Utrecht University

The role of proteasomes in the ERADication of MHC class I by HCMV US2 and US11

Author:
Arianne Brandsma
3383695

Supervisors:
Dr. Ir. Robert Jan Lebbink
Prof. Dr. Emmanuel Wiertz

Master Infection & Immunity
Virology, Department of Medical Microbiology
UMC Utrecht, the Netherlands
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Content

Abstract

Herpesviruses have developed powerful immune evasion strategies leading to lifelong persistence of the virus, although strong immune responses are present. Viral immune evasion proteins, including several proteins of human cytomegalovirus (HCMV), interfere with the MHC I presentation pathway at almost every step to prevent recognition by cytotoxic T cells. In particular, HCMV US2 and US11 are very effective in inducing rapid dislocation and degradation of MHC I. Because of this clear phenotype, US2- and US11-induced MHC I degradation has often been used to study ER-associated degradation (ERAD). The main steps in the ERAD pathway are substrate recognition, targeting of substrates to membrane-bound E3 ligases, dislocation into the cytosol and degradation by the proteasome. Studying the mechanisms of US2- and US11-induced dislocation and degradation of MHC I has led to the identification of many components of ERAD and a better understanding of general ERAD mechanisms. Our understanding of the mechanisms of US2 and US11 has also greatly increased. US2 and US11 both use distinct membrane complexes and pathways for the induction of MHC I dislocation and degradation. Proteasomes are essential for the degradation of ERAD substrates. Furthermore, there is convincing data arguing that proteasomes are involved in the dislocation of, at least some, ERAD substrates. Via the AAA-ATPase subunits of the proteasome a direct pulling force could be exerted on substrates but proteasomes could also be involved in another way. Increasing our understanding of the different ERAD pathways will ultimately lead to targeting these pathways for the treatment of HCMV infection and other ERAD-associated diseases.

Introduction

Viruses have developed ingenious ways to take over host cells and create optimal conditions for virus replication. Viruses have to escape anti-viral responses, mainly exerted by the immune system, in order to successfully infect a host. Adaptation of viruses to these immune responses is of vital importance for their existence. Using dedicated immune evasion proteins, herpesviruses are masters in evading or modulating the immune system.

Herpesviruses are large and enveloped double-stranded DNA viruses that can be classified into three families: the α-, β-, and γ-herpesviruses (1). Almost every adult is persistently infected with one or more herpesviruses. Still, herpesviruses are very species-specific and to date eight human herpesviruses have been identified. Herpes simplexvirus types 1 and 2 (HSV-1 and HSV-2) and varicella-zoster virus (VZV) belong to the α-herpesviruses, human cytomegalovirus (HCMV) and human herpesvirus types 6 and 7 (HHV-6 and HHV-7) are members of the β-herpesviruses, and Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) are γ-herpesviruses (2).

An estimated 400 million years ago herpesviruses emerged and have co-evolved since then with their hosts, which led to extensive adaption to their host's immune system (3). In most cases there is a balance between controlling the herpesvirus infection by the host and failing to totally eradicate the virus, leading to a life-long infection. Infections with herpesviruses are usually asymptomatic, but in some cases cause serious complications, especially in immunocompromised hosts (4-6). Furthermore, HCMV can cause congenital defects and EBV and KSHV are associated with developing certain malignancies (7).

Despite powerful immune responses against herpesviruses, these viruses are known to establish persistent infections. In addition, reinfection by the same viral strain may occur in immune individuals, indicating the presence of extensive immune evasion mechanisms (8). Herpesviruses target innate immunity by interfering with cytokine and chemokine signaling, preventing NK cell activation and subsequent killing of infected cells, blocking the complement system, and abrogating signaling of pathogen recognition receptors like TLRs and RIG-I-like receptors (9-12). Adaptive immune responses are manipulated by production of viral Fc receptors functioning as decoy receptors thereby inhibiting antibody-mediated effector responses, and by targeting MHC-II and in particular MHC-I antigen processing and presentation (13-16).

MHC class I molecules (MHC I) present antigenic peptides at the cell surface which can be recognized by cytotoxic T cells (CTLs) (17). These peptides are derived from proteasomal degradation of viral or cellular cytosolic proteins. MHC I is a membrane protein destined for expression at the cell surface and is therefore synthesized by a ribosome docked on the endoplasmic reticulum (ER) membrane. During translation, MHC I is N-linked glycosylated at one specific asparagine residue, which is important for protein folding (18). Calreticulin is an MHC I chaperone and is essential for correct MHC I folding. Upon folding of MHC I, β2 microglobulin (β2m) associates with MHC I (19). Peptides need to be transported into the ER lumen to bind MHC I molecules, which is facilitated by the Transporter associated with Antigen Processing (TAP). TAP is a heterodimer of TAP1 and TAP2 and is also part of the MHC class I peptide-loading complex (PLC) (20, 21). Besides TAP, heterodimers of MHC I and β2m, calreticulin, tapasin, ERp57 and protein disulfide isomerase (PDI) are part of the PLC (22). Tapasin stabilizes the expression of TAP and brings TAP close to MHC I molecules. Tapasin also retains MHC I in the ER when it is not yet loaded or loaded with a low affinity peptide. However, tapasin-associated MHC I loaded with low affinity peptides is sometimes able to escape the ER and move towards the Golgi. Now, tapasin functions as a cargo receptor for packaging the MHC I into

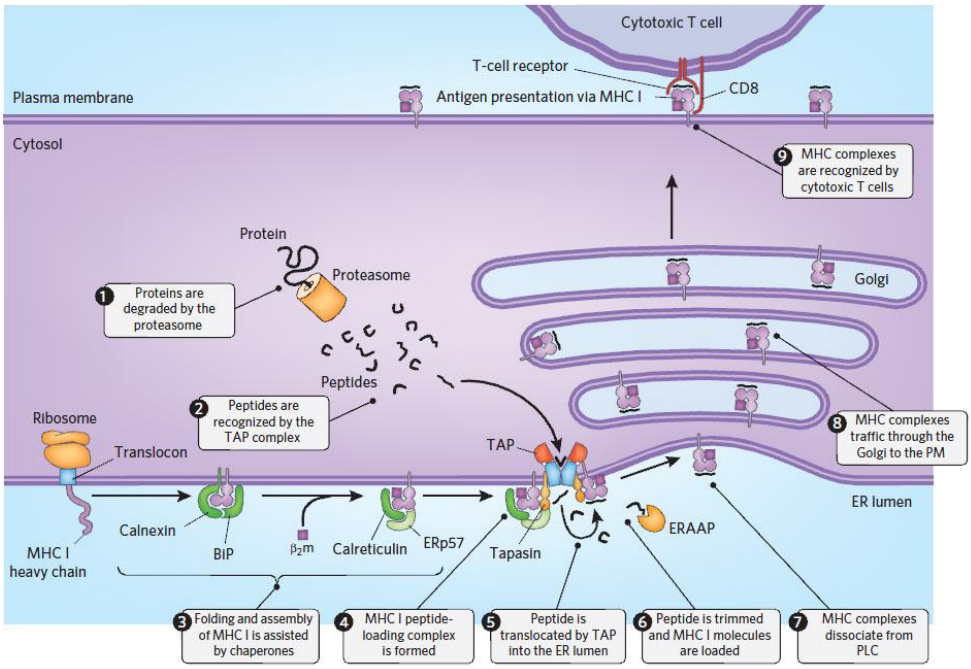

Figure 1. Role of the peptide-loading complex in MHC class I assembly and loading. Proteins are degraded by the proteasomes (1) and these peptides are recognized by TAP, which is part of the peptide-loading complex (PLC) and transports the peptides into the ER (2, 5). Newly synthesized MHC I heavy chains are folded by the aid of chaperones (3) and subsequently the PLC is formed (4). Peptides are edited and trimmed and loaded onto MHC I (6). Stable MHC I complexes trigger dissociation from the PLC and are transported via the Golgi to the plasma membrane, where the peptide can be recognized by cytotoxic T cells (7-9). Parcej *et al.* (24).

COP I coated vesicles, which are then transported back to the ER. In this way, MHC I can be in a cycle from the ER to the Golgi and back until it is loaded with a high-affinity peptide (23). Tapasin also binds the thiol oxidoreductase ERp57 and heterodimers of tapasin and ERp57 function in stabilizing the PLC and editing of peptides. The final component of the PLC, PDI, stabilizes the MHC I/β2m heterodimer in a conformation that enables peptide binding. Thus, the PLC ensures optimal MHC I folding and efficient peptide loading (Figure 1). Once the heterotrimeric complex of MHC I, β2m and peptide is properly assembled, it travels through the Golgi, where the N-glycan is further processed, until it finally reaches the plasma membrane (PM) (24).

Antigen presentation via MHC I to CTLs is a very important mechanism for the immune system to recognize virus-infected cells. The presented peptides are mainly derived from endogenous proteins, but can also be derived from virus-encoded proteins when a cell is infected. However, via a process termed cross-presentation exogenous proteins can be presented in MHC I molecules as well. This occurs exclusively in dendritic cells and is essential in priming T cell responses to viruses (25).

Herpesviruses encode a wide range of proteins that specifically interfere with MHC I presentation (Figure 2), indicating the importance of CTL recognition in eliminating infected cells. First, host shutoff proteins like the virion host shutoff (vhs) protein of HSV inhibit protein synthesis and thereby reduce MHC I expression (26). Likewise, recognition of infected cells by CTLs is abrogated when the host shutoff proteins of EBV and KSHV, BGLF5 and SOX respectively, are expressed (27-29). Second, MHC I trafficking is altered by several immune evasion proteins. HCMV has three proteins dedicated to target MHC I in the ER for degradation by the proteasome (30-32). HHV-6 and HHV-7 express U21, a protein capable of redirecting MHC I to lysosomal compartments for degradation (33, 34). In a similar way, KSHV K3 and K5 enhance endocytosis of MHC I and target it to the lysosomal compartment (35, 36). Transport from the ER to Golgi is delayed by the VZV ORF66 protein, affecting

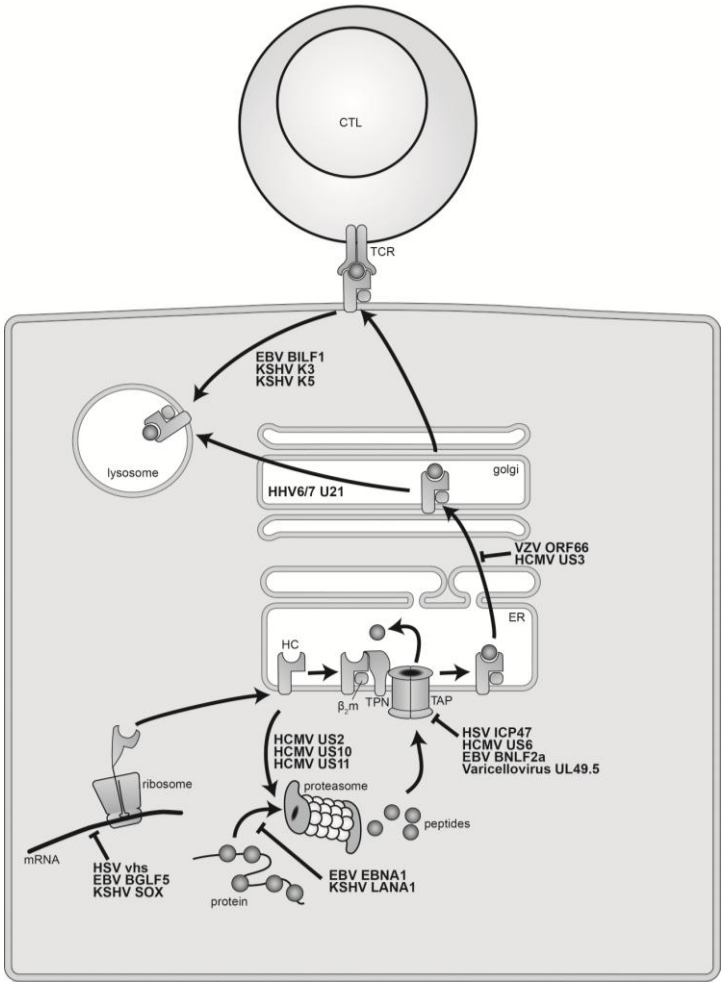

Figure 2. Interference of human herpesviruses with MHC I antigen presentation. HSV vhs, EBV BGLF5 and KSHV SOX all induce the degradation of host cell mRNAs, thus also degrading mRNA encoding MHC I heavy chains. HCMV US2, US10 and US11 induce dislocation of MHC I into the cytosol, thereby enhancing their degradation by the proteasome. U21, encoded by HHV-6 and HHV-7, redirects MHC I molecules to lysosomal compartments. EBV BILF1 and KSHV K3 and K5 induce endocytosis and target surface MHC I for lysosomal degradation. VZV ORF66 and HCMV US3 retain MHC I in the ER. EBV EBNA1 and KSHV LANA1 prevent their own degradation, in this way preventing the generation of peptides which can be presented in MHC I. HSV ICP47, HCMV US6, EBV BNLF2a and the UL49.5 proteins of several varicelloviruses inhibit TAP-mediated transport of peptides into the ER. β2m, β2 microglobulin; CTL, cytotoxic T lymphocyte; TCR, T cell receptor; TPN, tapasin. Horst *et al.* (41).

MHC I expression (37). Finally, TAP is a major target, since TAP inhibition prevents import of antigenic peptides into the ER. ICP47, a HSV protein, competes with peptides for TAP binding (38, 39) and UL49.5 of several varicelloviruses arrests TAP in a conformation incompatible with translocation of peptides (40). Thus, herpesviruses seem to inhibit every step of the MHC I presentation pathway.

Understanding more of the immune evasion strategies of viruses, and in particular herpesviruses, is of crucial importance in designing strategies for therapeutic intervention. Knowledge about MHC I evasion is used to improve herpesvirus vaccines and to improve vaccine vectors by deleting immune evasion molecules from the genome. Furthermore, targeting cancer cells through oncolytic viruses or inducing tumor-specific CTLs, but also novel strategies to enhance gene therapy and transplant protection all exploit MHC I evasion strategies by human herpesviruses (41). Besides that, the increasing repertoire of viral evasion molecules are an important toolbox for immunological and cell

biological studies and have led to the discovery of numerous proteins involved in the normal processes of the cell.

Here, we review the immune evasion strategies of the most studied human herpesvirus HCMV and we will focus on US2 and US11, immune evasion proteins targeting MHC I for proteasomal degradation. We will discuss the pathways of ER-associated degradation (ERAD) and will highlight the factors of ERAD essential for the function of US2 and US11. Proteasome structure and assembly, as well as the role of proteasomes in dislocation of ERAD substrates will be reviewed. Finally, we will present a model for US2- and US11-mediated MHC I degradation.

Human cytomegalovirus immune evasion

Like the other herpesviruses, HCMV has a large genome (~235 kb) which has recently been described to encode more than 700 open reading frames (42). Only ~40-50 of these viral genes are necessary for replication in the lytic phase of virus infection, suggesting that the rest of the genome is involved in host-interactions, including proteins that modulate or dampen host immune responses (43). Proteins of one well studied gene cluster, the unique short (US) region, are exceptional in immune evasion. The US2-US11 genes are thought to have originated from gene duplication and encode the viral proteins US2, US3, US6, US10, and US11. These proteins are known to interfere with MHC I antigen presentation.

US3 and US6 interfere with MHC I presentation

US3 is abundantly transcribed in the immediate-early (IE) phase and can already be detected one hour post-infection (p.i.), yet around 5 hours p.i. the transcription of US3 rapidly declines (44, 45). US3 is a type 1 membrane glycoprotein located in the ER and contains a signal peptide, an ER luminal domain, a transmembrane region and a short C-terminal tail. US3 proteins can oligomerize and bind to MHC I heavy chains (HCs) in a high affinity interaction (43). MHC I is bound to US3 before peptide loading; US3 complexes with β2m-associated MHC I HC in a high affinity interaction and this impairs further maturation of MHC I. In this way, intracellular transport to the Golgi and PM is prevented, leading to retention of MHC I in the ER. Expression of US3 is also sufficient to retain MHC I in the ER (46). Furthermore, US3 binds members of the PLC, including TAP, tapasin and PDI, leading to delayed maturation of MHC I by interfering with peptide loading (47, 48). US3 only affects tapasin-dependent HLA alleles, since tapasin-independent alleles could still be expressed on the surface of cells transfected with US3 (47).

Interestingly, US3 gene products can undergo alternative splicing, resulting in a single spliced (SS) 17 kDa protein or a double spliced (DS) 3.5 kDa protein, the latter of which no function has been assigned to (45). The 17 kDa protein is smaller because it lacks the transmembrane domain of the unspliced (US) 22 kDa US3. US3 SS also binds to tapasin, but does not induce MHC I retention in the ER. US3 SS actually competes with US3 US for tapasin binding. In the presence of US3 SS, the interaction between US3 US and tapasin decreases and MHC I peptide loading is restored (49). Thus, these different US3 isoforms function as novel auto-regulators of their own immune evasion capacity.

US6 is an ER-resident type 1 membrane protein and a well-characterized inhibitor of TAP (50, 51). US6 functions by inhibiting ATP binding to TAP1 and also by preventing the conformational changes of TAP that normally occur upon peptide binding (52), thereby abrogating peptide transport into the ER. The N-terminal ER-luminal part of US6 contains the functional domain and expressing this soluble part of US6 is sufficient for TAP inhibition (50, 53). US6 interacts with the ER-luminal loops of TAP1 and TAP2, indicating that US6 might induce conformational changes that block ATP-binding rather than directly obstructing the ATP-binding site itself (53). Furthermore, when DCs were exposed to soluble US6, this completely inhibited cross-presentation, indicating a crucial role for TAP in cross-presentation (54).

US2 and US11 induce MHC I dislocation

US2 and US11 are type 1 membrane proteins with an ER localization. US2 and US11 directly interact with MHC HC in the ER and target MHC I for degradation in the cytosol (30, 31). The transport of

MHC I from the ER to the cytosol is termed dislocation (or retrotranslocation). Once in the cytosol, MHC I is degraded in an ubiquitin- and proteasome-dependent manner. Expression of US11 alone induces rapid MHC I degradation and reduces the half-life of MHC I HC to <1 min (31). Despite the sequence similarity (45%) between US2 and US11 and the fact that they both induce MHC I dislocation, US2 and US11 seem to use different mechanisms for dislocation (55). Besides the ER luminal domain, both the cytosolic and TM domains of US2 are necessary to induce MHC I HC dislocation, while US11 only requires the TM domain (56). Furthermore, US2 targets properly folded MHC I HCs, whereas US11 can also target MHC I HCs of which folding is incomplete (57). When looking at kinetics, the degradation of MHC I induced by US2 appears to be slower than the degradation induced by US11. US2 and US11 also have distinct HLA class I haplotype preferences (58).

Degradation of proteins by the proteasome is, in general, ubiquitin-dependent and degradation of MHC I by US2 and US11 requires a functional ubiquitin system (59). Firstly, ubiquitin needs to be activated and this is carried out by E1, the ubiquitin-activating enzyme. ATP is used to form a thioester bond between the glycine residue of ubiquitin and the cysteine residue of E1. Secondly, the activated Ub is transferred to an E2 ubiquitin-conjugating enzyme. Finally, the Ub is transferred to a lysine residue of a specific substrate, after the Ub-E2 complex is recruited by an E3 ubiquitin protein ligase which specifically binds to substrates (Figure 3). In some cases, Ub is transferred to E3 prior to transfer of Ub to the substrate (60). Polyubiquitinated substrates occur after multiple ubiquitin-conjugation cycles and K48-linked polyubiquitination targets substrates for proteasomal degradation (61). Deubiquitination enzymes (DUBs) can remove Ub from a substrate. Lysine-independent ubiquitination of substrates has been reported, although it is much less frequent. Besides lysine residues, the ubiquitination of the cytoplasmic tail of MHC I can occur via serine, threonine or cysteine residues and this can also induce degradation of MHC I (62, 63).

US2 and US11 differ in their requirement of ubiquitinated MHC I for dislocation. Ubiquitination of MHC I HC via lysines is not required for the initial step of dislocation induced by US2, since HC that lack lysines in the cytosolic tail can still occur in the cytosol (57). However, when all lysines of the HC were substituted, US2 could no longer induce dislocation. US11, on the other hand, was still capable of dislocating these HCs into the cytosol (64), even though the dislocation of HCs still required a functional Ub system. This indicates that other residues of MHC I HC or possibly other substrates, i.e. not MHC I HC, need to be ubiquitinated for dislocation to occur. Overall, these data indicate that US2 and US11 use different mechanisms to mediate dislocation and degradation of MHC I.

More recently, US10, another ER-resident glycoprotein, was shown to downregulate surface expression of HLA-G, a specific subset of MHC I (32). Despite similar localization of US2, US11 and

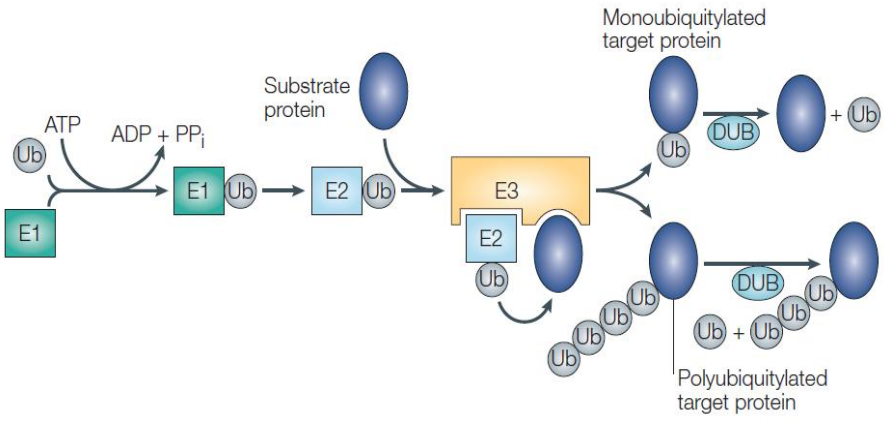

Figure 3. The ubiquitin-conjugation cycle. Ubiquitin (Ub) is activated by E1 and the activated Ub is transferred to E2. E3 recruits both Ub-E2 and the substrate protein and facilitates the transfer of Ub to the substrate. Multiple ubiquitin-conjugation cycles lead to polyubiquitinated proteins. Ub conjugated to substrates can be removed by DUBs. Liu *et al.* (60).

US10, the mechanism of dislocation and degradation used by US10 seems to be distinct from that of US2 and US11. More research is needed to gain insight into this mechanism of dislocation.

UL18

Interfering with MHC I presentation and reducing surface expression of MHC I can activate NK cells, leading to cytolysis of the virus-infected cells. To circumvent this, HCMV encodes UL18, a protein from the unique long region that is a non-functional MHC I homologue (65). Despite low sequence similarity between the extracellular domains of UL18 and MHC I (~21%), the secondary structure is very similar (Figure 4). UL18 can bind LIR-1, an inhibitory receptor, with a very high affinity compared to host MHC I (66, 67). LIR-1 is expressed on NK cells and binding of UL18 to LIR-1 induces an inhibitory signal that will prevent the NK cell from killing the host cell. Thus, downregulation of surface MHC I expression is compensated by encoding decoy MHC I molecules. UL18 can also bind LIR-1 expressed on other immune cells and might induce activating signals in some situations (68), yet these alternative roles of UL18 need to be further investigated.

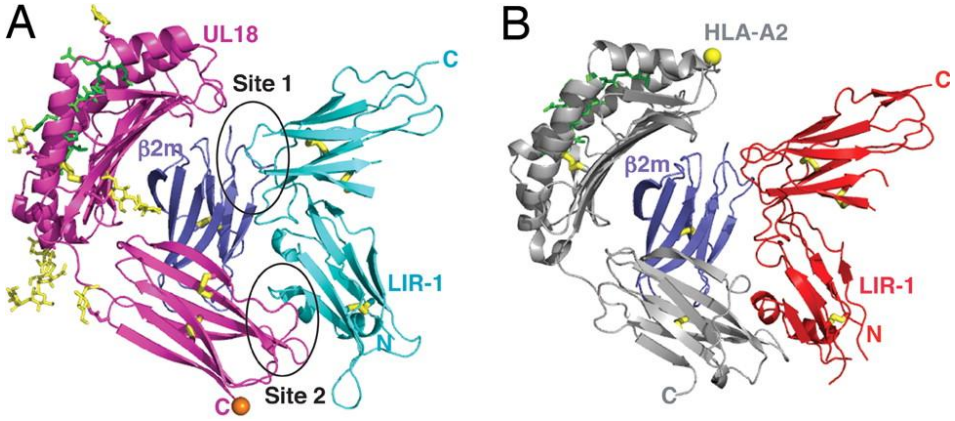

Figure 4. Structure of UL18 in comparison with HLA-A2, both associated with LIR-1. A and B. Structures of UL18 (A) and HLA-A2 (B) in complex with LIR-1. Note that UL18, like HLA-A2, interacts with β2m. Ordered carbohydrates on UL18 are thin yellow sticks, disulfide bonds are thick yellow sticks. A predicted O-glycosylation site of UL18 is indicated by an orange sphere, the single N-glycosylation site of HLA-A2 is indicated as a yellow sphere. Black open circles indicate the binding interaction sites of UL18 with LIR-1. N indicates the N-terminal end, C indicates the C-terminal end of the proteins. Yang *et al.* (67).

Viral miRNAs

Micro-RNAs (miRNAs) are small regulatory RNAs encoded by small genome regions and are non-immunogenic. Not surprisingly, herpesviruses encode for miRNAs that can function in immune evasion (69, 70). Primary miRNA transcripts are first processed by Drosha in the nucleus and subsequently further cleaved by Dicer in the cytoplasm, generating 20-25 nucleotide long dsRNA molecules. One of these miRNA strands binds to RISC (RNA-induced silencing complex) and if RISC binds a target mRNA with a complementary sequence, the target mRNA is cleaved by RISC leading to the degradation of the mRNA and silencing of the target gene (71). More often, the target mRNA is not a full match and only translation of the mRNA is inhibited by RISC (72).

HCMV encodes miR-UL112-1, the first identified miRNA targeting MICB for down-regulation. MICA and MICB are ligands for the NK cell activating receptor and upregulation of these ligands induces NK cell mediated cytolysis. Expression of miR-UL112-1 induced a reduction of MICB levels, leading to a decrease in binding to NKG2D, the NK cell activating receptor, and inhibition of NK cell-mediated lysis (73). Up to now, 11 miRNA precursors and 14 mature miRNAs of HCMV have been

identified (74) and further functional characterization is ongoing. It is not excluded that more miRNAs are encoded by HCMV and that besides host mRNA also viral mRNA is targeted as a mechanism of regulation.

Thus, the coevolution of HCMV with humans, and most importantly the human immune system, has led to extensive immune evasion strategies of HCMV. Presentation of viral peptides in the context of MHC I seems to be very important for the elimination of virus-infected cells, since this pathway is targeted by viral molecules at almost every level.

ER-associated degradation

Studying the mechanisms that underlie immune evasion by US2 and US11 has led to the discovery of common cellular processes. Dislocation of proteins appears to be a key feature in ERAD of misfolded proteins. Induction of dislocation of MHC I HCs by US2 and US11 is widely used to study ER protein dislocation and degradation, since this is a very rapid and effective process. This greatly contributed to the identification of constituents of ERAD, including p97, Derlin family members and ER-resident E3 ligases.

Secretory proteins pass through the ER on their way to membrane compartments or the cell exterior and approximately 20% of all proteins are secretory proteins. Numerous chaperones are present in the ER to aid folding and maturation of these proteins, yet folding is error-prone and about one-third of all newly synthesized proteins are degraded (75). ERAD is part of the quality control system of the ER, which retains immature proteins in the ER and is involved in initiating their degradation. The importance of ERAD is stressed by the growing list of human diseases associated with ERAD substrates, including cystic fibrosis, diabetes and Alzheimer disease (76). Furthermore, accumulation of misfolded proteins can induce the unfolded protein response (UPR), which upregulates ER chaperones and components of ERAD but can also lead to apoptosis when uncompensated (77).

Substrate recognition

The first step in ERAD is recognition of misfolded substrates and distinguishing them from folding intermediates. In principal, any secretory protein can become an ERAD substrate, creating a very heterogeneous group of protein substrates. Currently, it is unclear how terminally misfolded proteins are recognized, but some processes have been elucidated. A mannose-timer model has been described for the selection of glycoproteins (Figure 5), in which mannose acts as a molecular timer for the degradation of misfolded proteins (78-80).

Most secretory proteins are N-linked glycosylated in the ER and shortly after this, glucosidase I and II remove two glucose residues from the glycan structure. This triggers the recruitment of calnexin (CNX) and calreticulin (CRT), lectin-like chaperones, which promote folding of glycoproteins and retain them in the ER. When the third glucose residue is eventually removed by glucosidase II, the glycoprotein is released from CNX/CRT and travels to the Golgi (81). UDP-glucose glucosyl transferase (UGT1) is able to counteract glucosidase II by reglucosylating the glycans when proteins are incorrectly or immaturely folded. These proteins are again recognized by CNX/CRT and folding continues. If glycoproteins are terminally misfolded, sequential activity of ER mannosidase I (ERManI) and ER degradation-enhancing α-mannosidase-like protein (EDEM) removes the α(1,2)-mannose residues and exposes the terminal α(1,6)-mannose. This is a signal for ERAD: receptors with a mannose 6-phosphate receptor homology (MRH) domain, including OS-9 and XTP3-B, recognize these substrates and deliver them to ERAD ligases (82). Activity of ERManI and EDEMs also prevents re-glycosylation by UGT1. Removal of the α(1,2)-mannoses by ERManI is a rather slow process, which might result in creating a restricted time for folding attempts of glycoproteins (83). A difference seems to appear in EDEM1 targeting of soluble or membrane-bound glycoproteins. Soluble glycoproteins require the binding of OS-9 and XTP3-B to the glycan structure generated by EDEM1 for their delivery to ERAD ligases. On the other hand, membrane-bound glycoproteins are targeted by EDEM1 for ERAD mainly via preventing the reglycosylation by UGT1 and were shown to be independent of OS-9 and XTP3-B (84).

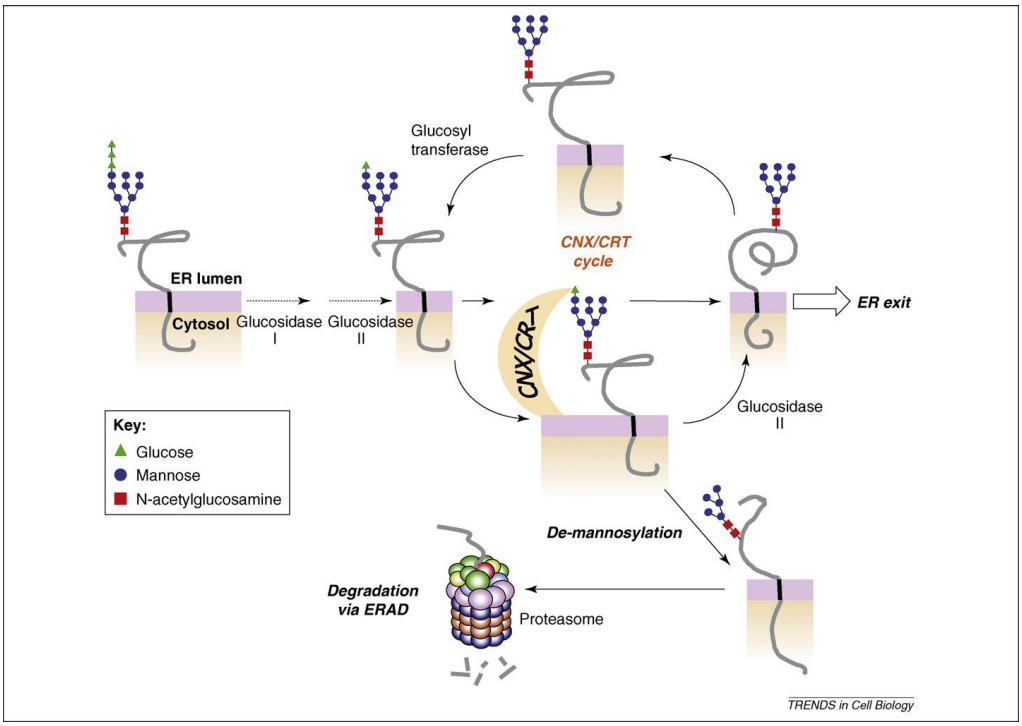

Figure 5. Glycoprotein quality control in the ER. Newly synthesized glycoproteins are core glycosylated in a cotranslational manner. Directly after this, Glucosidase I and II remove the outer two glucose residues. The mono-glucosylated glycoprotein is now a substrate for Calnexin (CNX) and Calreticulin (CRT), which are chaperones that facilitate protein folding. If the protein is properly folded, Glucosidase II removes the third glucose and the glycoprotein can exit the ER. If a protein is incompletely folded, it can be re-glucosylated by glucosyltransferase (GT) leading to rebinding of the protein to CNX and CRT to enhance folding. When a protein is terminally misfolded, it is demannosylated and targeted for dislocation and proteasomal degradation via ERAD. Feldman *et al.* (80).

The α(1,6)-mannose cannot be the only signal for glycoprotein ERAD, since some mature glycoproteins also expose this α(1,6)-mannose and are not targeted for degradation in the ER. Even less is known about the recognition of integral membrane proteins or non-glycosylated proteins, but future research might provide insight into these processes.

E3 ligases in ERAD

ERAD pathways are centered around E3 ligases, which form multi transmembrane protein complexes. Two main E3 ligases are involved in yeast ERAD: Hrd1 and Doa10 (85, 86). In mammals, many more E3 ligases for ERAD have been identified, including HRD1, gp78, TEB4 and RMA1 (87). HRD1 and gp78 are homologues of the yeast Hrd1 and HRD1 strongly interacts with SEL1L, forming protein complexes also including Derlin1-3, HERP, and OS-9 (88). Yeast Hrd3 (SEL1L homologue) regulates Hrd1 function and ascertains specificity to the substrate selection of Hrd1 (85, 89). SEL1L interacts with OS-9 and XTP3-B, thereby recruiting them to the ERAD ligases (82). However, the exact function of SEL1L in human ERAD is unclear. Derlins may be the most mysterious members of the E3 ligase complexes. Derlins are known to interact with HRD1 and SEL1L but also with substrates and have been suggested to, at least partially, function as adaptor proteins (90, 91). Luminal substrates typically are more dependent on Derlins, suggesting that Derlins assist these luminal proteins in passage across the membrane (92). HERP has been described as a specialized adaptor protein for non-glycosylated proteins, although it is unclear how luminal non-glycosylated proteins can interact with a HERP-containing E3 ligase complex (93). Besides functioning as a folding chaperone, the

luminal chaperone BiP has been suggested to be involved in delivering misfolded substrates to E3 ligases (94, 95).

Candidates for a dislocation channel

ERAD substrates are degraded by the proteasome in the cytosol and therefore translocation across the ER membrane into the cytosol needs to occur. This process is most likely facilitated by a channel in the ER membrane and several candidates have been suggested to form this channel (Figure 6).

The first suggested candidate was the Sec61 translocon, which mediates protein import into the ER after docking of a ribosome onto Sec61. Sec61 is a pore-forming protein complex and the only channel in the ER identified so far, therefore Sec61 is an attractive candidate. Sec61α, the central component of Sec61, was shown to interact with MHC I in US2-expressing cells upon proteasome inhibition (30). In a similar way, a misfolded version of pre-pro α-factor could be cross linked with Sec61 in yeast and Sec61 could also bind to a soluble, fully glycosylated short-lived ER protein (96, 97). Upon expression of Sec61-2, a temperature sensitive mutant of Sec61 deficient in post-translational translocation, fully glycosylated ERAD substrates accumulated in yeast while the amount of cytosolic precursor remained the same (98). The fact that fully glycosylated substrates were measured, indicates that these proteins were released from Sec61 during import into the ER and that dislocation is a separate process. When using Sec61-3, a mutant similar to Sec61-2 but with an additional cold-sensitive defect in co-translational translocation, the degradation of both soluble and membrane ER proteins was inhibited (99). The import of the substrate into the ER was not disturbed, yet this does not exclude reduced import of essential ERAD factors influencing the results. Sec61 was also indicated as the transporter of cholera toxin out of the ER into the cytosol (100).

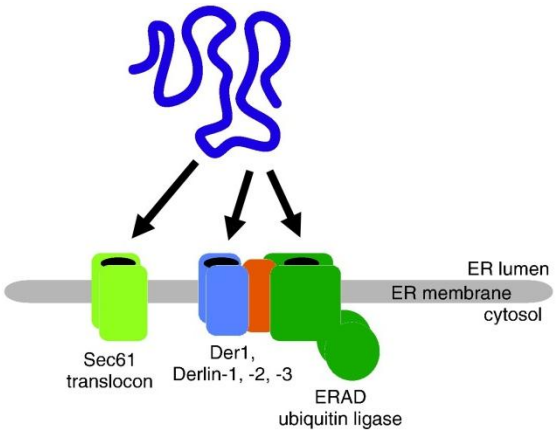

Figure 6. Candidates for a dislocation channel of ERAD. Different proteins have been described as forming a dislocon for proteins to pass through the ER membrane into the cytosol, including the Sec6 translocon, Derlins and ERAD ubiquitin ligases. Derlins and ERAD ligases are known to form complexes and might function together in forming transient pores for proteins to exit the ER. Adjusted from Bagola *et al.* (83).

There is also convincing evidence arguing that Sec61 is not the dislocon for all ERAD substrates. Firstly, the crystal structure of SecYEG, the bacterial translocon highly homologues to Sec61, indicates an hourglass shape creating a hydrophilic core with a maximal diameter of 20Å (101). This indicates that only (almost) unfolded proteins can dislocate via Sec61, while it has been shown that folded, mature proteins can also be dislocated into the cytosol (102). Secondly, at least some proteins are dislocated and degraded independently of Sec61. For example, yeast Ubc6 is a short-lived ER-membrane protein of which degradation is not reduced by mutations affecting Sec61 function, even though Sec61 mediated import is impaired (103). Thirdly, E3 ligase complexes containing the yeast E3 ligases Hrd1 and Doa10 do not contain Sec61 (104, 105), while another dislocon candidate does associate with Hrd1 (see below). Finally, it is still unclear if misfolded proteins stay attached to the translocon complex after import in the ER. Since glycosylation and other steps in the protein maturation occur cotranslationally and during import, aberrant structures might already attract components of ERAD while still attached to Sec61. Misfolding of proteins might

also increase the time that a protein is associated with Sec61. Consequently, binding of ERAD substrates and components to Sec61 could just result from prolonged association of misfolded proteins to Sec61 rather than from active involvement of Sec61 in dislocation (83).

Derlins have also been implicated to be involved in the formation of a dislocation channel for ERAD. Yeast Der1 strongly associates with Hrd1 and is required for the turnover of luminal proteins (106, 107). Mammalian Derlin-1, -2, and -3 also associate with ERAD ligases (108). Derlin-1 is involved in the degradation of both luminal and integral membrane proteins and associates with ERAD components in the cytosol and membrane but also with ERAD substrates, both before and after extraction from the ER (109). Similar functions have been suggested for Derlin-2 and Derlin-3 (108, 110). Depletion of Derlin-1 was shown to induce the UPR and obstructs the degradation of several substrates (111). When fluorescently labeled pre-pro α-factor was loaded onto isolated microsomes and incubated with cell lysate and ATP, significant amounts of fluorescence were released from the microsomes as measure for export of this protein (112). This effect was abrogated when antibodies against Derlin-1 were added, whereas antibodies specific for Sec61α were ineffective. Derlin-1 has also been implicated in the dislocation of cholera toxin from the ER (113). Furthermore, both yeast Der1 and Derlin-1 form oligomeric structures in the ER membrane, in line with the formation of an export channel (114, 115). Taken together, these data indicate that Derlins, in particular Derlin-1, are part of the dislocon or at least strongly involved in dislocon formation.

The ERAD E3 ligases have multiple transmembrane segments to which specific functions have not yet been assigned. Hrd1 and Doa10 are central players in yeast ERAD, therefore they might function as both E3 ligases and dislocation channels. Hrd1 can oligomerize upon interaction with the membrane protein Usa1, possibly indicating channel formation (116). Mammalian Hrd1 and gp78 have also been shown to dimerize, however the function of this dimerization is still unclear (117, 118). Moreover, substitution of some polar and hydrophilic residues in the transmembrane regions of Hrd1 resulted in abrogated degradation of integral membrane substrates (119). Thus, Hrd1 and probably also other ERAD ligases could be involved in the dislocation of membrane-bound substrates.

None of the candidates has been definitively excluded as forming (part of) the dislocation channel. One explanation could be that the dislocon is a combination of several candidates and/or that the dislocon only forms transiently. It might also be that another protein, possibly still unknown, constitutes the dislocon. However, if one protein forms the dislocon for all ERAD substrates, it seems unlikely that this protein has not been identified yet. Given the diversity of ERAD substrates that need to be exported out of the ER, it might be likely that all the above-mentioned candidates participate to create an environment permissive of dislocation and that different complexes are required for different substrates.

A point of discussion remains whether a protein channel could even form pores large enough for the dislocation of all ERAD substrates. Lipid droplets have been suggested as an alternative for the export of ERAD substrates from the ER (120). The involvement of lipid droplets in ERAD has been shown for the degradation of the secretory protein ApoB (121, 122). Accumulation of ubiquitinated ApoB in cytosolic lipid droplets could be measured and subsequent degradation of ApoB was proteasome- and autophagy-dependent. Upon ER stress, the formation of lipid droplets is increased which reduces the accumulation of misfolded proteins in the ER (123). However, it remains unclear whether export via lipid droplets is a normal ERAD pathway or rather a stress-induced response in specific cell types.

Delivery of substrates to the proteasome

Misfolded proteins that are selected for ERAD are dislocated from the ER, coupled with ubiquitin and delivered to the proteasome for degradation. The energy for dislocation of proteins across the membrane is provided by ATP hydrolysis, as was shown using a reconstituted *in vitro* system (124). p97 (also termed VCP; Cdc48 in yeast) is a homohexameric AAA-ATPase residing in the cytosol and seems to be the driving force for dislocation of ERAD substrates. Together with the cofactors Ufd1 and Npl4, p97 is essential for ERAD (125-127). Degradation of CPY* (a mutant carboxypeptidase) requires Cdc48, Ufd1 and Npl4 and release of CPY* into the cytosol is blocked in Ufd1-1 mutant cells (128). Furthermore, when cdc48-10 mutant strains were used, non-ubiquitinated CPY* accumulated in the ER, indicating that Cdc48 indeed drives dislocation from the ER membrane into the cytosol (129).

A strong reduction in export of MHC I heavy chains (HC) from the ER was observed in p97 dominant-negative mammalian cells. MHC I directly interacts with p97 and release of poly-ubiquitinated MHC I depends on p97 (125). A significant amount of p97 is also associated with ERAD ligases at the ER membrane (114, 130-132), which is in line with these findings. During nucleotide binding and ATP hydrolysis of p97, substantial conformational changes occur, converting ATP hydrolysis into mechanical forces. These mechanical forces are thought to facilitate pulling ERAD substrates out the ER and to underlie the unfolding and disassembling of proteins. However, several substrates have now been shown to dislocate independently of p97 (133-136), again indicating different requirements for different substrates.

After providing the energy for extraction of substrates out of the ER membrane, p97 might deliver the ERAD substrates to the proteasome by interacting with ubiquitin-binding molecules, including Ufd1, the ubiquitin chain-elongation factor Ufd2 and DUBs like YOD1 (137). Since deubiquitination is required prior to degradation by the proteasome, DUBs must be present. YOD1 interacts with p97 and is important for the degradation of at least several ERAD substrates (138). Binding of ERAD substrates by p97 is also thought to prevent accumulation and thereby aggregation in the cytosol. Thus, p97 may facilitate export of ERAD substrates from the ER and may also transport these substrates to the proteasome.

US2- and US11-induced ERAD of MHC I

As indicated above, US2 and US11 use different mechanisms for the induction of MHC I degradation. In general, the pathways of US2 and US11 are similar, since they both bind directly to MHC I HC and both require a functional ubiquitin-system, proteasomes and p97 (30, 31, 59, 133). However, dislocation of MHC I induced by US2 and US11 is mediated by different components of the ERAD machinery (Figure 7). For example, while BiP has been shown to be involved in both pathways in the lumen of the ER (139), PDI is only required for US2-mediated dislocation (140).

US11 recruits MHC I HCs to Derlin-1 via its transmembrane domain and Derlin-1 is essential for dislocation of MHC I by US11 (109). VIMP (VCP-interacting membrane protein) interacts with Derlin-1 and recruits p97 to the Derlin-1 complex. Furthermore, VIMP functions as a cofactor for p97 (111). On the cytosolic side of the ER, p97 in complex with Ufd1 and Npl4 has been shown to be involved in US11-mediated dislocation (125). Besides VIMP and p97, other components of the US11-induced Derlin-1 complex are SEL1L, AUP1 (ancient ubiquitous protein 1), UBXD8 and UBC6e (also termed UBE2J1) (133, 141). The latter protein is an E2 ubiquitin conjugating enzyme which has been suggested to act in concert with HRD1 (142). HRD1 and gp78 can be found in the Derlin-1 complex,

but up to now they have not been shown to be functionally important in US11-mediated degradation of MHC I.

Derlin-1 is not required for US2-mediaded degradation of MHC I, thereby also indicating that other members of the Derlin-1 complex are not essential. In line with this, it was demonstrated that

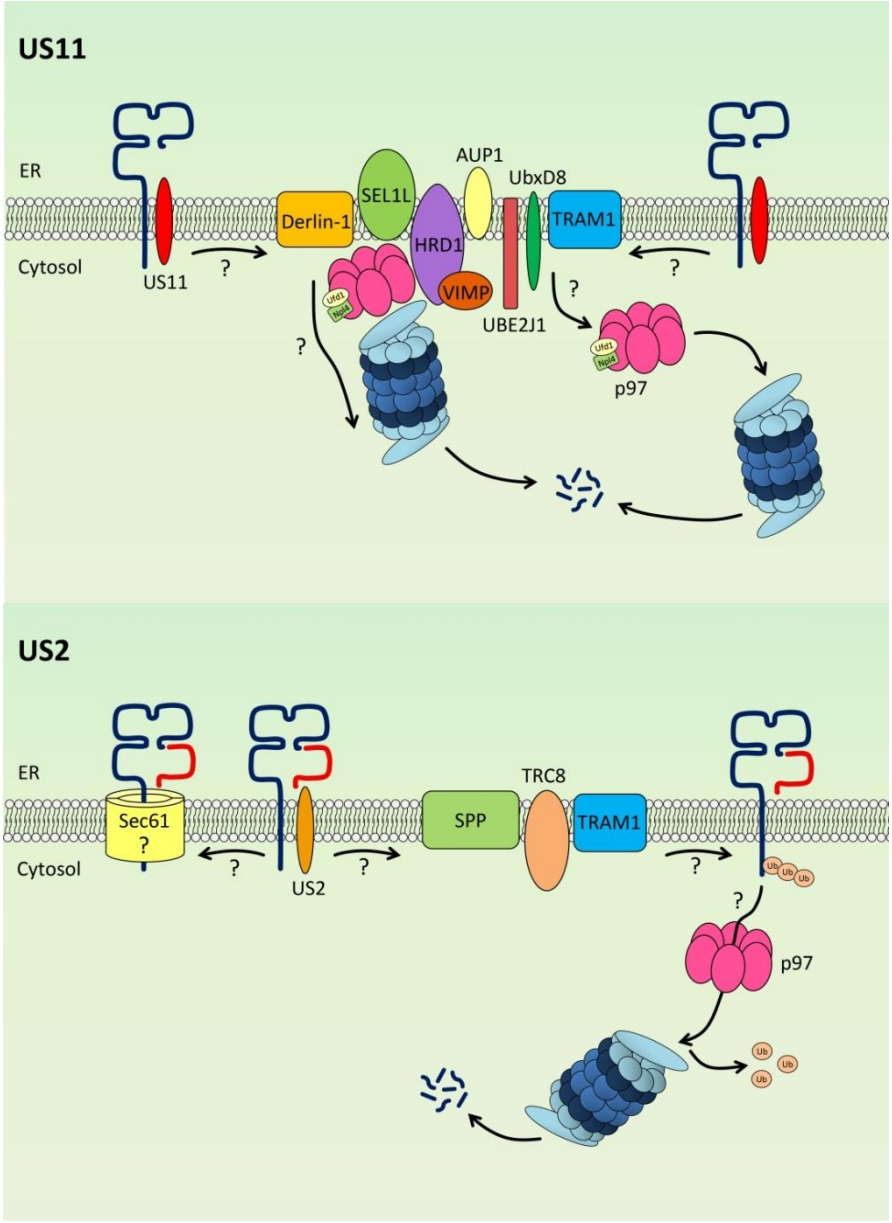

Figure 7. US11 and US2 induce dislocation and degradation of MHC I via distinct pathways. US11 targets partially folded or mature MHC I heavy chains (HCs) for dislocation (upper panel). US11 requires a complex of Derlin-1, including SEL1L, AUP1, UBE2J1, UbxD8, TRAM1, VIMP and p97 (associated with Ufd1 and Npl4). The direct involvement of HRD1 in US11-mediated dislocation has not yet been shown, but is depicted here because HRD1 strongly interacts with Derlin-1 and SEL1L and is most likely present in the Derlin-1 complex. It is still unclear if proteasomes are directly involved in the dislocation of MHC I HCs. Dislocation might also be initiated by the Derlin-1 complex and require p97 for the pulling force and delivering MHC I HCs to the proteasomes in the cytosol. The way in which US11 brings MHC I HCs to the Derlin-1 complex and if it has a function itself in this complex remains to be elucidated. US2 targets mature MHC I HCs (associated with β2m) via a distinct pathway (lower panel). US2 requires a complex of SPP, TRC8 and TRAM1 and MHC I HCs are ubiquitinated prior to degradation, but it is unclear exactly where this occurs. MHC I was shown to interact with Sec61 in the presence of US2, indicating that Sec61 might open laterally to facilitate entry and dislocation of MHC I. It has also been shown that p97 is involved in the dislocation and degradation of MHC I, but the exact mechanisms remain to be unraveled. MHC I must be deubiquitinated before entering the proteasome. Both the US11- and the US2-pathway lead to the degradation of MHC I.

US2 requires the signal peptide peptidase (SPP) and TRC8, a membrane bound E3 ligase, for dislocation and degradation of MHC I (143, 144). When TRC8 is depleted, ubiquitination and downregulation of MHC I in US2 expressing cells is abrogated (144). p97 is also involved in MHC I degradation by US2, albeit without Ufd1 and Npl4 as cofactors (133).

The ER-resident protein TRAM1 (translocating chain-associated membrane protein-1) is able to complex with both US2 and US11 as well as with deglycosylated and polyubiquitinated MHC I. Knockdown of TRAM1 in US2- and US11-expressing cells showed that TRAM1 is involved in the dislocation of MHC I for both viral proteins (145). Furthermore, TRAM1 is able to bind to both Derlin-1 and SPP. However, dislocation initiated by US11 was more sensitive to knockdown of TRAM1 than US2, in line with the different pathways of US2 and US11. Recently, it was shown that HRD1 and UBE2J1 are essential for the degradation of MHC I HCs in β2m-depleted cells (141). Depletion of β2m induces dislocation of MHC I HCs to the cytosol and intermediate, deglycosylated MHC I HCs can be detected in the cytosol (146). This indicates that HRD1 and UBE2J1 are important in the regulation of MHC I expression and that HCMV evasion proteins, in particular US11, can hijack normal cellular processes.

Thus, studying the mechanisms of US2- and US11-induced dislocation and degradation of MHC I has led to the identification of many components of ERAD and a better understanding of general ERAD mechanisms. Intensive research on US2 and US11 has also led to the characterization of different pathways for MHC I dislocation and degradation by these proteins. Using two different pathways is quite logical, because if US2 and US11 would both target the exact same ERAD components, they would be functionally redundant. However, a lot of questions regarding ERAD remain to be answered, including the main questions of how substrates are recognized for ERAD and which protein(s), if any, form the dislocation channel. Future research will focus on addressing these questions.

The role of proteasomes in dislocation

Proteasome structure

The 26S proteasome is a complex of ATP-dependent proteins and can exert protease function. The main function of the proteasome is degradation of proteins, which are marked for degradation by a ubiquitin tag (147). The proteasome is also a key player in ERAD to degrade misfolded proteins. Blocking the proteasome by using proteasome inhibitors is lethal for cells within hours, indicating the importance of a functional proteasome. Recently, it has been shown that amino acid scarcity, and not accumulation of protein waste, cause cell death upon proteasome inhibition (148). Assembly and proper function of the proteasome are therefore essential to life.

Proteasome inhibitors are often used to block degradation in order to study the accumulation of ERAD substrates. Several classes of proteasome inhibitors exist; all are known to bind and directly inhibit the active sites inside the 20S core particle. Peptide aldehydes such as MG132, PSI, and ALLN are substrate analogues or inhibit the transition state. Lactacystin and β-lactone are pseudosubstates which form a covalent bond with the active site of the proteasome. Peptide vinyl sulfones are another group of inhibitors and are described to act via a similar mechanism as lactacystin (149-151).

Interestingly, the proteasome has emerged as a therapeutic target for several diseases, including cancer, Alzheimer disease, type I diabetes and inflammatory bowel disease, because dysregulation of the proteasome has been implicated in the pathogenesis of these diseases (151). Despite concerns of nonspecific inhibition of basic cellular processes, several proteasome inhibitors are now being tested in clinical trials. Bortezombic, a reversible inhibitor of the catalytic β5 subunit, is the first proteasome inhibitor that the US FDA has approved for treating relapsed multiple myeloma and mantle cell lymphoma (152). Because there is great interest in proteasome inhibitors as drugs, it is not unlikely that more will follow to be used in the clinic. However, proteasome inhibitors should be tested extensively before being administered to patients and (long-term) side effects should be closely monitored.

The 26S proteasome consists of a 20S proteasome, termed the core particle (CP) which carries the catalytic activity, and one or two 19S regulatory particles (RP) attached to the end(s) of the 20S CP (153). The 20S CP is formed by axial stacking of two outer α-rings and two inner β-rings. Each ring is formed by seven structurally similar α- and β-subunits, resulting in an $\alpha_{1\text{-}7}\beta_{1\text{-}7}\beta_{1\text{-}7}\alpha_{1\text{-}7}$ structure. The β1, β2 and β5 subunits are catalytically active and the active sites are sequestered inside the cavity of the 20S CP to prevent random degradation of proteins (154). To cleave peptide bonds, the β1, β2 and β5 subunits are known to have caspase-like/PGPH (peptidylglutamyl-peptide hydrolyzing), trypsin-like and chymotrypsine-like activities, respectively (155). The crystal structure of the 20S CP

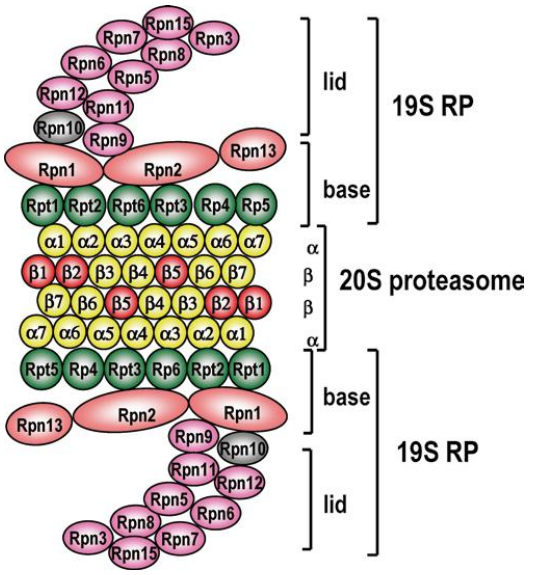

Figure 8. Schematic organization of the subunits of the 26S proteasome. The 20S proteasome consists of two rings with β subunits and two rings with α subunits. The β subunits indicated in red are the catalytically active subunits. The 19S regulatory particle (RP) is built of Rpn (RP non-ATPase and of Rpt (RP triple-ATPase) subunits, although the relative positions of these subunits has not been fully established. Rpn10 is indicated in grey, because this is thought to link the lid to the base of the 19S RP. Kaneko *et al.* (162).

indicates a narrow channel in the α-ring (~13 Å in diameter) and is almost completely closed. Therefore, proteins have to be unfolded to pass through the narrow pore in the α-ring before reaching the catalytic sites. Substrate proteins are degraded by the 20S CP into oligopeptides ranging from 3 to 5 amino acid residues. These peptides can then be further processed into amino acids by oligopeptidases and/or aminocarboxyl peptides.

The 19S RP regulates protein degradation by binding polyubiquitinated proteins, removing the polyubiquitin chains, unfolding proteins, opening the gate of the CP and facilitating transport of the protein into the CP. The 19S RP can be divided into two subcomplexes: a base and lid complex (156). Six homologues AAA-ATPase subunits Rpt1 – Rpt6 (Regulatory Particle Triple-A) and three non-ATPase subunits Rpn1, 2 and 13 (Regulatory Particle Non-ATPase) form the base of the 19S RP. The lid comprises nine non-ATPase subunits (Rpn 3, 5-9, 11, 12, 15) and the lid is connected to the base by the stabilizing Rpn10 subunit (Figure 8).

Upon IFNγ stimulation, specific β subunits can be induced and incorporated into newly synthesized proteasome, i.e. the β1i, β2i and β5i subunits (157). These three subunits replace their homologues β1, β2 and β5 and proteasomes containing these immunosubunits (i-subunits) are termed immunoproteasomes. Immunoproteasomes are induced in immune responsive cells and have higher trypsin-like and chymotrypsine-like activities. The immunoproteasomes specifically function in MHC I antigen processing and peptide generation, but also protect cells from oxidative stress induced by IFNγ (158). Peptides generated by the immunoproteasome are different than those generated by constitutive proteasomes and are also more efficient in activation of CTLs in the context of MHC I (159). Immunoproteasomes are beyond the scope of this review, but their function has been discussed elsewhere (160, 161).

Proteasome assembly

Many studies have focused on elucidating the assembly of the proteasome, since all the different subunits need to be quickly and correctly assembled. There are series of chaperones dedicated to proteasome assembly and it is commonly accepted that multi-step processes are required (Figure 9) (162, 163).

Four chaperones, termed PAC (proteasome assembling chaperone) 1-4, assist with the formation of the 20S CP α-ring. These PACs form functional homodimer pairs (PAC1-PAC2 and PAC3-PAC4). The α-ring then functions as a scaffold for β-subunit incorporation. β2 is recruited to the α-ring with the aid of Ump1/POMP, followed by sequential incorporation of β3, β4, β5, β6 and β1, forming a half-20S proteasome. Incorporation of β7 triggers the dimerization of two half-20S proteasomes, resulting in the degradation of β-propeptides and Ump1/POMP. The degradation of Ump1/POMP coincides with formation of the mature 20S proteasome and is followed by PAC1-PAC2 degradation (164). During CP assembly, the Ump1/POMP chaperone has a dual function (163, 165). On the one hand, it is required for the initiation of β-ring assembly in mammalian cells. On the other hand, it functions as a check point, preventing dimerization of half-20S proteasomes that do not contain all seven β subunits.

The mechanisms underlying 19S RP assembly are less well understood. The base and the lid most likely assemble independently of each other. The base assembly starts with the formation of three different precursor complexes: p28-Rpt3-Rpt6-Rpn14 (p28-module), S5b-Rp1-Rpt2-Rpn1 (S5b-module), and p27-Rpt4-Rpt5 (p27-module). The four chaperones (p28, Rpn14, S5b, and p27) interact with the C-terminal tail of the Rpt subunits and these C-tails are necessary for docking of the subunits on the CP (166). In this way, the chaperones prevent binding of premature Rpt subunits to the CP. With the aid of chaperones the p28-, S5b-, and p27-modules assemble with a Rpn2-Rpn13 complex

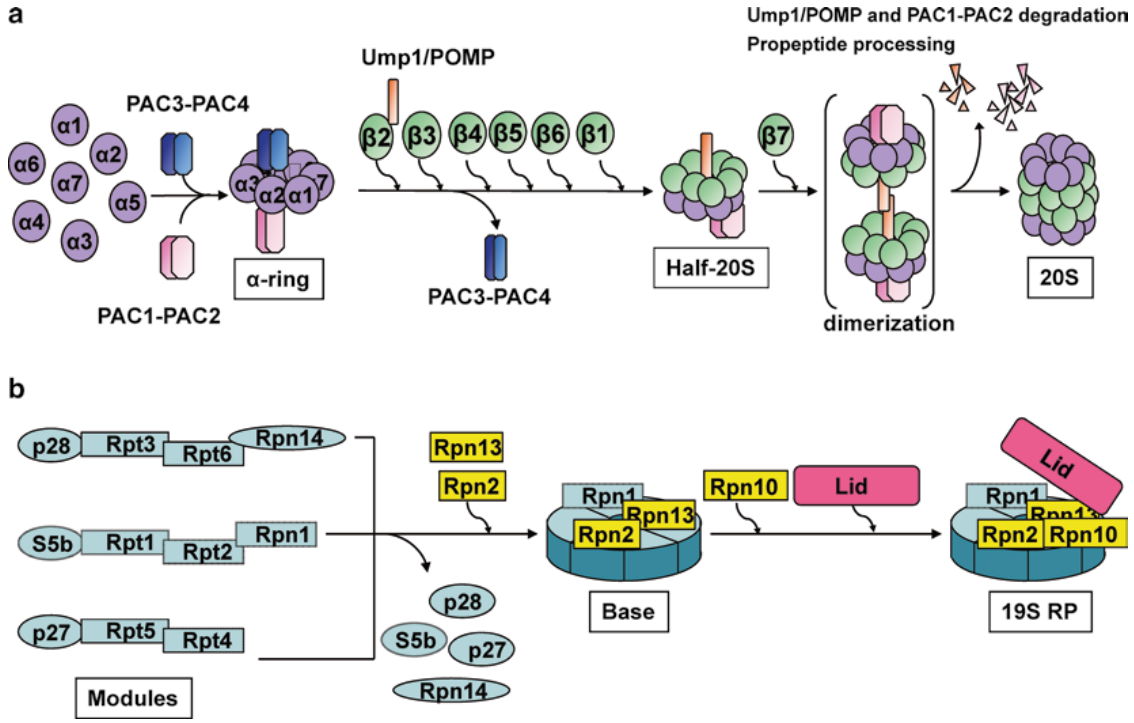

Figure 9. Current model for proteasome assembly. A. The assembly of 20S proteasomes. PAC1-PAC2 and PAC3-PAC4 chaperones are essential for α-ring formation. When β2 and Ump1/POMP are simultaneously incorporated on the α-ring, the assembly of the β-ring can start, forming a half-20S proteasome. Incorporating β7 into the half-20S proteasome induces the formation of a homodimer, which is the mature 20S proteasome, and the degradation of PAC1-PAC2 and Ump1/POMP. B. Assembly of the base complex of the 19S regulatory particle (RP). The base subunits form three different complexes and associate with each other, together with Rpn2 and Rpn13, leading to the formation of the base. Association of Rpn10 and the lid leads to the formation of the 19S RP. PAC, proteasome assembling chaperone. Kaneko *et al.* (162).

into the base complex. Binding of Rpn10 to the base complex allows association of the lid to form a mature 19S RP. Recently, the lid assembly process has also been described as a multi-step process (167). Firstly, Rpn5, 6, 8, 9 and 11 are assembled into a core complex. Secondly, another complex of Rpn 3, 7, and 15 associates to the core complex. Finally, the lid is properly folded after subsequent incorporation of Rpn10. It is still unclear whether specialized chaperon(s) exists for the assembly of the lid.

Interestingly, the proteasome-associated DUB Ubp6 has recently been reported to be involved in base assembly (168). Ubp6 can be found in mature 26S proteasomes but also in Rpn1-containing precursor complexes of the base. Rpn1 is known to directly or indirectly bind poly-ubiquitinated substrates. Upon Ubp6 depletion, base assembly is defective and poly-ubiquitinated proteins accumulate on the Rpn1-containing precursor complexes. Thus, to ensure proper assembly of the base, Ubp6 prevents binding of poly-ubiquitinated proteins to Rpn1 by removing ubiquitin from these proteins.

Involvement of proteasomes in dislocation

For a long time, it was assumed that the sole function of proteasomes in ERAD was the degradation of substrates in the cytosol. This theory was supported by the observation that the majority of the proteasomes are localized in the cytosol. Furthermore, incubation of US2-expressing cells with proteasome inhibitors resulted in the accumulation of MHC I in the cytosol (30). Similarly, when cells expressing US11 were incubated with proteasome inhibitors, an accumulation of deglycosylated MHC I in the cytosol was observed (31). These data indicate that dislocation of ERAD substrates into

the cytosol can still occur in the presence of proteasome inhibitors, but that the subsequent degradation in the cytosol is inhibited.

However, there is increasing evidence that proteasomes themselves can be directly involved in the dislocation of substrates from the ER. To study the direct role of proteasomes in dislocation, Sec62, a double membrane spanning ER protein, was fused to two protein A domains and Deg-1. Deg-1 mediates ubiquitination by ER-bound UBC6 and UBC7, creating a short-lived ubiquitinated model protein. Using pre1-1 mutants containing functionally attenuated proteasomes it was shown that Deg1-SEC62^{protA} intermediates accumulated at the ER membrane (169). Similar effects were seen with a cim5 mutant, which has a defective ATPase subunit in the 19S RP. It seems that proteolysis of the model protein in mutant cells occurs nearly as fast as in WT cells until the first membrane-spanning domain is reached, indicating that functional proteasomes are necessary for extraction of the substrate from the membrane *in vivo* (169). Upon proteasome inhibition by MG132, PSI, or lactacystin the half-life of κ_{NS1} (unassembled immunoglobulin light chains) significantly increased and both subcellular fractionation and protease protection strongly indicate that κ_{NS1} is localized at the ER membrane (170). Thus indicating that protease activity is tightly connected to dislocation of this soluble non-glycosylated protein.

One way by which the proteasome could be involved in dislocation is by directly pulling the substrates out of the ER, since the proteasome also contains AAA-ATPase subunits. Even though the majority of proteasomes reside in the cytosol, a significant part is associated with the ER and nuclear envelope (171), which would be essential for proteasomes to mechanistically pull ERAD substrates out of the ER. To study whether isolated proteasomes are sufficient in inducing dislocation, microsomes containing pαF (a mutated form of pre-pro α-factor) were incubated with isolated mammalian 19S particles. These isolated 19S particles were enough to induce dislocation of pαF from the membrane fraction into the cytosolic fraction. Adding 20S particles to the extracted pαF induced degradation of pαF which could be blocked by MG132 (172). Therefore, this elegant study shows that dislocation and degradation of a substrate can be uncoupled and that the 19S proteasome is sufficient for dislocation *in vitro*. It would be very interesting to investigate whether this is also true for other ERAD substrates and whether other proteins are also involved in dislocation *in vivo*.

Interestingly, US11 itself was also identified as an ERAD substrate, undergoing dislocation and proteasomal degradation. Treating US11 cells with a proteasome inhibitor (ZL_3VS) resulted in the accumulation of glycosylated and ubiquitinated US11, but also of deglycosylated intermediates. Deglycosylated US11 intermediates stayed integrated in the ER membrane, implying that after partial dislocation and ubiquitination the proteasome is involved in the final steps of dislocation (173).

Different proteasomal AAA-ATPase subunits might be involved in different steps of the ERAD pathway (128, 169). To systematically test the role of the six different 19S AAA-ATPases, mutant strains of each AAA-ATPase were generated and analyzed. Degradation of two substrates, luminal CPY*-HA and membrane 6myc-Hmg2, was only inhibited in Rpt4R and Rpt2RF mutants. The Rpt2 mutation affects global proteasomal activity, thereby inhibiting degradation of ERAD substrates. Rpt4, on the other hand, is involved in dislocation of CPY*-HA. Addition of excess Cdc48, which has also been shown to be involved in dislocation, partially restored CPY*-HA degradation in Rpt4 mutants, but not in Rpt2 mutant strains (174). It is still unclear whether Cdc48 and Rpt4 function in parallel, both pulling substrates across the ER membrane, or sequentially, where Cdc48 would pull ERAD substrates across the ER membrane and Rpt4 delivers the substrate from Cdc48 to the 26S proteasome.

As mentioned before, several studies have implied Sec61 being involved in dislocation of ERAD substrates, although a clear mechanism has never been described. It has been shown that proteasomes bind to Sec61 and compete with ribosomes for Sec61 binding. More specifically, the 19S base binds Sec61 with high affinity (171). Surprisingly, proteasomes and ribosomes bind distinct domains on the cytoplasmic tail of Sec61. Mutating the ATP-binding sites of each of the six AAA-ATPase 19S subunits all reduced the binding of 19S to the ER, indicating that the 19S base binds Sec61 in an ATP-bound state (175). This connection between the Sec61 translocon and the proteasome might explain why Sec61 has also been shown to be involved in dislocation. Sec61 could function as a platform on which ATP-bound proteasomes can dock and might pull ERAD substrates out of the ER. In line with this, components of the Sec61 translocon have been suggested to provide an extraction-supportive environment in the ER membrane (169). Proteasomes also interact with p97, which in turn binds to the ER membrane via interaction with VIMP and Derlin-1 (111). Besides that, proteasomes can bind ubiquitinated substrates with the 19S lid, which mainly occurs for cytosolic substrates (176). In a similar way, proteasomes could directly interact with ubiquitinated ERAD substrates at the ER membrane, provided that these substrates are integral membrane proteins with a ubiquitinated cytosolic tail or partially dislocated substrates. For some substrates,

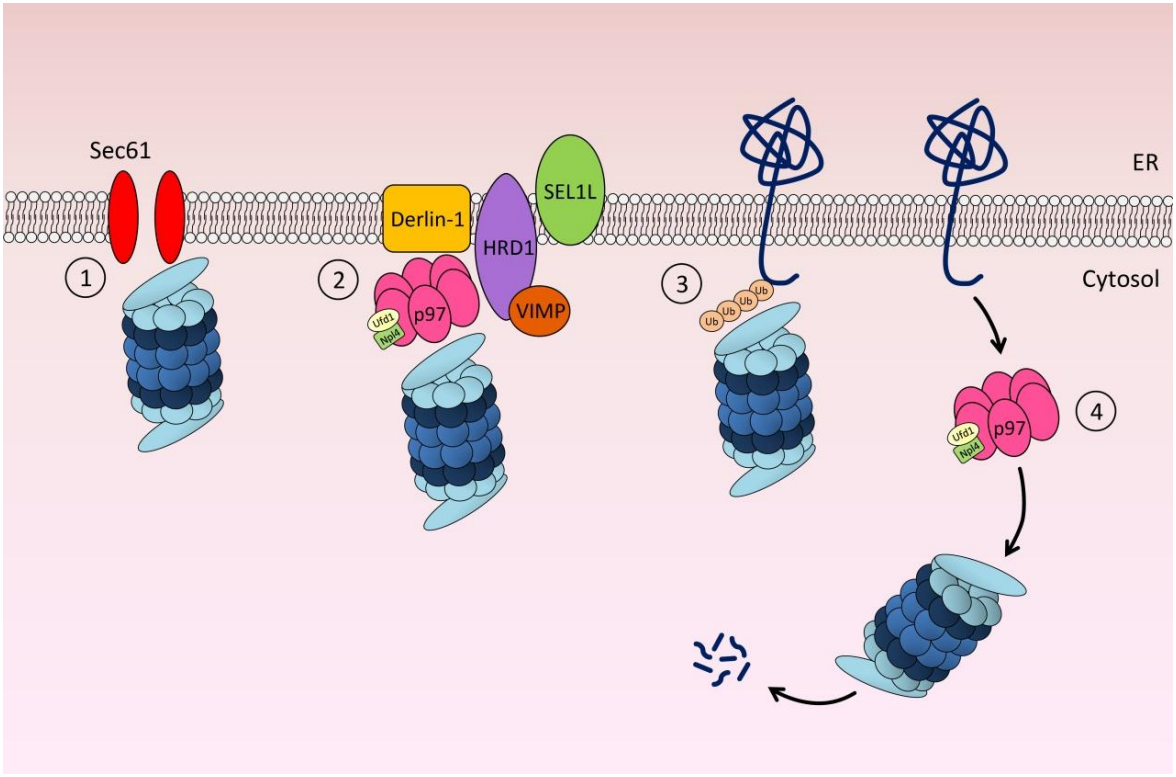

Figure 10. Association of the proteasome to the ER membrane. A significant amount of the proteasomes is associated with the ER membrane and several proteasome interaction partners at the ER membrane have been suggested. Proteasomes can associate with Sec61 (1) and in this way Sec61 could be involved in dislocation of substrates into the cytosol. The interaction between p97 and proteasomes is also well-established and could lead to recruitment of proteasomes to ERAD ligases via interaction of p97 with VIMP and Derlin1 (2). The 19S lid of the proteasome can interact with ubiquitin (Ub) and this could also occur at the ER membrane with ubiquitinated integral membrane proteins or partially dislocated substrates (3). Some substrates can accumulate in the cytosol upon proteasome inhibition, indicating that these substrates are transported via p97 to proteasomes in the cytosol (4). It is possible that these different mechanisms exist alongside of each other and that proteasomes associate to the ER membrane in different ways, depending on the substrate.

however, accumulation in the cytosol has been reported, indicating that substrates are transported to cytosolic proteasomes, most likely via p97. Thus, proteasomes can probably bind the ER membrane in multiple ways (Figure 10) depending on the ERAD substrate. Further research is required to establish if this hypothesis is true.

Tools for studying dislocation

In general, when studying dislocation, subcellular fractionation is commonly used as measure for dislocation (102, 170). In subcellular fractionation cells are homogenized and fractionated by centrifugation, resulting in a membrane and cytosolic fraction which can be analyzed for the presence of a certain substrate protein. This does not exclude, however, that proteins are bound to the ER on the cytosolic side. Trypsin treatment (or another protease) on the membrane fraction can be used as a means of differentiating between proteins bound to the ER and integral ER or luminal proteins, and should always be used as a control. Measuring ER-resident proteins can also be done by EndoH (endoglycosidase) treatment. EndoH is able to process high-mannose-type N-linked glycosylated proteins and results in a lower molecular weight of these proteins. Only ER-resident proteins are sensitive to EndoH, since transport to the Golgi ensures further processing of the glycans.

Dislocation of MHC class I HC can be visualized using eGFP-HC chimeric proteins in combination with fluorescence microscopy or pulse-chase labeling (102). More recently, *in vivo* biotinylation has been described as a novel tool for studying dislocation (177). The ERAD substrate of interest needs to be tagged with a 15 amino acid long biotin-acceptor-peptide (BAP) and co-expressed in a cell containing the biotin-ligase BirA (derived from *E. coli*). The BAP contains a single lysine residue which can efficiently be biotinylated by BirA. Since BirA is a cytosolic protein, it will only biotinylate ERAD substrates that are dislocated from the ER into the cytosol. This has been shown for MHC class I α-chain, NHK, HC and calreticulin (Crt) substrates and provides a tool for measuring the extent of dislocation *in vivo* (177). However, if dislocation is as tightly coupled to proteasomal degradation as some studies suggest, this method might not work for substrates which are not released into the cytosol but directly dislocated and degraded by the proteasome.

Thus, there is convincing data that the proteasome is involved in the dislocation of at least some ERAD substrates. However, it remains to be investigated whether the proteasomes are really able to exert a pulling force on substrates or that proteasomes are involved in dislocation in another way. One scenario might be that p97, which is most commonly accepted as providing the energy and pulling force for dislocation of ERAD substrates, requires the association of proteasomes for its function and dislocation of certain substrates. Further research is needed to test whether this is really true and whether this is the case for other ERAD substrates.

Discussion

Viral immune evasion proteins, including several of HCMV, interfere with the MHC I presentation pathway at multiple levels. In particular, US2 and US11 are very effective in inducing rapid dislocation and degradation of MHC I. Because of this clear phenotype, US2- and US11-induced MHC I degradation has often been used to study the ERAD pathway. After decades of research, many ERAD components and mechanisms have been discovered, but our understanding of the mechanisms of US2 and US11 has also greatly increased. The main steps in the ERAD pathway are substrate recognition, targeting of substrates to membrane-bound E3 ligases, dislocation into the cytosol and degradation by the proteasome.

The recognition of glycoproteins involves a 'mannose-timer', which limits the time for folding attempts and allows terminally misfolded proteins to be irreversibly targeted for degradation. However, not much is known about the selection of non-glycosylated proteins. It is reasonable to assume that some time-dependent mechanism exists for these proteins too, because folding attempts must be allowed before targeting proteins for ERAD.

For US2 and US11, recognition of the substrate is acquired via binding to MHC I HCs, either mature or also incompletely folded HCs, respectively. Combining the current knowledge of US2- and US11-induced MHC I dislocation from the ER and subsequent degradation in the cytosol leads to distinct models for US2 and US11 (Figure 7). US11 targets MHC HCs to a Derlin-1 complex containing many proteins which all seem to be involved in the dislocation of MHC I into the cytosol, although it is unclear if US11 is also incorporated in this complex.

Dislocation and degradation of MHC I via US2 involves SPP, TRC8 and TRAM1. However, MHC I was also shown to interact with Sec61 in the presence of US2, indicating that Sec61 might open laterally to facilitate entry and dislocation of MHC I. As mentioned before, it is still unclear if Sec61 is the main channel for dislocation and if Sec61 is then part of the E3 ligase complexes in the ER membrane. Ubiquitination of the cytosolic tail of MHC I is required for the dislocation and TRC8 is indicated as the E3 ligase for MHC I. After ubiquitination, MHC I HCs are dislocated and p97 is involved in this step. For US2-induced MHC I dislocation, there is no evidence that the proteasome is directly involved in the dislocation. This indicates that p97 transports MHC I from the ER membrane to proteasomes in the cytosol. MHC I is deubiquitinated and subsequently degraded by the proteasome.

It is debatable whether the models for US2- and US11-induced dislocation and degradation of MHC I can be applied to all ERAD substrates. A limited number of model substrates is used to study ERAD pathways and studies already indicate that each substrate requires a different combination of components for their degradation. Furthermore, the discrepancy between the results concerning the direct role of proteasomes in dislocation also indicates that this is indeed dependent on the substrate and the conditions in which dislocation is induced and measured. This is quite logical, considering the wide range of substrates targeted for ERAD and considering that a broad spectrum of proteins need to be involved in the fine tuning of this process for each substrate. Despite the differences in substrates, it will still be very interesting to investigate whether there is a uniform dislocation channel or whether dislocation through the ER membrane can also occur via dynamic complexes of proteins.

There is increasing evidence that the proteasome is directly involved in the dislocation of at least several ERAD substrates. Since p97 is also required for the dislocation of many ERAD substrates and has been implicated as the driving force for dislocation, it is likely that p97 and the proteasome

function together. It has not been excluded that p97 can facilitate dislocation and then targets substrates to proteasomes in the cytosol. In line with this are results showing that deglycosylated MHC I accumulates in the cytosol upon proteasome inhibition in US2- and US11-expressing cells (30, 31).

When using proteasome inhibitors which block the catalytic activity of the proteasome, it should be taken into account that this might not inhibit other functions of the proteasome. For example, the AAA-ATPase subunits of the 19S base are not targeted by proteasome inhibitors while these subunits have been implicated in the dislocation of several ERAD substrates. Therefore, proteasomes might still be involved in the dislocation of MHC I in the presence of proteasome inhibitors. Further research is needed to investigate this, for example by using shRNA to induce knockdown of proteasome subunits and measuring whether dislocation can still occur. It is also interesting to investigate whether the knockdown of proteasome subunits induces the formation of proteasome-assembly intermediates. This would imply that all subunits are required for correct proteasome assembly, which has recently been demonstrated (162), and knockdown of proteasome subunits might subsequently be used to test if intact proteasomes are essential for dislocation. Using an *in vitro* system with microsomes containing MHC I and US2 or US11 and adding isolated proteasomes, either pre-incubated with proteasome inhibitors or not, could be an elegant method to directly test the role of proteasomes in the dislocation of MHC I.

In conclusion, great progress has been made in the understanding of ERAD, although many questions remain unanswered, including how substrates are recognized, how dislocation is achieved and whether the proteasome is directly involved in dislocation. The use of proteasome inhibitors in clinical trials for the treatment of several diseases underscores how important it is to study ERAD and to fully understand these processes. Increasing insight in substrate specificity, e.g. the characterization of many E3 ligases targeting different substrate subsets, might lead to a shift from proteasome inhibitors to more specific E3 ligase antagonists as therapeutic drugs. Thus, future research should focus on elucidating the different ERAD pathways in order to target these pathways for the treatment of HCMV infections and other ERAD-associated diseases.

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