The plasmid pSRa1Neo-HLA-A2, provided by Dr J. Alejandro Madrigal (The Anthony Nolan Research Centre, The Royal Free Hospital, London, U.K.), was digested with XhoII, after which the fragment encompassing the HLA-A2 coding region was rendered blunt by digestion of the 5' protruding ends. The blunt-end ligated fragment was digested with HindIII and ligated with the large EcoRV/HindIII fragment of pSP72 (Promega). The HLA coding region was then re-isolated

as a BglII/XhoI fragment and cloned into BamHI/XhoI-digested pCDNA3 (Invitrogen). The resulting plasmid was designated pLUMC9901.

The plasmid containing US11 and the puromycin-resistance gene (pIE-puro US11) has been described elsewhere <sup>27</sup>.

#### **Stable transfection of HLA-A2 and US11 into E36 and ts20 cell lines**

The plasmids containing HLA-A2 and US11 were transfected separately or together into ts20 and E36 cells, which were both kept at 30–33°C, using Fugene (Roche) according to the manufacturer's directions. After 48h, G418 (Geneticin; Gibco BRL) or puromycin (ICN) was added to the transfected cells at amounts that killed untransfected cells within a few days. Clones were tested for expression of the genes of interest by radiolabelling and immunoprecipitation (see below).

#### **Pre-incubation of cells at different temperatures**

Prior to pulse–chase analysis cells were pre-incubated at 40°C (the non-permissive temperature) or other temperatures as indicated in the figures. Culture flasks containing cells were transferred from the stove to a closed waterbath at the chosen temperature, in which they were incubated for 2h. Starvation and pulse–chase incubations were all performed at the same temperature.

#### **Pulse–chase analysis, immunoprecipitation and SDS/PAGE**

After trypsinization, suspended cells were starved in RPMI 1640 medium (BioWhittaker) without methionine and cysteine for approx. 1h at either 33°C or 40°C. The proteasome inhibitor ZL3H was added where indicated. The cells were metabolically labelled with 250µCi of 35S-labelled Redivue Promix (a mixture of L-[35S]methionine and L-[35S]cysteine; Amersham) per 10<sup>7</sup> cells in starvation medium (pulse). For chase samples radioactive medium was replaced with RPMI 1640 medium supplemented with 1mM methionine and 0.1mM cysteine. Pulsed and chased cells were lysed in Nonidet P-40 (NP-40) containing lysis buffer as described in <sup>24</sup>.

Before immunoprecipitation, two subsequent preclears were performed using normal rabbit and normal mouse sera pre-coupled with mixed (1:1) Protein A–Sepharose and Protein G–Sepharose beads. Immunoprecipitation was performed on the precleared lysate for 2–4h at 4°C using specific antiserum pre-coupled to Protein A/G–Sepharose beads. Beads were washed with NET buffer [50mM Tris/HCl, pH7.4/150mM NaCl/5mM EDTA/0.5% (v/v) NP-40] supplemented with 0.1% SDS and subsequently boiled in sample buffer [40mM Tris/HCl, pH8.0/4mM EDTA/8% (w/v) SDS/40% (v/v) glycerol/0.1% Bromophenol Blue] for 5–10min. Samples



were loaded on SDS/polyacrylamide gels and run overnight. Gels were dried and exposed to a storage PhosphorImaging screen, which was scanned in a Personal Molecular Imager FX and analysed with Quantity One software (Bio-Rad).

#### **FACS analysis of cell-surface expression of MHC class I molecules**

FACS analysis of cell-surface expression of HLA-A2 was performed as described by Rensing et al. <sup>28</sup> using W6/32 antiserum and goat anti-mouse-FITC conjugate (Jackson, WA, U.S.A.).

#### **Infection with vaccinia virus**

Infection with vaccinia virus was performed as described previously <sup>29</sup>. Briefly, E36 and ts20 cells were infected simultaneously with recombinant vaccinia viruses expressing HLA-A2 (vvA2) and HCMV US11 (vvUS11), respectively. Cells (106/ml in RPMI without serum) were infected with virus at a multiplicity of infection of 1 for vvA2 and of 1.5 for vvUS11. After 1h of infection, complete medium was added to the infected cells. Cells were subsequently incubated at 40°C for 3h followed by a 1h starvation period in media lacking methionine and cysteine in the presence of proteasome inhibitor where indicated. Pulse-chase experiments were performed as described above.

#### **Subcellular fractionation**

Fractionation of cells was performed essentially as described by Wiertz et al. <sup>6</sup>. In brief, about 10<sup>7</sup> cells were starved and labelled for 30min with <sup>35</sup>S-Redivue Promix as described above. Cells were washed and resuspended in 1ml of homogenization buffer [0.25M sucrose/10mM triethanolamine/10mM potassium acetate/1mM EDTA, pH7.6, supplemented with protease inhibitors leupeptin (0.1mM) and 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF; 10mM)]. Cells were placed on ice and homogenized in a Dounce homogenizer (50 strokes) with a tight-fitting pestle. The homogenate was spun for 10min at 4°C and 1000g in an Eppendorf centrifuge. The pellet was saved and the supernatant was spun for 30min at 4°C and 10000g. The pellet was again saved and the supernatant spun for 1h at 4°C and 100000g. The latter two centrifugations were performed in a TLA 120.2 fixed-angle rotor, operated in a Beckman Optima® TLX ultracentrifuge. All pellets were resuspended in NP-40 lysis buffer. HLA-A2 and US11 were immunoprecipitated simultaneously from the solubilized pellets and the 100000g supernatant, and separated by SDS/PAGE. PhosphorImaging was performed as described above.

### **Proteinase K digestions**

After pre-incubation at 30°C or 40°C, cells were labelled with 35S-Redivue Promix as described above and subsequently resuspended in 200µl of cold permeabilization buffer (containing 25mM Hepes, pH7.2, 115mM potassium acetate, 5mM sodium acetate, 2.5mM MgCl<sub>2</sub> and 0.5mM EGTA). Proteinase K (Life Technologies) was added in concentrations as indicated and digitonin (Calbiochem) was added in concentrations that were always 100 times higher than the proteinase K concentrations used. The cells were incubated with the digestion mix at 4°C for 20min. Proteolysis was stopped by centrifuging cells at 14000g for 10min and resuspending the pellets in 1ml of NP-40 lysis buffer containing 2mM PMSF. For denaturing immunoprecipitations pellets were resuspended in 150µl of lysis buffer containing 2mM PMSF and 1% (w/v) SDS and boiled for 5min, after which 800µl of lysis buffer was added. Immunoprecipitations, SDS/PAGE and PhosphorImaging were performed as described above.

### **Acknowledgements**

This work was supported by grant no. 901-02-218 from the Council for Medical Research from the Netherlands Organisation for Scientific Research (to M.B.), grant no. RUL 1998-1791 from the Dutch Cancer Society (to G.H.), the Boehringer-Ingelheim Foundation (to C.H.) and grant no. R37 AI 33456 from the National Institutes of Health (to C.H.). We thank Dr Hidde L. Ploegh for critical reading of the manuscript, Dr Dominic Tortorella for helpful suggestions, Daphne van Leeuwen for assistance in the FACS study and Jan Beentjes for preparing the figures. We thank Dr J. Alejandro Madrigal for providing the pSRa1Neo-HLA-A2 construct.

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