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Downregulation of MHC class I molecules by human cytomegalovirus-encoded US2 and US11

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CHAPTER 7

Summary and Discussion

Summary and discussion

HCMV encodes several proteins that interfere with cross talk between infected host cells and immune effector cells. The viral gene products can reduce surface levels of antigen presenting MHC class I molecules. Several aspects may influence the degree of MHC class I down-modulation by HCMV, and thereby the success of immune escape by the virus.

First of all, the success of immune evasion is likely to be influenced by the specificity of MHC class I down-modulation, as a complete down-regulation of MHC class I surface expression would render cells susceptible to NK cell lysis. A selective down-modulation of only those alleles that present viral antigens to cytotoxic T cells (mainly HLA-A and –B locus products) could ensure both T and NK cell escape.

Several HCMV-encoded proteins contribute to the down-regulation of MHC class I molecules in the course of infection. They differ from each other in mechanism and selectivity in down-modulatory effect on the different MHC class I locus products. US3, an immediate early HCMV gene product, mainly affects surface expression of tapasin-dependent MHC class I alleles [1]. By blocking TAP, US6 prevents peptide transport into the ER and subsequent peptide loading at early and late times post infection [2,3]. This affects surface expression of all MHC class I alleles. In spite of this, surface expression of HLA-E molecules can be preserved by supplying it with a TAP-independent peptide [4,5]. US2 and US11, expressed at early times post infection, target newly synthesized MHC class I molecules for degradation [6,7]. The effect of these viral gene products on surface expression of different subsets of MHC class I molecules will be discussed below.

In the studies described in Chapters 2,3 and 4, we aimed to clarify how, and to what extent, US2 and US11 can contribute to the efficiency and specificity of MHC class I down-regulation. Previous studies had mainly focussed on the mechanism of interference with antigen presentation [6,8-11]. With the cell lines and antibodies used in these studies, it is difficult to deduce effects on individual MHC class I locus products. To enable detection of the effects of US2/US11 on individual MHC class I alleles, we introduced different class I heavy chain constructs into a murine cell line which co-expresses human β2m. We evaluated the effect on MHC class I surface expression using flow cytometry, as this is most relevant with respect to NK and T cell receptor interactions. Moreover, MHC class I molecules that appear stable in the presence of US2 and US11 in pulse-chase experiments, could nevertheless be withheld from the surface via other mechanisms than degradation. As shown in Chapter 2, US11 can cause ER retention of MHC class I molecules that are not targeted for degradation.

Flow cytometry studies showed that US2 selectively down-modulates HLA-A2, -B27, and HLA-G, but not HLA-B7, HLA-Cw3, or HLA-E (Chapter 3). Similar studies with US11 showed differences in selectivity, with HLA-A2, -B7, -B27, -Cw3 being sensitive, and HLA-G and -E being insensitive to US11-mediated down-regulation (Chapters 2 and 4). These flow cytometry studies complemented and refined previously performed pulse chase experiments evaluating stability changes of MHC class I molecules in the presence of US2 or US11 [9,12,13], as well as previously performed *in vitro* binding studies performed with US2 and various MHC class I alleles [14]. Not all HLA-C molecules are insensitive to US2, as HLA-Cw7 and -Cw2 were down-regulated in its presence [15]; our own unpublished results).

It is clear that there are sensitivity differences between the various MHC class I locus products, but also between gene products of a single locus subgroup, for down-modulation by US2 and US11. Our next step was to unravel the determinants for this selectivity. This would allow sensitivity predictions for a larger variety of MHC class I molecules.

Selectivity of US2-mediated degradation of MHC class I molecules

Based on crystal structure data of HLA-A2-β2m-US2 complexes and sequence alignments for the region of class I implicated in US2 binding, we hypothesized that allelic variation in the α2/α3 ER-lumenal region might explain the resistance of HLA-B7, -Cw3 and -E as opposed to the sensitivity of HLA-A2, -B27 and -G [16,17]. In Chapter 4, we investigated whether we could convert the resistance of HLA-B7 and HLA-E by replacing those residues that are assumed to prohibit an interaction with US2, with the corresponding residues found in US2-sensitive molecules. The results from experiments with HLA-B7 $^{ET(L)Q}$ and HLA-E ^{QRTD} mutants showed that residues in this region are

indeed important sensitivity determinants. Even a single mutation in HLA-E (H181R) significantly altered the sensitivity to down-modulation by US2.

HLA-Cw3 differs at positions 183 (E) and 184 (H) from many US2-sensitive class I molecules (carrying D and A/P at positions 183 and 184, respectively). However, this variation appeared not to be responsible for its resistance, as US2-sensitive HLA-Cw2 and -Cw7 also carry E183 and H184 residues. Nonetheless, sequence variation at this site may still affect the efficiency of down-modulation, as US2-resistant HLA-Cw3 molecules became somewhat more sensitive when E183D, H184P mutations were introduced (unpublished results). However, the presence of a positively charged lysine residue at position 173 is the most likely explanation for the resistance to US2-mediated down-regulation of HLA-Cw3, since US2-sensitive alleles, including the majority of HLA-C alleles, have a negatively charged glutamic acid at position 173.

The crystal structure data showed which region of the ER-lumenal part of HLA-A2 was implicated in US2 binding, but did not provide insights into possible interactions between transmembrane and cytosolic regions of class I heavy chains and the US proteins. Sequence alignments and studies using chimeras derived from US2-sensitive and -insensitive alleles make it unlikely that sequence variation outside the ER-lumenal part of HLA contributes to sensitivity differences to US2-mediated down-regulation (Chapter 4).

Sequence alignments of the region implicated in US2-binding show that HLA-A2, -B27, -E and -G are identical to their locus subgroup consensus sequences for this region. This makes it likely that the majority of HLA-A, B, -E and -G will be down-modulated in the presence of US2. However, not all HLA-B molecules are affected by US2, as exemplified by HLA-B7. Those residues that were shown to be responsible for the resistance of HLA-B7 can also be found in several other HLA-B alleles, including HLA-B8, -B40, -B41, -B42, and –B48. These HLA-B alleles, which are relatively common (found in 25-30% of the Caucasion population; F. Claas, unpublished observation), are likely to be insensitive to US2-mediated down-regulation.

We did not find any indication that sequence variation in the domains located outside the ER-lumenal region contributes to sensitivity differences between class I locus products for US2 (Chapter 4). The pulse chase experiments with HLA-G showed that class I heavy chains do not need to be equipped with a full-length tail in order to be a target for US2-mediated degradation (Chapter 4). However, heavy chains comprised of ER-lumenal regions only, are inadequate targets for US2, as illustrated by the resistance of the soluble HLA-G1 isoform (Chapter 4).

Requirements for US11-mediated degradation of MHC class I

For US11, the only MHC class I locus products that appear to be completely insensitive to down-modulation are HLA-G and HLA-E. Interestingly, all that is required to confer sensitivity to these two MHC class I locus products, is an extension of their cytoplasmic tail. HLA-G molecules naturally have a tail of 6 residues, and an extension of this tail with 27 residues derived from the tail of HLA-A2 resulted in a very efficient degradation of these mutants in US11-positive cells (Chapter 2). Surprisingly, a similar HLA-G mutant with 25 instead of 27 extra tail residues did not show this result. Apparently, the C-terminal lysine and valine residues were essential for degradation. HLA-E has a cytoplasmic tail that lacks only 4 residues compared to HLA-A molecules. HLA-E required only 2 extra residues (lysine and valine) to become sensitive to US11 (Chapter 4). This clearly shows that length and amino acid composition of the tail can be important determinants for sensitivity differences between MHC class I locus products.

However, lysine and valine residues are not the only requirements, nor are they essential in all cases. HLA-B alleles do not encode for C-terminal lysine and valine residues, but are nevertheless down-modulated by US11. Likewise, a mutant HLA-A2 with a tail as long as that of HLA-B molecules (HLA-A2delCKV) and HLA-A2 with the tail of HLA-E were still down-regulated by US11 (Chapter 4). These KV residues may, however, determine the effectiveness or threshold for down-modulation, as the mutant constructs that lacked these residues appeared to require higher levels of US11 than their wild type counterparts for a similar down-regulatory effect. Besides cytosolic residues, other regions of class I also play a role. Studies with HLA-A2/G and HLA-A2/E chimeras showed that the α1-α3 ER-lumenal residues influence sensitivity to US11 (Chapters 2 and 4). A crystal structure of complexed US11/MHC class I would be very helpful to narrow down the exact region and residues of class I heavy chains that are involved in US11 binding. The studies with HLA-A2/G

and HLA-A2/E chimeras did not reveal a role for the connecting peptide or trans-membrane regions in locus product sensitivity differences (Chapters 2 and 4).

The results from Chapters 2 and 4 give rise to the following model: In principle, all MHC class I cytoplasmic domains, with the exception of HLA-G, have an amino acid composition that can make them targets for US11-mediated degradation. However, a minimum of 29 class I tail residues has to go with either a favourable ER-lumenal region, or with lysine and valine tail residues in order to evoke sufficient down-modulation by US11. A favourable ER-lumenal region may bypass the function of the KV residues through a prolonged and/or stronger interaction with US11, thereby increasing the chances of getting dislocated to the cytosol for subsequent degradation by proteasomes.

The function of the KV, as well as of the other tail residues is still unclear. Addition of ubiquitin moieties to degradation substrates may provide a handle for extraction of proteins across the ER membrane. Enhancement of MHC class I down-regulation by KV residues may rely on the lysine, functioning as a potential ubiquitination site.

Using a cell line carrying a temperature-dependent defect in the E1 ubiquitin-activating enzyme, we have shown that ubiquitination is essential for US11-mediated dislocation of MHC class I molecules (Chapter 5). At the non-permissive temperature, MHC class I molecules remained in the ER lumen. These data clearly showed the importance of ubiquitination for the dislocation of MHC class I molecules induced by US11. However, it remains unclear which protein(s) need to be ubiquitinated to trigger the dislocation process.

A study by Shamu et al. showed that lysine residues in the tail of HLA-A2 are not essential for US11-mediated degradation. However, elimination of a mutant HLA-A2 lacking lysine residues in the cytoplasmic tail appeared to be retarded compared to wild type HLA-A2 [18].

Alternatively, the KV residues may merely facilitate access of components of the dislocation and degradation machinery to essential residues residing within another region of the tail. Phosphorylation can be an important signal for docking of E3 ligases, which in turn can ubiquitinate their substrates [19]. The tail of MHC class I molecules encodes several potential as well as acknowledged phosphorylation sites [20-25]. Substitutions of the known Y321 and S335 phosphorylation sites did not alter the sensitivity to US11-associated degradation (Chapter 2 and un-published results). More research will be required to unravel why the tail is essential for US11-mediated dislocation of MHC class I molecules.

Clearly, sequence variation in MHC class I molecules is an important determinant for their sensitivity to down-modulation by several of the US proteins. At present, it is unknown whether the sequence of the US region of laboratory HCMV strains differs from that in strains circulating in the population.

Besides the specificity, the degree of MHC class I down-regulation is likely important for a successful immune escape of HCMV. *In vivo*, MHC class I expression is upregulated in the presence of cytokines. When the expression of MHC class I was elevated *in vitro*, higher levels of US proteins were required for a sufficient down-modulatory effect (Chapter 3). Also, cells infected with a higher HCMV copy number show a higher degree of MHC class I down-regulation, compared to cells infected with lower copy numbers [26].

Why would HCMV encode for so many proteins with the same purpose, namely to prevent immune detection through down-modulation of MHC class I molecules? In part, this may reflect the fact that these proteins show temporal expression patterns. Moreover, expression of more than one immune evasion gene at a given time during infection could ensure a more efficient down-modulation of one particular MHC class I locus product. A concerted action of US proteins could also affect a broader range of MHC class I locus products. Moreover, by using different mechanisms, an exhaustion of one particular route can be prevented.

Two of the HCMV-encoded proteins, US2 and US11, show similar temporal expression patterns, and seem to act very similarly. We evaluated the need for expression of both these proteins.

Differences between US2 and US11

Whereas specificity of US2-mediated down-modulation seems to rely mostly on a region at the junction of the α2/α3 domain, our data indicate that the conditions are different and more complicated for US11. Although ER-lumenal residues of HLA class I

do play a role, replacement of residues LHLE in the HLA-E α2/3 region by QRTD did not affect its sensitivity to US11 (unpublished results). Association of US2 and US11 with distinct domains of class I molecules may contribute to a broader defense of HCMV. Together, US2 and US11 could attack a wider variety of MHC I molecules.

Whereas US2 does not require MHC class I tail residues, US11-mediated down-modulation depends largely on this region. Interestingly, the opposite has been observed for US2 and US11 deletion mutants. When US2 was deprived of its tail residues it was no longer functional, while tailless US11 could still target class I molecules for degradation [27,28].

Whereas US2 accompanies its targets to the cytosol, US11 seems to remain behind in the ER [7,29]. This is accompanied by a prolonged stability of US11, compared to US2. In the presence of proteasome inhibitors, a deglycosylated cytosolic intermediate can be observed for US2.

At present, several proteins and protein complexes have been identified that could form a link between the dislocation process and proteasomal degradation of ER lumenal proteins in general, and MHC class I molecules in particular. The cytosolic ATPase p97 (also called VCP or Cdc48 in yeast) and its cofactor, a dimer of Ufd1 and Npl14, have been implicated in the retrograde transport of ER substrates [30,31].

Recently, a new protein has been identified, which interacts with the transmembrane region of US11 [32,33]. This protein, named Derlin-1, is a homologue of yeast Der1, which was shown to be required for degradation of misfolded lumenal ER proteins [34]. Derlin-1 is predicted to have 4 transmembrane regions and cytosolic N- and C-termini. Derlin-1 is essential for US11-mediated dislocation of class I molecules, but not for dislocation of class I heavy chains induced by US2. Interestingly, dislocation of US2 itself is derlin-1 dependent [35].

Derlin-1 interacts with another protein called VCP-interacting membrane protein (VIMP). VIMP is predicted to have a single transmembrane spanning domain, a short ER lumenal domain and a relatively long (~132 aa) cytosolic region. The cytoplasmic region of VIMP links the p97/VCP complex to the ER membrane [36]. The p97-Ufd1-Npl4 complex can recognize poly-ubiquitin chains, and ATP hydrolysis by p97 could provide the driving force to finally pull the substrate into the cytosol [37].

It is unknown if other ER-localized proteins facilitate the disposal of US2 and US11 targets. It is tempting to speculate on US2 and US11 making use of proteins normally engaged in quality control and disposal of misfolded ER proteins. It is known that in the absence of other complex components, e.g. the β2m light chain or peptides, free class I heavy chains cannot meet the quality standards in the ER and are targeted for degradation [38]. Different ER chaperones are assisting different stages of the folding and assembly of MHC class I molecules. In Chapter 6, we investigated if US2 and US11 can act at relatively early stages of MHC class I folding and complex formation, i.e. before association with β2m. We found that both US2 and US11 can target free HCs for degradation, as evaluated in a β2m-negative cell line. They did so equally well, in cells lacking β2m and in β2m-reconstituted cells.

Interestingly, the presence of the proteasome inhibitor ZL_3H severely decreased the efficiency of heavy chain dislocation, but only when mediated by US11 and only in cells lacking β2m. This was not observed with a similar experimental set-up including US2, nor in the absence of proteasome inhibitor, nor in $US11^+$, $\beta2m^+$ cells supplemented with ZL_3H.

The ER chaperones that are generally found in association with free class I HCs are BiP, calnexin, and the oxidoreductase ERp57. BiP is known to retain many misfolded proteins in the ER [39]. Studies with mutant Kar2p (the yeast homologue of BiP), have shown an association between the ATPase activity of Kar2p with release of malfolded proteins into the cytosol [40]. In another report, in which the release of misfolded immunoglobulin light chains from BiP was studied, the dislocation seemed to be tightly coupled to proteasome activity [41].

Calnexin serves as a chaperone for many glycoproteins [42,43]. It may also contribute to oxidative folding, as it acts in conjunction with the oxido-reductase ERp57 [44]. It is possible that class I heavy chains require a more reduced oxidation status for efficient dislocation. There are indications that proteasome inhibitors may interfere with calnexin / oxidoreductase interactions [45,46]. In cells treated with lactacystin, a redistribution of ER chaperones was observed. Upon proteasome inhibition, CNX, CRT, and ER degradation substrates (but not BiP, PDI,

glucosyltransferase, or ERp57) accumulated in a pericentriolar quality control compartment derived from the ER [45,46].

The fact that we found an abrogation of US11-mediated dislocation in the presence of proteasome inhibitor and exclusively in cells lacking β2m-expression may imply that US11 uses partially similar mechanisms for discarding HCs as the endogenous pathway used by FO-I cells to dispose of unassembled class I molecules. It also implies that it uses a mechanism distinct from that used by US2. More research will be required to elucidate the exact pathways of protein disposal.

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