Antigen Presentation and the Ubiquitin-Proteasome System in Host–Pathogen Interactions

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Abstract

Relatively small genomes and high replication rates allow viruses and bacteria to accumulate mutations. This continuously presents the host immune system with new challenges. On the other side of the trenches, an increasingly well-adjusted host immune response, shaped by coevolutionary history, makes a pathogen's life a rather complicated endeavor. It is, therefore, no surprise that pathogens either escape detection or modulate the host immune response, often by redirecting normal cellular pathways to their advantage. For the purpose of this chapter, we focus mainly on the manipulation of the class I and class II major histocompatibility complex (MHC) antigen presentation pathways and the ubiquitin (Ub)-proteasome system by both viral and bacterial pathogens. First, we describe the general features of antigen presentation pathways and the Ub-proteasome system and then address how they are manipulated by pathogens. We discuss the many human cytomegalovirus (HCMV)-encoded immunomodulatory genes that interfere with antigen presentation (immuno-evasins) and focus on the HCMV immunoevasins US2 and US11, which induce the degradation of class I MHC heavy chains by the proteasome by catalyzing their export from the endoplasmic reticulum (ER)-membrane into the cytosol, a process termed ER dislocation. US2- and US11-mediated subversion of ER dislocation ensures proteasomal degradation of class I MHC molecules and presumably allows HCMV to avoid recognition by cytotoxic T cells, whilst providing insight into general aspects of ER-associated degradation (ERAD) which is used by eukaryotic cells to purge their ER of defective proteins. We discuss the similarities and differences between the distinct pathways co-opted by US2 and US11 for dislocation and degradation of human class I MHC molecules and also a putatively distinct pathway utilized by the murine herpes virus (MHV)-68 mK3 immunoevasin for ER dislocation of murine class I MHC. We speculate on the implications of the three pathogen-exploited dislocation pathways to cellular ER quality control. Moreover, we discuss the ubiquitin (Ub)-proteasome system and its position at the core of antigen presentation as

proteolysis and intracellular trafficking rely heavily on Ub-dependent processes. We add a few examples of manipulation of the Ub-proteasome system by pathogens in the context of the immune system and such diverse aspects of the host–pathogen relationship as virus budding, bacterial chromosome integration, and programmed cell death, to name a few. Finally, we speculate on newly found pathogen-encoded deubiquitinating enzymes (DUBs) and their putative roles in modulation of host–pathogen interactions.

1. Host–Pathogen Interactions

The vertebrate immune system is equipped to deal with invading pathogens, whether by means of mechanical barriers such as the skin and other epithelial surfaces or by means of innate immunity. Innate immunity comprises the phagocytic and inflammatory systems, with phagocytes like macrophages and neutrophils, dendritic cells (DCs), and natural killer (NK) cells, as well as soluble mediators such as cytokines and complement. Phagocytes are the immune system's first line of defense: they recognize, engulf, and clear the pathogen and are the main cellular component of the innate antibacterial response. NK cells can directly recognize and kill pathogen-infected cells that fail to express MHC molecules and secrete cytokines that affect the immune response. NK cells are the main cellular effectors of the innate response against viruses. The complement system can lyse infected cells or simply coat the surface of the pathogen or pathogen-derived material, resulting in its neutralization and opsonization. To counteract pathogen infection, host cells also have extracellular and intracellular pathogen recognition receptors to alert the immune system, such as toll-like receptors (TLRs) at the cell surface, and protein kinase R (PKR) and nucleotide-binding oligomerization domain (NOD) proteins in the cytosol (Akira *et al*., 2006; Inohara *et al*., 2005) that can detect pathogen-associated molecular patterns (PAMPs) such as bacterial peptidoglycan or viral dsRNA. These "danger" signals initiate the synthesis of cytokines like interferons to induce inflammation, a crucial component of the innate defense against pathogens. Because innate immunity is not always successful at recognizing or eliminating the infectious agents, a more sophisticated line of defense, adaptive immunity, is also in place.

The adaptive immune system includes cells originated in the *t*hymus, the T lymphocytes, and the *b*one marrow–derived B lymphocytes (B cells), DCs, and macrophages. The two subsets of T lymphocytes, CD8$^+$ and CD4$^+$ T cells, possess distinct T cell receptors (TCRs), CD8 and CD4, respectively, that interact with their coreceptors on the surface of the target cell, the polymorphic class I and class II MHC molecules (Ploegh, 1998). Class I MHC molecules are expressed by nearly all nucleated cells, whereas class II MHC molecules are constitutively expressed only by professional antigen-presenting cells (APCs),

such as macrophages, B cells, and DCs. Class II MHC expression however, can be induced in many cells, in particular by IFN-γ treatment. APCs can endocytose, process, and display antigen in the context of class II MHC products at their cell surface to activate CD4$^+$ T cells. In the presence of antigen displayed by the APC and the appropriate lymphocyte costimulatory molecules, CD4$^+$ T helper (T$_H$) cells produce cytokines that "help" activate other cells: T$_H$1 (or inflammatory T cells) activate macrophages to kill the phagocytosed pathogens; T$_H$2 cells (or helper T cells) trigger T and B cell proliferation and activate the B cell differentiation program into antibody-producing plasma cells. Furthermore, activation of CD4$^+$ T$_H$ cells is carefully regulated by a small subset of T cells, the regulatory T cells (Tregs). Regulatory T cells play an important role in downregulation of the host immune response, limiting the immunopathology resultant from antipathogen reactions, and preventing autoimmune disease (Beissert et al., 2006; Mills, 2004). In addition to making antibodies, B cells are a special kind of APCs. Unlike DCs and macrophages, B cells are not actively phagocytic. However, stimulation of the membrane immunoglobulin (mIg) antigen-recognition component of their B cell receptor (BCR) with cognate antigen triggers the B cell to capture and deliver the antigen to class II MHC compartments, culminating with antigen presentation for activation of T cells. Bone marrow–derived professional APCs include macrophages and DCs. Macrophages are phagocytic APCs with a low basal antigen-presenting capacity—owing to low surface expression of class II MHC and costimulatory molecules—is induced on macrophage activation, for instance, by IFN-γ. Macrophages reside in (or are recruited to) peripheral tissues, where they phagocytose and clear pathogens. Phagocytosis, in turn, induces release of proinflammatory cytokines like IFN-γ that turn macrophages into potent APCs, resulting in initiation of CD4$^+$ T cell activation.

Dendritic cells, the consummate professional APCs, travel through the periphery, sampling all tissues for prospective invaders. Immature DCs phagocytose pathogens and home to the nearest lymphoid organ to "educate" (prime) naïve CD8$^+$ T cells by cross-presenting antigen in the context of class I MHC molecules—a process described in more detail later. Mature DCs can also prime naïve CD4$^+$ T cells. Like resting macrophages, immature DCs have very low antigen-presenting capability, and only on exposure to maturation signals [such as lipopolysaccharide (LPS) on bacterial surfaces] does internalized antigen get loaded into class II MHC products and get displayed at the cell surface to CD4$^+$ T cells (Bryant and Ploegh, 2004; Stockwin et al., 2000). "Educated" (antigen-specific) CD8$^+$ T lymphocytes survey all cells in the body, ready to destroy any that displays signs of the presence of cellular alterations (such as viral and tumor peptides) within their surface class I MHC molecules (Andersen et al., 2006; Castelli et al., 2000). Antigen-specific CD4$^+$ T

lymphocytes can coordinate macrophage bactericidal properties, activation of T and B lymphocytes, and antibody production.

Not only do the cells of the adaptive immune system provide a more elaborate defense, but also an increased level of protection from a subsequent reinfection with the same pathogen, the bedrock principle of vaccine strategies (Crotty and Ahmed, 2004; Pulendran and Ahmed, 2006). Adding to the complexity of the immune system is the cross talk between innate and adaptive immunity, which is crucial in eliciting an effective immune response (Zingoni et al., 2005). As mentioned, phagocytes release cytokines that stimulate the adaptive response. Conversely, on activation by antigen recognition, T cells synthesize and secrete cytokines that activate macrophages, increasing their ability to kill ingested microbes, an innate immune response (Munz et al., 2005; Salazar-Mather and Hokeness, 2003). The vertebrate immune system, therefore, is the appropriate battleground for microbial pathogens, selecting for those that devise successful strategies to avoid detection and elimination (Hilleman, 2004; Ploegh, 1998).

2. Manipulation of the Host Response by Pathogens: Some General Considerations

Intracellular pathogens have evolved sophisticated mechanisms to subvert host processes to ensure their own replication and transmission. The initial hurdle is entry into the host cell, which poses great challenges for avoiding immune detection before establishing infection. To promote entry into host cells without alerting the immune system, bacteria possess capsular surfaces that have evolved to minimize antibody and complement deposition while in circulation through the body. On the other hand, filamentous adhesins (like fimbriae and pili) that protrude through the bacteria's capsule enable binding to host cell receptors, which enables secretion systems to deliver bacterial effectors to modulate uptake and invasion (Finlay and McFadden, 2006; Galan and Collmer, 1999). Virus particles are very often coated with highly variable capsid (nonenveloped viruses) or envelope (enveloped viruses) proteins to avoid detection and clearance by antibody-mediated responses. These capsids or envelopes can also be studded with immunomodulatory molecules of viral or even host origin and promote attachment to the host cell membrane, fusion and delivery of the virus internal core. Alternatively, they may act as signaling devices and induce intracellular cascades required for virus uptake. Ultimately, intracellular release of the viral DNA or RNA occurs (Marsh and Helenius, 2006; Skehel and Wiley, 2000). The establishment of an infection critically depends on bacterial and viral genes dedicated to manipulation of host functions. A number of reviews have covered the bacterial and viral genes involved in manipulation of the host immune system, from control of apoptosis, cytokine signaling, to the antibody response, so the

reader is referred to Alcami (2003); Alcami and Koszinowski (2000); Bowie *et al.* (2004); Finlay and McFadden (2006); Hengel *et al.* (2005); Hilleman (2004); and Tortorella *et al.* (2000). For the purpose of this chapter, we will focus on pathogen manipulation of antigen presentation pathways and the Ub-proteasome system.

3. Antigen Presentation

Antigen presentation involves the conversion of protein antigens into peptide ligands that can bind to MHC products that are displayed at the cell surface for recognition by T cells. In a simplified view of antigen presentation, the class I and class II MHC pathways have evolved to sample different sources of antigen to which they have access: the class I MHC pathway usually deals with cytosolic antigens and is crucial for activation of $CD8^+$ T cells, whereas the class II MHC pathway deals with exogenous antigens and the activation of $CD4^+$ T cells (Bryant and Ploegh, 2004; Cresswell *et al.*, 2005; Pamer and Cresswell, 1998). Antigen presentation is, of course, not as simple and clear-cut, as we shall discuss later. There are, however, common principles that apply to the discrete steps of antigen processing and presentation by class I and class II MHC molecules: antigen must be acquired, it is subjected to proteolysis, delivered to MHC^+ compartments, and properly assembled with the MHC product. The complex is then subject to sorting through the secretory pathway and delivered to the cell surface (Fig. 1). Because each of these steps affords a target for interference by pathogens, we shall survey them for each pathway.

4. Class I MHC Antigen Presentation

The class I MHC is a trimeric complex composed of the class I MHC heavy chain (HC), the β_2-microglobulin (β_2m) or light chain, and the antigenic peptide. The structure of the fully assembled complex and its interactions with antigen-specific receptors on T cells have been extensively reviewed (Alam *et al.*, 1996; Rudolph and Wilson, 2002; von Boehmer, 2006). The class I MHC HC is inserted into the ER membrane and *N*-glycosylated and binds in its course of synthesis to the membrane-associated chaperone calnexin (CNX), at which point folding and intrachain disulfide bond formation take place. Once dissociated from CNX, the HC binds its soluble partner subunit, β_2m, and enters the peptide-loading complex (PLC). The PLC is composed of two MHC-encoded components, TAP and tapasin, and two "housekeeping" ER proteins, calreticulin and ERp57. The *t*ransporter *a*ssociated with antigen *p*resentation (TAP) is an ATP-dependent pump with two subunits, TAP1 and TAP2 that transports peptides into the ER. Tapasin, a transmembrane glycoprotein, mediates the interaction between the TAP transporter and peptide-free HC/β_2m dimers. The soluble calreticulin and ERp57, a chaperone and a thiol oxidoreductase, respectively, normally involved in
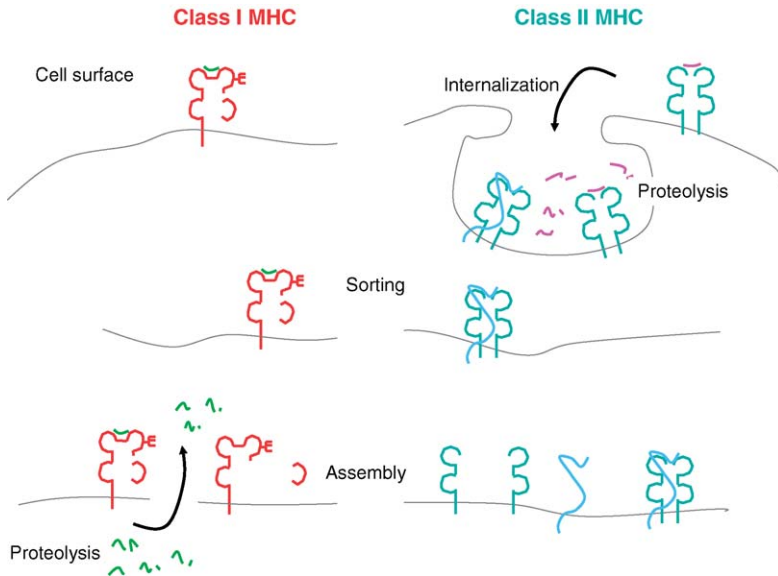
Figure 1 Common principles in antigen processing and presentation by class I and class II MHC molecules. In the class I pathway, endogenous antigens are derived from cytosolic proteolysis and delivered to the ER lumen, where loading onto class I MHC products takes place. The assembled complex is then sorted to the cell surface. In the class II pathway, exogenous material is internalized from the extracellular space and delivered to the lysosome, where processing and loading onto MHC products occur. Sorting through the secretory pathway then delivers the class II complex to the cell surface.

folding of nascent glycoproteins, promote assembly of the class I MHC complex. The peptide antigen cargo for class I MHC originates from proteasomal proteolysis in the cytosol. The array of proteasome-generated peptides is subject to trimming by cytosolic endopeptidases and delivered to the ER lumen by the TAP transporter. Further trimming by ER-resident endopeptidases can also occur to guarantee a custom-fit of the peptide antigens, typically 8–10 amino acids long, into the peptide-binding groove on the HC/β_2m dimer associated with the PLC. Empty HC molecules are detained in the ER by virtue of interaction with tapasin, until assembly with β_2m and peptide takes place, at which point the HC/β_2m/peptide trimeric complex is released from the PLC and allowed to exit the ER and enter the secretory pathway. Once displayed at the cell surface, the antigen-loaded class I MHC complex is ready for inspection by the T cell receptor (TCR) on circulating cytotoxic CD8^{+} T cells (Cresswell *et al.*, 2005; Heemels and Ploegh, 1995; Rammensee, 2002, 2004).

5. Pathogen Recognition by CD8$^+$ T Cells and NK Cells

Class I MHC products on most cells present exclusively "self" peptides, derived from the cell's own proteins, the majority of which results from protein synthesis on free ribosomes in the cytoplasm. Because of the intrinsic error-prone nature of protein synthesis and folding, a sizable fraction of translation products (estimated at up to ∼30%) may never result in a finished product. These *d*efective *ri*bosomal *p*roducts (DRIPs) are destroyed within 30 min of their synthesis by the cytosolic proteasomal pathway and enter the class I MHC antigen presentation pathway (Yewdell *et al.*, 2001). In tumor cells or cells infected by a virus, mutated forms of endogenous proteins or viral proteins will compete with the host's own proteins for presentation by class I MHC products. As "non-self" (tumor- or virus-derived) peptides displayed in the context of class I MHC products accumulate at the cell surface, their chance of triggering activation of CD8$^+$ T cells with a cognate receptor increases. The activated cytotoxic CD8$^+$ T lymphocytes will then lyse the target cell by releasing perforin and granzymes or by Fas ligand engagement. Secretion of IFN-γ and tumor necrosis factor-α (TNF-α) also aids in elimination of infected and tumor cells by cytotoxic T lymphocytes (CTLs) (Andersen *et al.*, 2006; Castelli *et al.*, 2000).

The selective pressure imposed by immune surveillance has made loss of class I MHC expression a hallmark of some tumors and virus-infected cells, as this allows them to be invisible to CTLs. There is, however, a backup system for when lack of class I MHC expression impairs the CD8$^+$ T cell cytotoxic response: NK cells. NK cells display both activating and inhibitory receptors at their surface, which recognize different ligands at the surface of target cells. NK cell activity is ultimately determined by the integration of signals that are perceived by the NK cell surface receptors (Lanier, 2005). All NK cells express at least one inhibitory receptor, which engages class I MHC molecules on the surface of the target cell, resulting in downregulation of NK cell effector functions. Low levels or absence of class I MHC products on the surface of the target cell relieve the inhibitory signals and lead to NK cell cytotoxicity, resulting in clearance of the virus-infected or tumor cells. NK cell recognition has been extensively revised and the reader is referred to Backstrom *et al.* (2004); Kumar and McNerney (2005); and Lanier (2005).

There is an exception to the rule that the class I MHC pathway is devoted to display of peptide antigens from endogenously generated proteins: the so-called professional APCs, DCs, and macrophages can acquire and process *exogenous* material and present it at the cell surface in class I MHC products, a process called cross-presentation. Cross-presentation allows noninfected professional APCs to prime naïve T cells with pathogen- or tumor-derived peptides acquired through endocytosis of infected cells/cell remnants. This "cross-priming"

is essential for development of CD8$^+$ T cell immunity to viruses and tumors *in vivo*, since only professional APCs can present viral/tumor antigens in the context of class I MHC products without being themselves infected/tumorigenic. There is considerable controversy as to the exact nature of the antigen acquired and modes of antigen acquisition, as well as the intracellular mechanisms leading to cross-presentation and the subsets of APCs endowed with this property (Cresswell *et al.*, 2005; Groothuis and Neefjes, 2005; Guermonprez and Amigorena, 2005; Jutras and Desjardins, 2005; Shen and Rock, 2006). This controversy is, however, beyond the scope of this discussion.

6. Class II MHC Antigen Presentation

The class II MHC antigen presentation pathway deals with antigens that reside in extracellular space and are internalized into the endolysosomal pathway. All mammalian cells internalize their own cell surface proteins by constitutive endocytosis. In class II MHC$^+$ cells, this allows class II MHC access to self-proteins as a source of peptides. Professional APCs, such as B cells, macrophages, and DCs, also engage in receptor-mediated endocytosis to acquire extracellular antigen: the antigen from the extracellular milieu is bound by cell surface receptors, internalized, and delivered to the class II MHC antigen processing machinery. Antibodies, complement system factors, and common bacterial or viral components that coat the surface of pathogens or their toxic products bind receptors on APCs that allow them to recognize and internalize this foreign material. Of the many receptors used by professional APCs for this purpose, the mannose receptor, which recognizes mannose residues and glycoproteins on viral and bacterial products, and the scavenger receptor, which recognizes very promiscuously many different classes of macromolecules, are among the most important. Professional APCs also have complement receptors and receptors for the Fc region of antibodies, the Fc receptors, which can assist in the acquisition of opsonized antigen and in its delivery to the proper intracellular destination. B cells can also use their surface immunoglobulin or BCR, to acquire antigen (Bryant and Ploegh, 2004; Cresswell, 1994; Kim *et al.*, 2006c).

Class II MHC loading with antigenic peptides takes place mostly in the endocytic vesicles of professional APCs. Class II MHC αβ dimers assemble in the ER and associate with the chaperone invariant chain (Ii), which inserts its *cl*ass II MHC-associated *I*i *p*eptide (CLIP) portion in the peptide-binding groove of the αβ dimer, preventing its premature (prelysosomal) loading. Ii is also important for correct assembly and transport of class II MHC in the endocytic pathway. Further class II MHC maturation and peptide loading takes place in acidified compartments of the endolysosomal pathway of APCs, since low pH favors an "open" conformation of the class II MHC molecule and

hence peptide exchange, as well as the action of specific cysteine proteases that displace Ii from the class II MHC–Ii complex, and that of the class II MHC-like molecule HLA-DM which facilitates peptide loading. The many hydrolase activities present in the endolysosomal compartments of APCs, such as the IFN-γ-inducible lysosomal thiol reductase (GILT), numerous cysteine proteases of the cathepsin (Cat) family, like CatB, CatS, CatL, and asparaginyl endopeptidase (AEP), produce the peptide ligands that are loaded onto class II MHC products. Peptides bound by class II MHC molecules are usually 13–25 residues long. The end result is a mature class II MHC–peptide complex at the cell surface, consisting of a class II MHC αβ dimer loaded with peptide, which interacts with the TCR on $CD4^+$ T cells. The result of this interaction is dependent on class II MHC–TCR contacts and also on the context provided by lymphocyte costimulatory molecules at the immunological synapse. The $CD4^+$ T cell response may be cytolytic, but generally these antigen-specific $CD4^+$ T lymphocytes function as helper cells, releasing cytokines to enhance the overall immune response by inducing macrophage activation, T and B cell proliferation, and B cell differentiation to produce antigen-specific antibodies and different immunoglobulin isotypes with different effector functions (Bryant and Ploegh, 2004; Chapman, 2006; Cresswell, 1994; Honey and Rudensky, 2003; Hsing and Rudensky, 2005; Stern et al., 2006; Villadangos et al., 1999).

7. Ubiquitin-Proteasome System

All cellular proteins, regardless of their half-life, are subject to turnover. The main pathway for degradation of short-lived proteins in the cytoplasm of eukaryotic cells is the Ub-proteasome system (Hershko and Ciechanover, 1992). Since the discovery of Ub and Ub-dependent proteolysis in the late 1970s, it has become increasingly clear that the Ub-proteasome system is pivotal to numerous cellular processes: cell cycle control, transcriptional regulation, signal transduction, antigen presentation and induction of the inflammatory response, degradation from the ER, membrane trafficking, receptor endocytosis and downregulation, apoptosis, and development (Hershko and Ciechanover, 1992; Pickart, 2001).

8. The Ubiquitin Conjugation Cascade

Ubiquitin is a small 76-amino acid protein, synthesized as a precursor that is processed by deubiquitinating enzymes (DUBs) to expose the glycine–glycine sequence at the Ub C-terminus, its site of attachment to target molecules. ATP-dependent Ub activation is catalyzed by the E1 (Ub-activating) enzyme, which adenylates the Ub C-terminus, allowing the subsequent formation of a
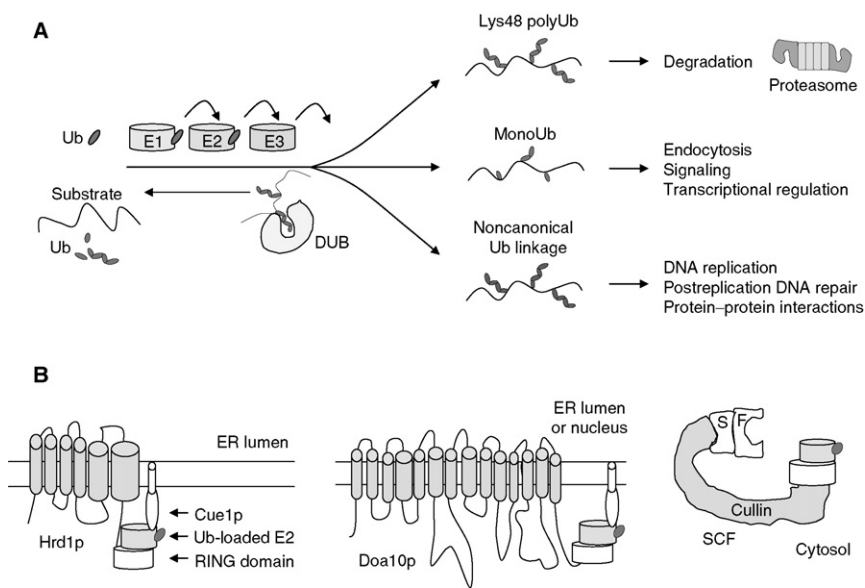
Figure 2 Overview of the Ub-proteasome system. (A) Ubiquitin-conjugation cascade and how Ub chain linkage type and length influence substrate fate. E1, Ub-activating enzyme; E2, Ub-conjugating enzyme; E3, Ub-ligase enzyme; and DUB, deubiquitinating enzyme. (B) Diversity in E3 ligases. E3s play crucial roles in substrate selection and can be regulated by localization, oligomerization, associated E2s, posttranslational modifications, and degradation. Hrd1p and Doa10p are yeast E3 ligases that are multispanning membrane proteins of the ER and, in the case of Doa10p, nuclear envelope. The mammalian SCF family of E3 ligases are mainly cytosolic and can recruit substrate adaptor proteins, the F-boxes, with very diverse substrate specificities.

high-energy thioester bond between the glycine residue of Ub and the cysteine residue on the E1 active site. Ub is then transferred from the E1 cysteinyl side chain to a cysteinyl group on one of several E2 (Ub-conjugating) enzymes. Finally, one of hundreds of E3 (Ub-ligase) enzymes, binds the Ub–E2 complex and the substrate, thus facilitating the transfer of Ub to a lysine residue in the substrate via an amide (isopeptide) bond (Hershko and Ciechanover, 1992). The functions of E3 ligases, in particular, are tightly regulated by signal-induced mechanisms, such as localization, oligomerization, degradation, and posttranslational modifications, which makes E3s the master orchestrators of specificity in the Ub conjugation cascade. This multistep mechanism, much like phosphorylation, endows protein ubiquitination with a high degree of specificity and flexibility, which is paramount to its important biological functions (Haglund and Dikic, 2005; Hershko and Ciechanover, 1998; Pickart, 2001, 2004; Varshavsky, 2005) (Fig. 2A).

9. Ubiquitin Ligases

We elaborate on Ub E3 ligases to some extent, as they are key players in several aspects of the immune system, including immune evasion (Liu, 2004) and also in ER quality control and degradation (Hirsch *et al.*, 2004; Kostova and Wolf, 2003; Romisch, 2005), that yields to some of the ligands on class I MHC products. E3 ligases can be divided into two broad classes: the *h*omologous to *E*6-AP *c*arboxyl *t*erminus (HECT)-domain ligases or the really interesting new gene (RING)-like domain ligases. The first HECT E3 described, E6-associated protein (E6-AP), was shown to be required for ubiquitination and degradation of p53, mediated by the human papillomavirus protein E6 (Scheffner *et al.*, 1993). In HECT E3s, Ub is transferred from the E2 to a conserved cysteine residue in the HECT domain, followed by attack of this thioester by a lysine on the substrate (Pickart, 2001). The RING-CH domain is a ring finger motif with a *c*ysteine residue in the fourth zinc-coordinating position and a *h*istidine residue in the fifth. RING-type E3s are more abundant and do not form an obligatory thioester intermediate with Ub; rather they bring the Ub-loaded E2s and the substrate into proximity, thus facilitating the Ub transfer from the E2 to the substrate (Pickart, 2001). RING-type E3s can be single subunit E3s, which have both a RING-finger domain and substrate recruitment domain(s) on the same protein, like MDM2, a key regulator of p53. Multisubunit E3s include the very diverse *C*ullin-*R*ING *l*igases (CRLs) (Petroski and Deshaies, 2005). CRLs are composed of a catalytic core that recruits the Ub-loaded E2—formed by a nucleating Cullin protein and a RING finger protein—as well as a substrate recognition complex. The archetypal CRLs are the *S*kp-1-*C*ullin-1-*F*-box protein complexes or SCF E3s. The Cullin subunit (any one of Cullin-1, -2, -3, -4A, -4B, -5, or -7) forms an elongated bent backbone for the multisubunit ligase. The Cullin N-terminus binds the S-phase kinase-related protein-1 (Skp-1), an adaptor that recruits any one of a number of substrate-specific adaptor subunits called F-box proteins. The F-box protein is the main determinant in substrate specificity, as it binds the substrate through its particular substrate recognition domain (Jin *et al.*, 2004a), although the RING box protein may participate (Jin and Harper, 2002). The Cullin N-terminus binds the catalytic core composed of the *R*ING-*b*ox (Rbx) protein with its associated Ub-loaded E2 (Zheng *et al.*, 2002). This arrangement allows the F-box protein to bring its bound substrate close to the ubiquitination machinery of the complex (Fig. 2B). Phosphorylation of the substrate very often regulates the F-box protein–substrate interaction, converting the substrate into a form susceptible to E3 activity, adding an extra layer of control to the process (Joazeiro and Weissman, 2000; Schulman *et al.*, 2000; Zheng *et al.*, 2002).

CRLs assemble with numerous substrate receptors. Cullins 2 and 5, for example, recruit substrates through *s*uppressor *o*f *c*ytokine *s*ignaling/elongin-BC

(SOCS/BC) boxes and form the so-called SCF2s and SCF5s complexes. In SCF2s and SCF5s, Skp-1 is substituted by the Skp-1-like protein elongin C which binds the Ub-like elongin B that binds the substrate adaptor subunit (Petroski and Deshaies, 2005). Many of these E3 complexes have important roles in the immune system (Liu, 2004; Liu et al., 2005). The nuclear factor-κB (NF-κB) transcription factor is a master organizer of both innate and adaptive immunity. NF-κB is activated in response to TLR signaling on recognition of pathogen-associated molecules like bacterial peptidoglycan (Liu et al., 2005) in a process that is crucially dependent on ubiquitination. One of the steps requires the cytosolic SCF$^{β\text{-}TrCP}$ E3 complex. The SCF$^{β\text{-}TrCP}$ substrate adaptor component is the F-box protein β-transducin repeat-containing protein (β-TrCP) that possesses WD repeats that bind to phosphorylated inhibitor of NF-κB (IκB), inducing its ubiquitination and degradation. NF-κB is thus released from the IkB-NF-κB dimer and translocates into the nucleus, activating downstream transcription. The elongin-C-elongin-B-Cullin-5-SOCS (ECS) complex uses SOCS proteins as the substrate adaptors. SOCS boxes bind Janus kinases (JAKs), which are recruited and activated in response to IFN and cytokine signaling, promoting ubiquitination and degradation of JAKs by the ECS complex. This, in turn, inhibits phosphorylation and activation of the signal transducer and activator of transcription (STAT) family of transcription factors that are crucial for the immune response following IFN and cytokine signaling and following viral infections (Liu et al., 2005). There are other families of E3s with noncanonical RING-domains, like the K3 homologues and the Ufd2-homologous box (U-box) E3s (which we discuss in more detail later). For a more comprehensive review of HECT and RING E3s and different classifications read (Ardley and Robinson, 2005; Coscoy and Ganem, 2003; Hatakeyama et al., 2001; Petroski and Deshaies, 2005; Sharrocks, 2006).

10. Ubiquitin Chains and Ubiquitin-Like Modifiers (Ubls)

Originally believed to always deliver a "kiss of death" and target the substrate for proteasomal degradation, the much more wide-ranging effects of Ub conjugation are beginning to be appreciated. Chain length and linkage type also influence the outcome of the Ub-conjugated substrate. The multiple Ub moieties in a polyUb chain (chains of 4 or more Ub moieties) are linked to one another by an isopeptide bond between a lysine residue on one Ub molecule (usually on Lys48) and the C-terminal carboxyl group of the next Ub on the chain. At times, extension of a polyUb chain on a substrate conjugated with 1–3 "initiator" Ub moieties requires a special subclass of E3s, the UFD2-homology box (U-box) E3s (once called E4s) (Hoppe, 2005). Targeting of proteins for proteasomal proteolysis generally requires polyubiquitination in a lysine (Lys)

48-type linkage. By contrast to polyUb, substrate monoubiquitination or attachment of noncanonical Ub chains—Ub chains with non-Lys48 linkages such as Lys63 and Lys29 linkages—usually have nonproteolytic functions in DNA repair, endocytosis, signal transduction, transcriptional regulation, and ribosomal function (d'Azzo *et al.*, 2005; Pickart and Eddins, 2004).

Monoubiquitination can occur on a single lysine residue or on several lysine residues in a substrate (multiubiquitination). Monoubiquitination is extremely important as a sorting signal in the endocytic pathway. For example, monoUb attachment is sufficient to induce endocytosis of growth hormone receptor and sorting to the lysosome for degradation (Hicke, 2001; Hicke and Dunn, 2003). Direct modification of the cargo (*cis*-regulation) or modification of the protein-trafficking machinery (*trans*-regulation) by monoUb could have many consequences for antigen presentation, as these processes rely heavily on events that take place in endolysosomal compartments. Noncanonical ubiquitin chains play many diverse roles in signaling pathways, in DNA replication and post-replication DNA repair, and modulating protein–protein interactions. For instance, activation of NF-κB is tightly regulated by a balance between Lys48- and Lys63-mediated ubiquitination of different components of the NF-κB pathway. Triggering of many cell-surface receptors leads to assembly of signaling complexes that recruit tumor necrosis factor receptor-associated factor 6 (TRAF6), an E3 ligase that binds UBC13, promoting Lys63-linked polyubiquitination of the γ subunit of the inhibitor of NF-κB kinase (IKK) complex, IKKγ. This leads to activation of the IKK complex, which in turn results in IkB phosphorylation. Phosho-IkB then recruits the SCF complex that catalyzes Lys48-linked polyubiquitination of IkB and consequently activates NF-κB (Karin and Ben-Neriah, 2000). For comprehensive reviews see (Finley *et al.*, 2004; Pickart and Eddins, 2004; Varshavsky, 2005).

Ubiquitin-like molecules or modifiers (Ubls) share structural homology with Ub and can also be conjugated onto protein substrates, mostly with outcomes other than proteasomal degradation. Ubls like the *s*mall *u*biquitin-like *mo*difier SUMO, *ne*uronal precursor cell-expressed *d*evelopmentally *d*own-regulated 8 (NEDD8) or *I*FN-*s*timulated *g*ene product of *15* kDa (ISG15), to name but a few, are implicated in important physiological processes like nuclear transport, maintenance of chromosome integrity, transcriptional regulation, cell cycle control, signaling and regulation of proteolysis (Hochstrasser, 2001; Schwartz and Hochstrasser, 2003). Ubls may regulate Ub-mediated proteolysis or signaling through comodification of a substrate, thus modulating the effects of Ub conjugation (Lamsoul *et al.*, 2005; Sobko *et al.*, 2002) or by regulating the activity, specificity, localization, or stability of enzymes in the Ub-conjugating cascade, as is the case for NEDD8 modification of Cullin-RING E3s (Kawakami *et al.*, 2001; Petroski and Deshaies, 2005; Wu *et al.*, 2005).

11. Deubiquitinating Enzymes

Deubiquitinating enzymes can cleave isopeptide bonds to remove Ub from the substrate or from polyubiquitin chains. There are about 100 DUBs in the human genome, organized in five classes according to their catalytic domain structure: the ubiquitin-specific proteases (USPs), the ubiquitin C-terminal hydrolases (UCHs), the Machado-Joseph disease proteases (MJDs), the ovarian tumor proteases (OTUs), and the JAB1/MPN/Mov34 proteases (JAMMs). The first four classes comprise cysteine-type proteases, whereas JAMMs are metalloproteases. For a more detailed inventory of DUBs, read Nijman *et al.* (2005). DUBs have very diverse specificity properties, in terms of the ubiquitin or Ubl moiety itself (substrate specificity), in terms of the target protein to which the Ub or Ubl is attached (target specificity), and possibly in terms of the context provided by target and attached modification. DUB specificity *in vivo* can be further regulated by subcellular localization or association with different binding partners (Amerik and Hochstrasser, 2004; Li and Hochstrasser, 2003; Reyes-Turcu *et al.*, 2006; Soboleva and Baker, 2004). DUB functions are therefore also extremely diverse, ranging from regulation of proteasome function, to regulation of chromatin structure, to membrane protein trafficking, and with obvious implications in processes such as cancer and neurodegeneration (Amerik and Hochstrasser, 2004; Nijman *et al.*, 2005; Soboleva and Baker, 2004).

12. The Proteasome

The proteasome, very abundant in the cytosol, is a multisubunit protease composed of the 20S and 19S proteasome complexes. The 20S proteasome (or central core particle) has the general architecture of a barrel, formed by four stacked rings of seven subunits each, the outer two rings being composed of α subunits and the innermost two rings of β subunits (Groll *et al.*, 1997). The β subunits, which line the proteasome's inner cavity, carry out the catalytic activity. For mammalian proteasomes, only three of the seven β subunits in each ring are catalytically active. Access to this cavity occurs through narrow pores (with a diameter on the order of 10–15 Å) at both ends of the barrel, so it is usually assumed that protein substrates must be unfolded prior to their delivery to the catalytic chamber (Groll *et al.*, 1997, 2000; Kohler *et al.*, 2001). Also at both ends of the core particle there is the 19S cap complex, whose functions range from recognition of poly-Ub chains on target proteins, to unfolding of the substrate to facilitate entry into the catalytic cavity, to deubiquitination activity (Adams, 2003; Heinemeyer *et al.*, 2004; Rivett *et al.*, 1997; Schmidt *et al.*, 2005; Seeger *et al.*, 1997).

As mentioned earlier, the proteasome plays an instrumental role in class I MHC antigen presentation and activation of peptide-specific CD8$^+$ T cell responses. This process requires not only generation of peptides of the right

quality—that is, right size and sequence to allow a correct fit into the peptide-binding cleft—but also in the right quantity to trigger a successful response, which is no small endeavor due to many destructive aminopeptidase activities in the cytosol (Shastri *et al.*, 2005; Strehl *et al.*, 2005). IFN-γ, a crucial component of the innate and adaptive antiviral immune responses, affords the immune system a competitive edge. IFN-γ induces expression of auxiliary β subunits, β1i, β5i, and β2i [also known as *low* *m*olecular weight *p*rotein 2 (LMP2), LMP7 and multicatalytic endopeptidase-like complex 1 (MECL1), respectively], as well as synthesis of the proteasome activator PA28, and of the proteasome maturation protein (POMP) (Strehl *et al.*, 2005). The immuno-subunits LMP2, LMP7, and MECL1 are incorporated into nascent proteasomes, replacing their endogenous counterparts and constituting the so-called immuno-proteasome. The proteasome activator PA28 (or 11S proteasome) binds to the outer rings of the 20S proteasome, thereby opening the central gate and facilitating substrate entry. POMP is important for assembly and maturation of the proteasome (Strehl *et al.*, 2005). This IFN-γ-induced proteolytic cascade, mediated by immunoproteasomes and PA28, might be induced to respond to a demand for high proteasome activity when the constitutive cascade is no longer sufficient, altering the proteolytic activity of the proteasome for maximal efficiency in production of the class I MHC peptide repertoire. IFN-γ treatment also activates a transcriptional program that increases the synthesis of class I MHC molecules themselves and that of components of the peptide-loading complex, thus increasing cell surface presentation (Kloetzel, 2004; Kloetzel and Ossendorp, 2004; Kruger *et al.*, 2003; Rivett and Hearn, 2004; Van den Eynde and Morel, 2001). Therefore, even though class I MHC presentation is constitutive, it can be modulated in the course of an immune response, with a proposed role in the early stages of a cytotoxic response. It is thus not surprising that viruses have targeted the IFN-γ signaling cascade so aggressively (Alcami and Koszinowski, 2000; Hengel *et al.*, 2005; Salazar-Mather and Hokeness, 2006).

13. ER Quality Control and Degradation

Although tightly controlled, ER protein synthesis is not always successful. Proteins may sustain damage or fail to complete their synthesis early during biogenesis, or be trapped in an irreversible nonnative conformation, or a mutation may result in a structural alteration that leads to misfolding, as is the case for the cystic fibrosis conductance regulator (CFTR) (Jensen *et al.*, 1995; Ward *et al.*, 1995), mutant plasma α1-antitrypsin (Teckman and Perlmutter, 1996), or tyrosinase (Halaban *et al.*, 2000). They may also be expressed in the absence of their cognate subunits, as is the case for unassembled subunits of

TCRα (Huppa and Ploegh, 1997; Yang *et al*., 1998). ER quality control is a homeostatic process that involves an elaborate machinery that recognizes and retains newly synthesized misfolded or misassembled proteins and targets many of them for degradation by the Ub-proteasome system (Ellgaard and Helenius, 2003). This mode of degradation therefore samples sets of proteins otherwise targeted to extracellular space, where the degradation products are available as peptide ligands for class II MHC products. In addition to its role in ER quality control, this ER-associated degradation (ERAD) can also be employed in the physiological regulated proteolysis of normal ER proteins whose degradation is subject to metabolic cues, such as hydroxymethylglutaryl-coenzyme A reductase (HMGR) (Hampton, 2002; Hampton and Bhakta, 1997).

A feature of this ER-associated protein degradation is the spatial separation between targeting of substrates and their proteolysis, which requires substrate export from the ER lumen or membrane to the cytoplasm by a process termed dislocation (also called retrograde translocation or retrotranslocation) (Werner *et al*., 1996). Dislocation is a complicated multistep process that involves substrate recognition, targeting for dislocation, removal from the ER membrane, deglycosylation, ubiquitination, and finally proteolysis (Kostova and Wolf, 2003; Meusser *et al*., 2005; Romisch, 2005). The proteasome is usually considered to be a nonselective degradation apparatus, with selection of ERAD substrates being mediated mostly by the Ub ligases. However, the ER quality control E3 enzymes are mostly cytosolic or membrane-associated and thus are separated from their substrates at least by the ER membrane. This invokes the existence of mechanisms, present in E3 ligases themselves or in upstream factors, which facilitate coupling of ERAD substrate recognition to ubiquitination by E3 ligases in the cytoplasm.

14. ERAD Substrate Recognition

Owing to the extremely diverse nature of proteins that must be examined by the ER quality control machinery, a unifying model for how recognition of ERAD substrates takes place remains intractable. Nonetheless, misfolded proteins cleared from the ER enter the class I MHC-processing pathway, and hence this route of degradation is an important aspect of the generation of class I MHC epitopes. Instead, ERAD is likely to be custom-fitted to the client protein in question.

For glycoproteins, a possible mechanism is the recognition of terminally misfolded proteins by the calnexin/calreticulin (CNX/CRT) lectin-type chaperones, which retain immature glycoproteins in the ER until productive folding takes place (Ellgaard and Helenius, 2003; Hammond, 1994; Helenius and Aebi, 2004). Terminally misfolded proteins—that is, proteins that after

extensive CXN/CRT cycle folding attempts still fail to acquire their native conformation—are trimmed by ER mannosidase I, leading to recognition by ER *d*egradation-*e*nhancing alpha-*m*annosidase-like protein (EDEM), which presumably targets them for degradation (Eriksson *et al.*, 2004; Jakob *et al.*, 2001; Molinari *et al.*, 2003). Most ER lumenal proteins require the lumenal chaperone BiP for degradation, while transmembrane proteins with large cytosolic domains usually rely on cytosolic chaperone systems, like the heat shock protein (HSP) complexes Hsp70/Hsp90 and Hsp40/Hsp70 (Ellgaard and Helenius, 2003; Romisch, 2005). Yet it appears that a protein's lumenal or ER membrane localization matters less than the localization of the folding alteration within the polypeptide itself. CFTR whose cytoplasmic domains are recognized first by the Hsp70/Hsp90 cytoplasmic chaperone system may be targeted for degradation by the cytosolic Hsp70/Hsp90-interacting CHIP E3 ligase cochaperone (Connell *et al.*, 2001; Murata *et al.*, 2001), even if the protein also has a misfolded lumenal domain (which might also target it to a BiP-dependent degradation pathway). If the cytoplasmic domain is properly folded, then the lumenal domains are inspected, and if then the protein is recognized as misfolded, it is degraded in a process that involves BiP (Connell *et al.*, 2001; Meacham *et al.*, 2001; Vashist and Ng, 2004). This suggests that ER quality control uses sequential checkpoints to select degradation substrates and target them to the appropriate degradation pathway.

In the case of nonglycosylated substrates, protein disulfide isomerase (PDI), one of a large number of ER-resident oxidoreductases that catalyze disulfide bond formation and isomerization, can play a role in ER quality control by unfolding certain substrates prior to degradation. Another oxidoreductase, ERp57, interacts with CNX and CRT to facilitate folding, but in the event of a terminally misfolded protein may aid in transfer of proteins with improper disulfide bonds to the EDEM pathway (Hirsch *et al.*, 2004). Another possibility, at least in yeast, is that misfolded proteins actually escape to the Golgi and are then recycled to the ER (Taxis *et al.*, 2002; Vashist *et al.*, 2001). This ER–Golgi shuttling model was proposed because mutations in several secretory pathway genes (like Ufe1p, Sec23p, and Erv29p) compromise degradation of ERAD substrates (Taxis *et al.*, 2002; Vashist *et al.*, 2001), invoking a functional secretory pathway for efficient degradation of misfolded proteins from the ER. The Golgi apparatus could presumably endow misfolded proteins with a signal for destruction, but such a modification has not been found. Since the Ufe1p and Sec23p have since been shown to be required for maintenance of proper ER structure (Prinz *et al.*, 2000), the effects on protein degradation may simply be pleiotropic consequences of perturbing the normal arquitecture of the ER (Hammond *et al.*, 1994; Romisch, 2005). Ubiquitination at the ER membrane is yet another mode of ERAD substrate selection.

15. ERAD E3 Ligases

Most ERAD E3s are at the ER membrane, as is the case for the yeast Hrd1p/Der3p and Doa10p, or can be brought to the ER membrane on demand, as is the case for cytosolic SCF complexes and CHIP, for example (Kostova and Wolf, 2003; Meusser *et al.*, 2005; Romisch, 2005). An E3 ligase can recruit distinct E2 Ub-conjugating enzymes and/or distinct adaptor proteins (as we have seen for the SCF and ECS E3 complexes and will discuss later for ERAD E3s), thus conferring specificity in substrate selection. The yeast Hrd1p/Der3p E3 ligase was first discovered in a genetic screen for *Saccharomyces cerevisiae* genes involved in *H*MG-CoA *r*eductase *d*egradation (Hrp) (Hampton *et al.*, 1996) and is an ER-resident protein with six predicted transmembrane domains and a C-terminal RING-finger motif facing the cytosol. Hrd1p can act in a complex with Ubc7p and Ubc6p to ubiquitinate substrate proteins (Fig. 2B). Ubc7p is a soluble protein that becomes active only when tethered to the ER membrane by the Ubc7p cofactor Cue1p membrane protein. *D*egradation *o*f transcription factor Matα2–10 protein (Doa10p) is a transmembrane protein of the ER/nuclear envelope, which participates in yeast ERAD (Swanson *et al.*, 2001). Doa10p is predicted to span the membrane 14 times (Kreft *et al.*, 2006) and, like Hrd1p, uses Cue1p/Ubc7p and Ubc6p to ubiquitinate its substrates (Fig. 2B). However, the two ligases target different sets of substrates for degradation (Bays *et al.*, 2001; Swanson *et al.*, 2001).

Hrd1p ubiquitination activity can be directed to a specific subset of ER degradation substrates, by virtue of its association of Hrd1p with Hrd3p and Der1p. Hrd3p is a single-spanning ER membrane protein with a large ER luminal domain that can recognize misfolded proteins, thus possibly functioning as a substrate recruitment factor for the Hrd1p ligase complex (Gauss *et al.*, 2006). The *d*egradation from the *ER*-1 protein (Der1p) spans the ER membrane four times and is required for degradation of some misfolded glycoproteins (Knop *et al.*, 1996). Der1p may function as a substrate adaptor protein or even as a channel for ejection of degradation substrates from the ER membrane. Hrd3p can associate with Der1p, presumably enabling substrate delivery to downstream components. Hrd3p can even regulate Hrd1p activity, which is necessary for substrate extraction from the membrane and delivery to the proteasome (Gauss *et al.*, 2006) (Fig. 3). These multifunctional protein complexes can therefore function in substrate selection in the ER lumen or membrane and even facilitate subsequent steps that lead to proteasomal degradation. Similarly, Doa10p can catalyze ubiquitination of both membrane and soluble proteins, yet the mechanisms of subsequent proteasome targeting differ (Ravid *et al.*, 2006), presumably due to association with other regulatory proteins. The many layers of E3-mediated regulation of ERAD substrate selection are most likely just emerging.
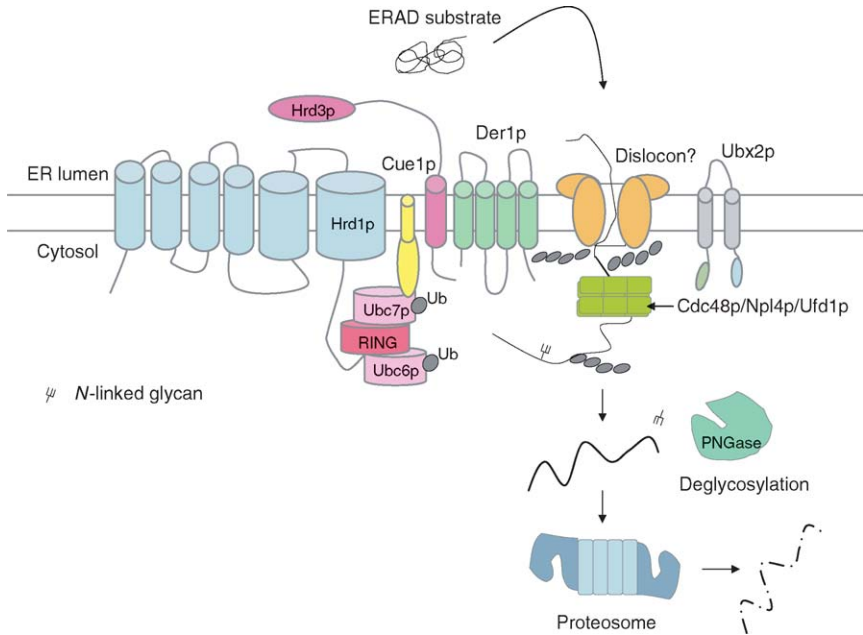
Figure 3 The yeast Hrd1p/Der3p-Hrd3p E3 ligase participates in multiple steps of ERAD, from substrate selection in the ER membrane or lumen to ubiquitination in the cytosol. PNGase, peptide-*N*-glycanase; Ub, ubiquitin. See text for details.

16. Mammalian ERAD E3s

In mammals, there are two predicted homologues of Hrd1p/Der3p, the HRD1 and the gp78 E3 ligases. Like its yeast counterpart, human HRD1 is involved in degradation from the ER (Kikkert *et al.*, 2004). HRD1 may associate with UBC7 to catalyze ubiquitination of a subset of substrates, like TCRα and CD3δ. HRD1 is not involved in the regulated degradation of the mammalian HMGR (Kikkert *et al.*, 2004). HRD1 may also associate with SEL1L, a homologue of Hrd3p, as well as other ER membrane and ER membrane-associated proteins, including Cdc48p(p97)/NPL4/UFD1, forming multisubunit complexes that seem to coordinate steps that range from substrate selection to delivery to the proteasome for at least a subset of ER degradation substrates (Lilley and Ploegh, 2005a; Ye *et al.*, 2005). gp78 was identified as the tumor autocrine motility factor receptor (AMFR) (Nabi *et al.*, 1992) and later as an E3 ligase due to its homology to Hrd1p and involvement in degradation of CD3δ and apoliprotein B100 (Fang *et al.*, 2001; Liang *et al.*, 2003). gp78 is an ER-resident

protein, predicted to span the membrane five times, with a C-terminal cytosolic RING-domain and an additional UBC7 E2-binding site, the Cue domain, arranged in tandem. While Hrd1p recruits UBC7 through the transmembrane Cue1p protein, it seems that convergent evolution has made the E3- and the E2-docking protein come together in a single human protein. Curiously, gp78 is involved in sterol-regulated Ub–dependent degradation of HMGR (Song et al., 2005), suggesting it constitutes the true functional homologue of yeast Hrd1p.

Homocysteine-induced endoplasmic reticulum protein (HERP) is a single-spanning ER membrane protein that is induced by the unfolded protein response (UPR) and also required for ERAD. HERP is proposed to improve ER protein folding and decrease protein load, protecting cells from ER-stress–induced apoptosis (Kokame et al., 2000). Furthermore, HERP has an N-terminal ubiquitin-like domain (ULD) and is required for the degradation of conexin and CD3δ (Hori et al., 2004; Sai et al., 2002). It forms a complex with HRD1, p97, Derlin-1, and VIMP (Schulze et al., 2005), a VCP (p97)-interacting membrane protein, that recruits p97 to Derlin-1 (Ye et al., 2004). HERP may function as another adaptor protein in these multiprotein complexes at the ER membrane, influencing substrate selection.

The Doa10p mammalian homologue, TEB4 (or MARCH-VI), is a multiple-transmembrane-domain-containing protein of the ER membrane that functions as an E3 ligase: it has an N-terminal noncanonical RING-domain in the cytosol that catalyzes Ub conjugation and TEB4 self-ubiquitination and degradation (Hassink et al., 2005), but its regulation is poorly characterized. Parkin is a cytosolic E3 ligase with a C-terminal noncanonical double-RING-finger (RING-IBR-RING), and an N-terminal Ub-binding domain, believed to mediate proteasomal degradation of aggregation-prone proteins (Imai et al., 2000), typical of Parkinson's disease. Both phosphorylation (Yamamoto et al., 2005) and ER stress-induced association with C-terminus of Hsc70-interacting protein (CHIP), involved in cytosolic chaperone-dependent folding, regulate Parkin E3 ligase activity (Imai et al., 2002; Sahara et al., 2005), which could be beneficial for reduction of protein aggregates and cellular pathology.

How specificity in substrate selection is conferred can be illustrated by E3s involved in glycoprotein turnover. The F-box proteins Fbs1 and Fbs2 bind high mannose N-linked glycoproteins (Winston et al., 1999). By using Fbs1/Fbs2 as its substrate adaptor(s), the cytosolic SCF^{Fbs1,Fbs2} E3 ligase is rendered specific for glycoproteins that have been dislocated from the ER (Yoshida et al., 2002, 2003) (Fig. 4A). The CHIP U-box E3 ligase which is involved in the degradation of CFTR (Meacham et al., 2001) and glucocorticoid hormone receptor (Meacham et al., 2001), can be "manipulated" to function in ER glycoprotein turnover. CHIP usually serves as a cochaperone
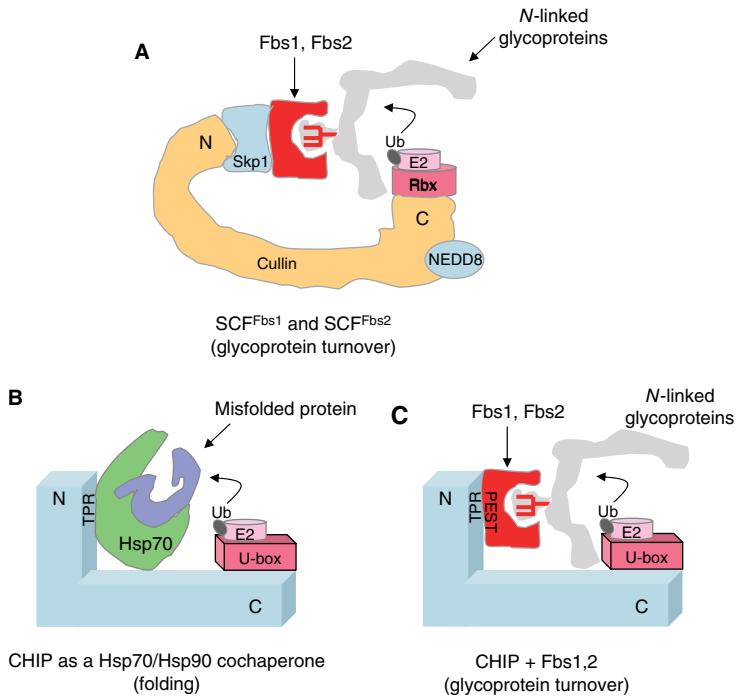
Figure 4 Substrate specificity in mammalian ERAD E3s involved in glycoprotein turnover. (A) The cytosolic SCF E3 ligase catalyzes ubiquitination of dislocated *N*-linked glycoproteins when complexed with the F-box proteins Fbs1 and Fbs2; Rbx, RING-box domain. (B) CHIP, C-terminus of *H*sc70-*i*nteracting *p*rotein, is usually a cochaperone for the heat shock protein (Hsp) chaperone system Hsp70/Hsp90, ubiquitinating misfolded proteins bound to Hsps. (C) When complexed with Fbs1 or Fbs2, CHIP ubiquitinates dislocated glycoproteins. E2, Ub-conjugating enzyme; Ub, ubiquitin.

for the cytosolic *h*eat *s*hock *p*rotein Hsp70/Hsp90 chaperone system. The CHIP N-terminal TPR motif recruits Hsp chaperones loaded with misfolded proteins, whereas its C-terminal U-box RING domain recruits E2 enzymes (Murata *et al.*, 2003) (Fig. 4B), effectively linking protein folding with ubiquitination. The CHIP E3 ligase activity can be directed to ER glycoprotein turnover by binding to Fbs2 (Nelson *et al.*, 2006), through an interaction between its TPR motif and a PEST motif in Fbs2 (Fig. 4C). N-linked glycans can, therefore, function in ER quality control not only to regulate ER retention in the folding cycle, but also to function in ERAD substrate selection and ubiquitination, adding an additional layer of complexity and specificity to glycoprotein quality control (Nelson *et al.*, 2006; Yoshida, 2003).

17. The Elusive Dislocon

Export of proteins through the ER membrane most likely takes place via an aqueous channel that allows the passage of polypeptides through the highly hydrophobic ER membrane environment while maintaining proper ionic balance between the ER and the cytoplasm. The Sec61 channel, the very same channel responsible for protein import into the ER, was initially thought to mediate transport in the reverse direction (hence the name retrograde translocation or retrotranslocation also coined for the dislocation process) (Pilon *et al*., 1997; Plemper *et al*., 1997, 1998; Wiertz *et al*., 1996b), with accessory factors regulating directionality and specificity of the channel. The extent to which Sec61 is involved in ER dislocation or the identity of the "dislocon" are not without controversy, and the search for a dislocation channel(s) is a subject of intense research (Meusser *et al*., 2005; Romisch, 2005).

Mammalian Derlin-1, a member of the *Der*1p-*Li*ke (Derlin) family of yeast Der1p homologues, is involved in dislocation from the ER (Lilley and Ploegh, 2004; Ye *et al*., 2004) and was proposed to constitute a channel for protein export from the ER membrane to the cytosol (Lilley and Ploegh, 2004; Ye *et al*., 2004). Like their yeast homologue, Derlins 1, 2, and 3 are tetraspanning ER membrane proteins that can homo- and heteroligomerize and could presumably form higher order structures with channel-like properties (Lilley and Ploegh, 2004; Ye *et al*., 2004). Conclusive evidence for a role of Derlin-1 as a channel is still unavailable, and in any case, Derlin-1 is unlikely to be the only channel, as turnover of some ERAD substrates does not rely on Derlin-1 function (Kreft *et al*., 2006; Lilley and Ploegh, 2004). Derlins 2 and 3 are obvious candidates that could function in place of Derlin-1. Alternatively, Derlins may act to deliver a particular substrate to a channel/another adaptor in its cognate dislocation pathway. In fact, Derlins form a large, multiprotein complex with p97 and the Hrd1p and Hrd3p mammalian homologues HRD1 and SEL1L, respectively (Lilley and Ploegh, 2005a; Ye *et al*., 2005), suggesting a very intimate connection between substrate recognition, export through the membrane, ubiquitination, and extraction into the cytoplasm. The existence of such a complex that would integrate all of these different functions, including formation of a channel or dislocon, would offer obvious advantages in terms of control of both specificity and directionality of the dislocation process. We shall return to this substrate "guidance" theme.

18. Driving Dislocation and the Ub-Binding Route to the Proteasome

A cytosolic complex containing the AAA ATPase Cdc48p (yeast) [valosin-containing protein (VCP)/p97 (in mammals)] and its cofactors nuclear protein localization 4 (Npl4p) and ubiquitin-fusion degradation 1 (Ufd1p), was recently shown

to participate in ER degradation (Lord *et al*., 2002; Romisch, 2005; Ye *et al*., 2001). Cdc48p/p97 is an essential protein of the AAA ATPase (*ATPases associated with various cellular activities*) family, conserved from archaea to mammals, whose functions include mitotic spindle disassembly, membrane traffic and fusion, nucleic acid repair and replication, and Ub-proteasome degradation (Woodman, 2003). Cdc48p/p97 is a motor protein that generates energy from ATP binding and hydrolysis; it forms a homohexameric barrel structure, with each subunit containing two AAA domains that contain the Walker motifs essential for ATPase activity, and the 6 subunits arranged in a ring with a pore in the center (Zhang *et al*., 2000).

Cdc48p/p97 interacts with many different adaptor proteins, which regulate its function (Dreveny *et al*., 2004). P97 can recognize denatured proteins nonspecifically (Thoms, 2002) and has an affinity for polyubiquitin chains (Ye *et al*., 2003). When complexed with the polyUb-binding Ufd1p and Npl4p, Cdc48p/p97 activity is directed to ER degradation (Ye *et al*., 2001). Both in yeast and in mammals, the trimeric Cdc48p(p97)/NPL4/UFD1 complex is proposed to function in a postubiquitination, preproteasomal step (Bays and Hampton, 2002; Jarosch *et al*., 2002), in one of two fashions: the ATP-hydrolytic activity of the AAA ATPase p97 may provide the driving force to extract substrates through the ER membrane, or may be required to liberate already dislocated substrates from the cytosolic face of the ER membrane (Braun *et al*., 2002; Flierman *et al*., 2003; Hirsch *et al*., 2004; Kostova and Wolf, 2003; Meusser *et al*., 2005). The proteasome presumably interacts with the ER membrane (Hirsch and Ploegh, 2000), either directly or through a receptor that docks the proteasome to the ER membrane, perhaps Sec61 (Kalies *et al*., 2005). Notwithstanding, the Cdc48p(p97)/NPL4/UFD1 complex or other accessory factors might aid substrate feeding to the proteasome (Hartmann-Petersen and Gordon, 2004a; Richly *et al*., 2005).

Ubiquitin-binding factors, such as Rad23p and Dsk2p (in yeast), have a ubiquitin-associated (UBA) motif that binds polyubiquitin chains and a ubiquitin-like (UBL) motif that binds to the 19S proteasome, and these are required for efficient degradation of a model ERAD substrate (Elsasser *et al*., 2004; Schauber *et al*., 1998; Wilkinson *et al*., 2001). The yeast Ub regulatory X domain-containing Ubx2p/Sel1p protein, an integral ER membrane protein, was recently shown to recruit the Cdc48p/Npl4p/Ufd1p complex to the ER membrane, thereby facilitating the transfer of polyubiquitinated substrates from the E3 ligases Hrd1p and Doa10p to Cdc48/p97 (Neuber *et al*., 2005; Schuberth and Buchberger, 2005). These dual function Ub-binding factors effectively serve as bridges between the p97/NPL4/UFD1 complex and the proteasome. As more of these Ub- and proteasome-binding proteins are discovered (Buschhorn *et al*., 2004; Decottignies *et al*., 2004; Medicherla

et al., 2004; Mullally et al., 2006), a "guidance" model, in which ERAD substrates are escorted from a dislocation channel to the proteasome by a cascade of Ub-binding factors, gains strength (Hartmann-Petersen and Gordon, 2004b; Hartmann-Petersen et al., 2003; Hendil and Hartmann-Petersen, 2004; Richly et al., 2005).

These interactions could be responsible for maintaining the substrate in a proteolysis-competent state and protect it from premature deubiquitination, as well as contribute to directionality of dislocation (Hendil and Hartmann-Petersen, 2004; Meusser et al., 2005; Romisch, 2006). In fact, it seems Cdc48p/p97 may even be capable influencing substrate fate (Rumpf and Jentsch, 2006). Cdc48p/p97 can simultaneously bind Ufd2p, a U-box E3 that catalyzes polyubiquitin chain extension, and one of two factors that can counteract its action: Otu1p, a DUB, and Ufd3p protein, a WD40 repeat protein of unknown function that has been shown to be required for Ub-dependent proteolysis (Ghislain et al., 1996; Johnson et al., 1995). Otu1p can disassemble the polyUb chains, whereas Ufd3p competes with Ufd2p for the same docking site on Cdc48p/p97. Presumably, Cdc48p/p97 can selectively recruit different substrate processing cofactors and thus tip the balance toward substrate degradation or release from the degradation cascade (Rumpf and Jentsch, 2006), suggesting a very tight regulation of proteasomal proteolysis. These aspects are important not only to understand how these pathways contribute to class I MHC–peptide epitope presentation, but also how viruses manipulate these routes to avoid detection.

19. Peptide N-Glycanase

PNGase is a cytosolic deglycosylating enzyme that presumably removes N-linked glycan chains from misfolded substrates prior to proteasomal degradation (Hirsch et al., 2003; Suzuki et al., 2000). Both in yeast and mammals, there is generally a tightly knit relationship between PNGase and the proteasome: PNGase interacts with (at least) the S4 and S5 subunits of the mammalian 19S proteasome and the Ub-binding factor Rad23p (HR23B in mammals), which seems to recognize only deglycosylated degradation substrates (Katiyar et al., 2004). This suggests that misfolded protein substrates may first be deglycosylated by ER-associated or free PNGase, then identified by the HR23B adaptor protein, and subsequently targeted to the nearby proteasome (Katiyar et al., 2004). Mammalian PNGase also associates with the ER membrane gp78 E3 ligase and the cytosolic p97 and Y33K, a UBA/UBX domain protein (Li et al., 2006a). A gp78-Y33K-p97-PNGase-HR23B complex could therefore be formed that recruits PNGase to the cytosolic face of the ER membrane that couples the activities of dislocation, ubiquitination, and deglycosylation and escorts misfolded glycoproteins to the proteasome (Kim et al., 2006a; Li et al., 2005, 2006a).

20. Viral Interference with Class I MHC Antigen Presentation

Viruses keep evolving and developing sophisticated immune evasion strategies. In particular, they have targeted virtually every step of the class I MHC antigen presentation pathway, inhibiting proteolysis and generation of the antigenic peptide [Epstein–Barr virus (EBV) nuclear antigen-1 or EBNA-1, HCMV E protein pp65, and HIV Tat], inhibiting peptide loading and assembly in the ER (HSV ICP47, HCMV US6, bovine herpes-virus-1 UL49.5), retaining class I MHC molecules in the ER (adenovirus E3/19K and HCMV US3), blocking their exit from the ER-to-Golgi complex (ERGIC) (MCMV m152), misdirecting MHC complexes to lysosomal compartments (MCMV m06 and HHV-7 U21), internalizing MHC complexes from the cell surface (KSHV K3 and K5 and HIV Nef), encoding homologues of class I MHC as decoys for NK cells (HCMV UL18 and UL142 and MCMV m04), and causing degradation of class I MHC products by the Ub-proteasome system (HCMV US2 and US11 and MHV-68 mK3) (Fig. 5). Because these topics have been the subject of numerous
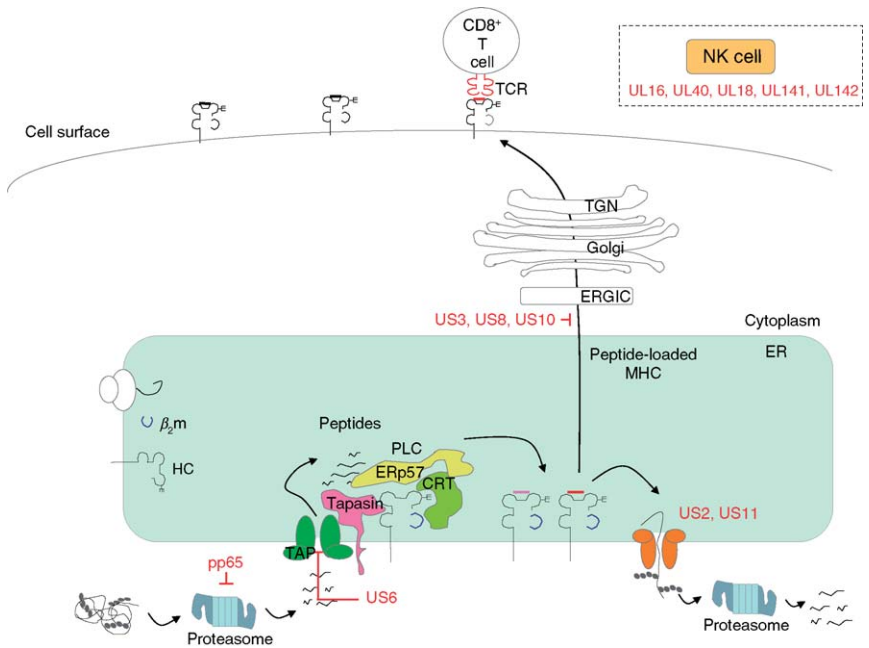
Figure 5 HCMV interference with class I MHC antigen presentation. HCMV immunoevasins aimed at inhibition of cytotoxicity by CD8+ T cells and NK cells are in red. TCR, T cell receptor; PLC, peptide-loading complex; TAP, transporter associated with antigen presentation; CRT, calreticulin.

reviews (Alcami and Koszinowski, 2000; Ambagala *et al*., 2005; Hengel and Koszinowski, 1997; Hengel *et al*., 1998, 1999; Lybarger *et al*., 2003; Mocarski, 2004; Yewdell and Hill, 2002), we shall discuss only a few of these mechanisms in more detail, particularly those exploited by HCMV.

21. Human Cytomegalovirus

The β-herpesvirus HCMV is extremely successful in evolutionary terms: it is a ubiquitous, highly species-adapted pathogen that is able to establish a life-long persistent infection with minimal or no disease symptoms in the immunocompetent host. Prolonged latency periods (a dormant state with minimal production of viral proteins and absence of viral progeny) and controlled sporadic reactivation ensure transmission to a new host, and thus survival of both host and virus. Perturbation of this delicate balance leads to life-threatening infections in immunocompromised patients, transplant recipients and infected newborns and illustrates how the outcome of this host–virus relationship is dependent on viral manipulation of the host immune response (Hengel *et al*., 1998; Klenerman and Hill, 2005). The several HCMV-encoded immunoevasins (Jones *et al*., 1995) are presumably aimed primarily, but not solely, at control of the CD8$^+$ T cell and NK cell responses (Falk *et al*., 2002; Mocarski, 2004; Pinto and Hill, 2005; Yewdell and Hill, 2002). Here we will discuss the HCMV immunoevasins that interfere with class I MHC antigen presentation, US3, US6, US10, US2, US11, UL16, UL18, UL40, UL141, and UL142. In light of our most recent findings, we will elaborate on the mechanism of ER dislocation co-opted by the HCMV US2 and US11 immunoevasins. More specifically, we discuss the similarities and the differences between the two cellular ERAD pathways that US2 and US11 have allowed us to uncover and the possible implications for ER dislocation. We will extend this by comparing the HCMV US2- and US11-mediated dislocation of human class I MHC HC molecules with dislocation of murine HCs by the MHV-68 mK3 immunoevasin.

22. HCMV Interference with Class I MHC Antigen Presentation

If one goes back to the steps we depicted for antigen presentation (Fig. 1) and then examines the immunoevasins encoded by HCMV, we will find that this herpesvirus exploits many aspects of the antigen presentation pathway thus defined. The HCMV phosphoprotein pp65 tegument protein mediates the phosphorylation of the HCMV immediate early antigen-1 (IE-1) during HCMV infection. Phosphorylation of IE-1 interferes with the presentation of IE-1-derived antigens (Gilbert *et al*., 1996). The US3 protein binds to and retains some class I MHC locus products in the ER membrane (Ahn *et al*., 1996; Jones *et al*., 1996). US3 is a type I membrane glycoprotein with an Ig-like

lumenal domain that is essential for its own retention, albeit transient, in the ER (Lee et al., 2003). US3 eventually travels to the lysosome where it is degraded (Gruhler et al., 2000). Some evidence suggests that ER retention of class I MHC complexes by US3 depends on the ER localization signal on US3 and perhaps the ability of the US3 luminal domain to oligomerize (Misaghi et al., 2004b) as determinants of retention of both molecules (Lee et al., 2003). Another model suggests that association of US3 with tapasin, which inhibits tapasin, is sufficient to mediate ER retention (Park et al., 2004). Class I MHC alleles that require peptide optimization by tapasin may be retained in the ER, whereas tapasin-independent locus MHC products are spared from retention. In fact, there is a perfect correlation between tapasin-dependence and US3 sensitivity (Park et al., 2004). Both mechanisms, tapasin inhibition and direct binding, may be in place, perhaps allowing US3 to retain a larger repertoire of class I MHC locus products.

US6 inhibits peptide loading of the class I MHC molecules by blocking the TAP transporter (Ahn et al., 1997; Hengel et al., 1997; Lehner et al., 1997). US6 is an ER-resident type I membrane glycoprotein with a bulky lumenal domain that binds the core transmembrane domains of the TAP subunits, TAP 1 and TAP 2, from within the ER lumen, inhibiting ATP binding (Hewitt et al., 2001; Kyritsis et al., 2001) and thus TAP-mediated peptide translocation into the ER. The US6 luminal domain oligomerizes and may form a bridge between the TAP 1 and TAP 2 subunits to effectively block TAP activity (Halenius et al., 2006). US10 delays trafficking of class I MHC HCs and stalls them in the ER; although the block is not absolute and the mechanism is poorly characterized, US10 expression results in downregulation of class I MHC from the cell surface (Furman et al., 2002a). US2 and US11 catalyze destruction of class I MHC HCs from the ER membrane by targeting them to the Ub-proteasome system (Wiertz et al., 1996a,b), a process we discuss in detail in a later section. Expression of each individual immunoevasin results in reduction of cell surface expression of class I MHC peptide-loaded complexes and evasion of CD8^{+} T cell-mediated lysis (Ahn et al., 1996; Jones et al., 1995).

Besides CD8^{+} T cell recognition, HCMV can frustrate NK cell recognition (Orange et al., 2002). Protection of HCMV from NK cell-mediated lysis can be mediated by several immunoevasins, HCMV UL16, UL40, UL18, UL141, UL142, and pp65 (Lodoen and Lanier, 2005; Orange et al., 2002; Rajagopalan and Long, 2005; Reyburn et al., 1997; Wills et al., 2005). The activating receptor NKG2D on the NK cell recognizes divergent families of class I MHC-related ligands, like the MIC and ULBP products. HCMV UL16 retains the ULBP 1, ULBP 2, and MIC-B NKG2D ligands in the ERGIC compartment of the target cell, preventing NKG2D recognition and thus NK cell activation (Dunn et al., 2003). HCMV UL141 and pp65 act at the level of the NK effector cell rather than the APC. The result is, nevertheless, the same: prevention of

NK cell activation. UL141 downregulates the NK cell-activating receptors CD226 (DNAM-1) and CD96 (TACTILE) (Tomasec et al., 2005). The pp65 tegument protein engages the activating receptor NKp30 and antagonizes its effects, dampening NK cell-mediated cytotoxicity. How a tegument protein gains access to the receptor on the NK cell is unknown, but pp65 engagement of the NKp30 receptor causes dissociation of a receptor-associated signaling module (Orange et al., 2002), disrupting the activating signaling pathway that would lead to NK cell activation (Arnon et al., 2005).

NK cell responses also rely on receptors that recognize class I MHC locus products. The killer cell immunoglobulin-like receptor (KIR) genes encode a family of activating and inhibitory receptors that recognize human leukocyte antigen (HLA)-A, -B, and -C. The CD94/NKG2 receptors recognize the nonclassical class I MHC molecule HLA-E. HLA-E presents fragments derived from the signal sequences of classical class I MHC molecules, which delivers an inhibitory signal to CD94/NKG2 receptors on NK cells. UL40 encodes a peptide whose sequence is exactly homologous to the HLA-E binding leader peptide from HLA-C locus products. UL40 therefore loads HLA-E and maintains HLA-E on infected cells (Tomasec et al., 2000; Ulbrecht et al., 2000) even when other class I MHC products are downregulated. UL18 encodes a class I MHC-like molecule that engages the inhibitory CD85j/LIR-1/ILT-2 receptor on the NK cell, thus inhibiting NK cell effector functions (Cosman et al., 1997; Reyburn et al., 1997).

However, UL18 and UL40 expression may be insufficient to confer target cell protection (Falk et al., 2002; Leong et al., 1998). UL18 and UL40 may, in fact, be more relevant for control of viral infection by T cells, with HLA-E-restricted CD8^{+} T cells playing a role in lysis of cells expressing UL40, and non-MHC-restricted CD8^{+} T cells playing a role in lysis of UL18-positive cells (Pietra et al., 2003; Romagnani et al., 2004; Saverino et al., 2004). CD85j is an invariant receptor expressed by many T cells and responsible for transduction of inhibitory signals that downregulate antigen-specific T cell functions (Merlo et al., 2001; Saverino et al., 2000). CD85j interaction with UL18 on CD8^{+} T cells occurs in a TCR-independent manner and leads to activation (not inhibition) of non-MHC-restricted CD8^{+} T cells (Saverino et al., 2004). This expands the repertoire of T cell activation mechanisms and is probably a viral strategy of ensuring survival of the host, by allowing some level of protection from the initial wave of NK cell-mediated antiviral response. In vivo HCMV-infected APCs are faced with multiple immunoevasins displaying allelic preferences and expression patterns that are both spatially and temporally regulated. The many immunoevasins expressed by HCMV are thus likely to have both synergistic and antagonistic interactions (Ahn et al., 1996; Farrell et al., 2000; Klenerman and Hill, 2005; Mocarski, 2004; Reddehase, 2002; Yewdell and Hill, 2002), as has been experimentally verified for murine cytomegalovirus (Wagner et al., 2002).

23. Dislocation from the ER: HCMV US11 and US2

Evidence of ER-to-cytosol transport or dislocation, a crucial ER quality control step now considered of general importance in dispensing with misfolded or misassembled ER proteins, was initially provided by studying the mechanism of action of the HCMV US2 and US11 immunoevasins (Wiertz *et al.*, 1996a,b). The viral proteins appropriate this cellular quality control process to extract (dislocate) class I MHC HCs from the ER membrane. On arrival in the cytoplasm, the dislocated HC molecules are destroyed by the proteasome; destruction of the HC component of the class I MHC complex by US2 and US11 abolishes cell-surface expression of class I MHC complexes and, consequently, presentation of viral peptides to CD8^{+} T cells, allowing HCMV to remain undetected (Wiertz *et al.*, 1996a,b).

US2- and US11-mediated HC dislocation from the ER membrane is not only an ingenious viral immune evasion strategy, but also a useful case study in ER quality control and degradation. One theme that arises from the characterization of this process over the 10 years that have passed since its discovery is that HC dislocation is unique in many respects: HC molecules do not meet the requirement of being either misfolded or misassembled, yet their dislocation takes place by virtue of the presence of US2 or US11; the speed of HC degradation is unrivaled by that of any other ER-associated degradation substrates: HC half-life is reduced from hours to a mere 2–5 min in cells infected by HCMV or in cells expressing either US2 or US11 (Wiertz *et al.*, 1996a,b); both US2 and US11 have stringent requirements in terms of which HLA alleles (Barel *et al.*, 2003, 2006; Machold *et al.*, 1997) or assembly, folding and ubiquitination status of the class I MHC complex (Blom *et al.*, 2004; Furman *et al.*, 2003; Gewurz *et al.*, 2001) either viral protein is able to target for dislocation and proteasomal destruction.

Notwithstanding the unique nature of this virus-mediated process, knowledge from the US11 pathway, and in particular the identification of the Derlin proteins, has widened our understanding of the cellular factors involved in ER dislocation more generally (Lilley and Ploegh, 2004, 2005a). The US11 transmembrane domain (TMD) is crucial for US11 function: more specifically, mutation of a polar amino acid, glutamine (Q) 192, within the US11 TMD to a hydrophobic leucine (L) residue renders this US11 Q192L mutant inactive in dislocating HCs from the ER membrane. In a screen for proteins that interact specifically with the active version of US11, work from our laboratory showed that the aforementioned Derlins, the mammalian Der1p homologues, are involved in HC dislocation mediated by US11, but not by US2 (Lilley and Ploegh, 2004). The fact that the dislocation mechanism used by US2 is not dependent on the Derlins prompted us to investigate what other ERAD

pathway is being co-opted by the HCMV US2 immunoevasin and allowed us to uncover an unexpected ERAD player.

24. Signal Peptide Peptidase Is Required for Dislocation from the ER

US2 is an ER-resident type I membrane glycoprotein of only 199 amino acids, with a noncleavable signal sequence (Gewurz et al., 2002), a lumenal domain that dictates an allele-specific association with the lumenal domain of HC (Gewurz et al., 2001), a transmembrane segment, and a short cytosolic tail of only 14 amino acids (residues 185–199), with no obvious sequence homology to known cellular proteins. The US2 tail is essential for dislocation: $US2_{186}$, a cytosolic tail deletion mutant of US2, is dislocation incompetent (Furman et al., 2002b). By using an affinity purification approach similar to that used for US11 (Lilley and Ploegh, 2004), signal peptide peptidase (SPP) was found as a specific interacting partner for dislocation-competent (active) US2 (Loureiro et al., 2006), an interaction that relies solely on the presence of the highly hydrophobic US2 tail. More importantly, reduction of SPP levels by RNA interference led to inhibition of class I MHC HC dislocation and to the discovery of SPP as a necessary factor for the US2-mediated ER dislocation pathway.

SPP is an ER-resident protein of approximately 45 kDa that is predicted to span the ER membrane seven to nine times (Friedmann et al., 2004), and a member of the presenilin (PS)/SPP-Like (SPPL) superfamily of intramembrane-cleaving aspartic proteases (Weihofen et al., 2002). These proteases are characterized by the ability to cleave substrate polypeptides within a transmembrane region and by possessing two active site aspartate (D) residues (italicized) within the conserved motifs YD and LGLGD in adjacent membrane-spanning regions (Martoglio and Golde, 2003; Wang et al., 2006a; Weihofen et al., 2002). There are seven related members of the PS/SPPL superfamily in the human genome: PS-1, PS-2, SPP and four SPP-Like proteins, SPP2a, SPP2b, and SPP2c, and SPP3 (Martoglio and Golde, 2003). Presenilins 1 and 2 are the catalytic components of γ-secretase, a tetrameric complex containing PS and three other subunits. PSs play a role in processing of the β-amyloid precursor protein (APP) into Aβ40 and Aβ42, peptides that constitute the principal components of the β-amyloid plaques in Alzheimer's disease (AD); PSs are also required for development due to processing of the Notch receptor by γ-secretase (Selkoe and Kopan, 2003) and might be involved in intracellular trafficking (Sisodia and St George-Hyslop, 2002; Wang et al., 2006c). The function of the four SPP-like proteins is, at this point, unknown, but the role of intramembrane-cleaving proteases is usually to liberate signaling molecules from membrane-bound precursors with consequent activation or repression

of signaling cascades (Fortini, 2002; Kopan and Ilagan, 2004; Martoglio and Golde, 2003; Parent *et al.*, 2005; Xia and Wolfe, 2003).

25. SPP and Generation of HLA-E Epitopes

In humans, SPP performs an important immunological function as it generates the peptide ligands for the nonclassical class I MHC molecule HLA-E. On insertion of secretory or type II membrane proteins into the ER, their signal sequence is cleaved by the ER luminal protein signal peptidase, leaving the signal peptide anchored in the ER membrane. The ER membrane–anchored signal peptide is subsequently cleaved by the intramembrane-cleaving SPP within the transmembrane region. The resulting signal peptide fragments are released into the cytosol (N-terminal portion) or into the ER lumen (C-terminal portion). The latter peptides may easily bind to class I MHC molecules in the ER lumen. The HLA-A2 molecule, for instance, is known to bind signal sequence-derived peptides. The N-terminal signal sequence fragments are TAP-transported into the ER lumen and bind to HLA-E (Lemberg *et al.*, 2001). By presenting fragments derived from the signal sequences of classical class I MHC molecules, HLA-E monitors the presence of classical class I MHC molecules. This is, as we discussed earlier, of crucial importance for NK cell recognition.

26. SPP and Processing of the Hepatitis C Virus Core Protein

SPP is involved in processing of the hepatitis C virus (HCV) core protein (McLauchlan *et al.*, 2002). HCV is a single-stranded RNA virus with a single open reading frame encoding a large polyprotein. The N-terminal portion of the HCV polyprotein encodes the structural components of the HCV virion, the core protein (thought to constitute the virion capsid), and the E1 and E2 envelope glycoproteins. The mature structural components of the HCV virion are produced through a series of cleavage events catalyzed by cellular proteases. The core protein is the most N-terminal portion of the polyprotein and is followed by the signal sequence of the E1 envelope glycoprotein. The E1 signal sequence targets the polyprotein to the ER membrane and induces transloca-tion of E1 into the ER lumen. Cleavage by signal peptidase liberates the N-terminal end of E1, leaving the core protein anchored (by the E1 signal peptide) in the ER membrane. SPP-mediated intramembrane proteolysis of the E1 signal sequence then results in release of the HCV core protein from the ER membrane, thus freeing the mature core protein for incorporation into lipid droplets (Martoglio and Golde, 2003). SPP-mediated HCV core protein matu-ration and trafficking to lipid droplets and the outer mitochondrial membrane

may be critical for viral assembly and life cycle (Ait-Goughoulte *et al.*, 2006) and may also affect cellular lipid metabolism and apoptosis, as HCV core protein-transgenic mice display liver pathologies, mitochondrial injury, and enhanced oxidative stress (Chou *et al.*, 2005; Korenaga *et al.*, 2005; Meyer *et al.*, 2005; Okuda *et al.*, 2002; Omura *et al.*, 2005; Schwer *et al.*, 2004; Suzuki *et al.*, 2005). SPP-mediated cleavage of the HCV core protein may thus modulate these important cellular functions of HCV.

27. SPP and Calmodulin Signaling

SPP may regulate the interaction of signal peptide remnants of HIV gp160 envelope protein and preprolactin (p-Prl) with calmodulin. A characteristic feature of a signal sequence is its tripartite structure: a polar N-terminal n-region, a hydrophobic core (h-region) of 7–15 residues, and a polar C-terminal c-region that contains the consensus sequence for signal peptide cleavage (von Heijne, 1985). The n-region of most signal sequences comprises only a few residues. However, some signal sequences have extended n-regions of up to 150 residues. The function of such long n-regions is not known. Both the p-Prl and the gp160 signal sequence have an extended basic n-region that can potentially form a basic amphiphilic alpha-helix, a feature of CaM-binding domains (O'Neil and DeGrado, 1990), not found in the majority of signal sequences. SPP-mediated cleavage releases this CaM-binding domain on the N-terminal fragment of the p-Prl and gp160 signal peptides into the cytosol. The functional and physiological significance of an interaction between the p-Prl and p-gp160 signal peptide fragments that are released into the cytosol and calmodulin (CaM) could be due to a regulatory function of the signal peptide fragments. CaM-dependent processes could be enhanced or inhibited depending on the amounts of CaM-binding signal peptide fragments generated and released into the cytosol (Martoglio *et al.*, 1997).

28. SPP Peptide Peptidase and Development

The SPP orthologues in *Drosophila melanogaster*, *Spp*, and in *Caenorhabditis elegans*, *Imp-2*, play an essential role in development: SPP protease activity seems to be essential for larval development both in the fly and in the nematode (Casso *et al.*, 2005; Grigorenko *et al.*, 2004). Although the mechanism by which SPP mutations impairs developmental processes in the fly is currently unknown, in *C. elegans* the molting defect induced by *imp-2* deficiency was mimicked by cholesterol depletion and by deficiency in *Irp-1*, a homologue of mammalian lipoprotein receptor-related protein (LRP) receptors suggesting a role in cholesterol and lipid metabolism (Grigorenko *et al.*, 2004).

29. SPP and ER Quality Control

The possibility of a role for SPP in ER quality control was first advanced by High and colleagues, who reported an association between SPP and a truncated version of a polytopic ER protein (opsin) in an *in vitro* system (Crawshaw *et al*., 2004) and proposed SPP to be implicated in the recognition of misassembled transmembrane domains during membrane protein quality control at the ER. US2-mediated dislocation of class I MHC HC molecules presents the first functional evidence of such a role for the intramembrane-cleaving protease SPP. The US2 tail is necessary and sufficient to recruit SPP, and structural predictions suggest that the US2 tail suggests may adopt a 3_{10} helical conformation that could form a protein–protein interaction domain (Oresic *et al*., 2006). SPP is crucial for dislocation by US2, as reduction of its levels by RNA interference blocks HC degradation. The question remains as to the detailed mechanism of its involvement.

An obvious possibility is involvement of the catalytic activity of SPP. This would imply a cleavage event during dislocation, such as within the TMD of US2 or HC or another factor, unknown at this point; a postcleavage function of one of these protein fragments could play a regulatory role in the process. SPP-mediated intramembrane proteolysis requires, among other things, a membrane protein substrate to have access to its catalytic core in a type II orientation (Lemberg and Martoglio, 2002; Martoglio and Golde, 2003). Both US2 and HC are type I membrane glycoproteins, so the topology of their transmembrane and tail segments is opposite to that of predicted SPP substrates. SPP-mediated intramembrane cleavage within the HC TMD is inconsistent with the observed recovery of full length HC in the dislocation reaction (Blom *et al*., 2004; Misaghi *et al*., 2004a; Wiertz *et al*., 1996a,b). For US2, while a suggested protein–protein interaction domain in the US2 tail (Oresic *et al*., 2006) may mediate binding to SPP, intramembrane cleavage of the US2 TMD by SPP in this inverted orientation would presumably not occur, as seen for other proteases (Roques *et al*., 1983; Tarasova *et al*., 2005). However, the proposed bent-helix conformation on the US2 tail (Oresic *et al*., 2006) might allow US2 to conform to the requirements for SPP cleavage. Whether SPP-mediated US2 cleavage takes place is unknown at this point. One can speculate that this putative processing of US2 by SPP, as for the HCV core protein (McLauchlan *et al*., 2002), could be necessary for "maturation" of US2 into a dislocation-active form. Binding of US2 to SPP could still modulate SPP enzymatic activity and thus affect dislocation. The possibility remains that SPP mediates cleavage *in trans* of an unidentified factor whose function is important. Experiments with SPP inhibitors and catalytic mutants of SPP should allow an assessment of the contribution of the proteolytic properties of SPP to dislocation.

The involvement of SPP need not be related to its catalytic activity. Substrate recruitment and subsequent cleavage by SPP may be separable events, as shown for the related PS (Kornilova et al., 2003; Lemberg and Martoglio, 2004). Some intracellular cleavage products of γ-secretase are proposed to be intermediates that are destined for degradation (Kopan and Ilagan, 2004; Parent et al., 2005). γ-secretase-mediated cleavage of a large number of type I transmembrane proteins releases their C-terminal fragments (CTFs). PS1 deficiency causes delayed turnover and subsequent accumulation of some γ-secretase substrates as full-length proteins (Esselens et al., 2004; Wang et al., 2006c; Wilson et al., 2004c), suggesting cleavage of the CTFs as a prelude to degradation. Treatment with γ-secretase inhibitors, however, does not phenocopy PS deficiency (Wang et al., 2006c), suggesting that this effect is independent of γ-secretase activity. Therefore, SPP may be crucial for HC dislocation irrespectively of its catalytic properties. Binding of US2 to SPP could presumably modify the SPP structure in a way that affects dislocation. Alternatively, recruitment of SPP by US2 could perhaps nucleate assembly of a dislocation complex, much like US11 and the Derlins (Lilley and Ploegh, 2005a). SPP may be a component of an ERAD pathway for a subset of ER degradation substrates that includes misfolded transmembrane proteins, such as truncated opsin (Crawshaw et al., 2004), and that is recruited by US2 to dislocate HC (Fig. 6, A arrow). Experiments to address the identity of SPP-associated proteins may prove informative.

Curiously, another class of intramembrane-cleaving proteases, the rhomboid serine proteases, share a homology domain of unknown function with the Derlins (Lemberg et al., 2005). It is tempting to speculate that our observations extend the connection from regulated intramembrane proteolysis to a direct involvement in ER dislocation. An involvement of SPP with the UPR is also a possibility. Cells deficient for the X-box binding protein-1 transcription factor (XBP-1) show upregulated levels of SPP transcripts (Shaffer et al., 2004), but this aspect of the process remains to be explored. Although removal of signal peptide remnants from the ER membrane, assigned to SPP in animals and plants, is not a function exclusive to higher eukaryotes, a gene that encodes an orthologue of this enzyme is absent from the yeast genome (Martoglio, 2003; Weihofen et al., 2002). The role of SPP in higher eukaryotes might therefore not be limited to signal peptide processing but extend to processes such as protein dislocation from the ER. PS and the other SPP-like members of the PS/SPPL superfamily of intramembrane-cleaving aspartic proteases, so far of unknown function (Martoglio and Golde, 2003), and some of which may not reside in the ER (Krawitz et al., 2005), may likewise be involved in disposal of different degradation substrates. HCMV might just be exploiting a cellular degradation pathway that involves intramembrane-cleaving proteases for disposal of class I MHC HC.
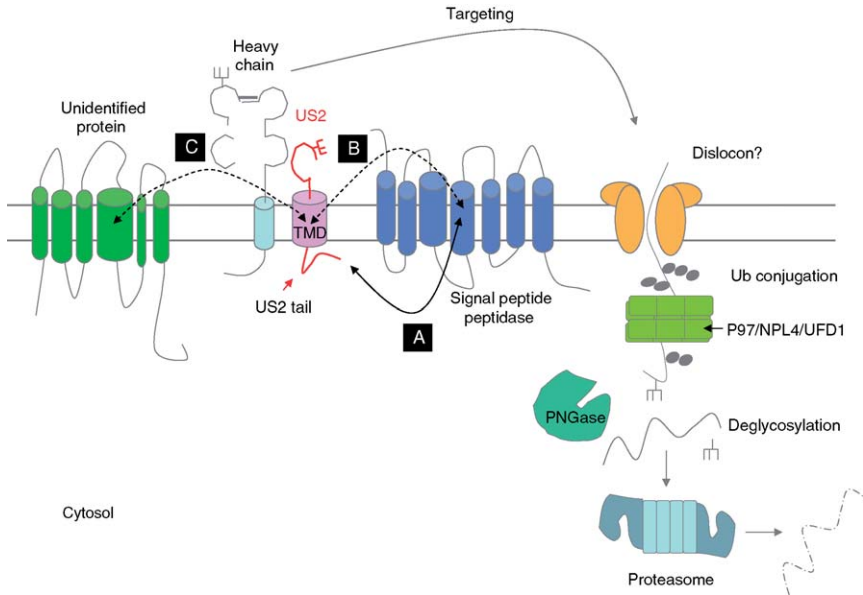
Figure 6 The US2 dislocation pathway requires signal peptide peptidase. The US2 cytosolic tail recruits SPP (A), which is required for dislocation of class I MHC HC. An additional step critical for dislocation is dependent on interactions involving the US2 transmembrane domain (US2 TMD) and SPP (B) or other protein(s) so far unidentified (C). Ub, ubiquitin; TMD, transmembrane domain. PNGase, peptide-*N*-glycanase.

   The US2 TMD, although dispensable for interaction with SPP, is also required for HC dislocation (Loureiro *et al*., 2006). HC dislocation is therefore dependent not just on (US2 tail-mediated) recruitment of SPP, but also on additional (US2 TMD-mediated) interactions within the plane of the membrane. The US2 TMD may be involved in further engagement of SPP (SPP-mediated cleavage or otherwise) (Fig. 6, B arrow), or alternatively, in the recruitment or engagement of other protein(s) involved in dislocation (Fig. 6, C arrow). ER membrane E3s or their adaptor subunits are likely candidates. SPP is most certainly not the sole host-derived component of the US2 dislocation pathway, and a putative multiprotein complex (cascade of adaptor proteins) analogous to that found for US11 is likely to be found that evokes many (complicated) links between the ERAD machineries at the level of the ER membrane and the cytosol. Uncovering the identity of the additional cellular partners of this HCMV US2 immunoevasin will certainly give us insight into the dislocation mechanism.

30. Three Routes of Pathogen-Mediated ER Protein Disposal

A theme that arises from analysis of the US2- and US11-mediated HC dislocation processes is that these are only superficially similar: although HC dislocation by US2 and US11 has the same outcome (proteasomal degradation) of HC and shares many if not all of the steps that take place after extraction of the HC from the ER membrane (such as deglycosylation and degradation kinetics), the differences between the pathways are rather striking in terms of the steps prior to dislocation. As we mentioned earlier, US2 and US11 have different allele and substrate folding, assembly, and ubiquitination requirements. Derlin-1 is crucial for US11- but not US2-mediated dislocation of HCs (Lilley and Ploegh, 2004; Ye et al., 2004), and, conversely, SPP is required by US2 but not by US11 (Loureiro et al., 2006). This suggests that the HCMV immunoevasins US2 and US11 are targeting HC molecules to distinct ER dislocation pathways, perhaps by serving as adaptor molecules aiding in recruitment and/or assembly of distinct multiprotein complexes at the ER membrane (Lilley and Ploegh, 2004, 2005b).

The murine γ-herpesvirus 68 (MHV-68) mK3 also targets newly synthesized murine class I MHC HC for dislocation from the ER and proteasomal degradation (Boname and Stevenson, 2001; Lybarger et al., 2005; Wang et al., 2005; Yu et al., 2002). MHV-68 mK3 belongs to a family of structurally related molecules, the K3 homologues, which have E3 ligase activity. K3 homologues are present in several different γ-herpesviruses and poxviruses (Coscoy and Ganem, 2000; Ishido et al., 2000; Mansouri et al., 2003; Stevenson et al., 2000). All K3 homologues possess a noncanonical RING-finger domain with ubiquitin ligase (E3) activity [also called a plant homeodomain (PHD) or leukemia-associated protein (LAP) domain], and a conserved integral membrane topology, with the transmembrane domains and cytosolic C-terminal tails mediating interaction with the substrate (Coscoy and Ganem, 2003). The mK3 PHD/LAP-family E3 is a type III ER membrane protein with the PHD/LAP RING-related domain facing the cytosol (Boname and Stevenson, 2001; Sanchez et al., 2002). mK3-mediated degradation of murine HCs is absolutely dependent on components of the PLC: association of mK3 with TAP and tapasin presumably imposes the necessary proximity and/or orientation of the mK3 RING domain that allows mK3 to specifically ubiquitinate class I MHC HC as they enter the PLC (Lybarger et al., 2005; Wang et al., 2004, 2005).

mK3-mediated dislocation of murine HCs is dependent on the ATPase activity of p97 and physical association with Derlin-1 and VIMP (Wang et al., 2006d). This is reminiscent of the pathway that HCMV US11 is proposed to co-opt for dislocation of mammalian class I MHC molecules. In a sense, it appears that mK3 may be a more evolved immunoevasin that can couple the

ability to recruit other components of the dislocation machinery, like US11 and presumably US2, with E3 ligase activity, in one viral polypeptide. There are several mammalian K3 homologues, the *m*embrane-*a*nchored *R*ING-*CH* (MARCH) proteins (Bartee *et al*., 2004; Goto *et al*., 2003), which are likely to be the cellular ancestors of MHV-68 mK3.

mK3-mediated ubiquitination of the murine class I MHC HC cytosolic tail, the portion of the HC molecule more likely to come into contact with the cytosolic RING-CH domain of mK3, is not required for dislocation even though ubiquitination and presence of the cytosolic tail are essential for dislocation (Wang *et al*., 2005). In fact, like mK3, HCMV US2 and US11 also induce ubiquitination-dependent degradation of class I MHC molecules in a cytosolic tail lysine-independent fashion (Furman *et al*., 2003; Shamu *et al*., 1999). How, then, does mK3 access the luminal domain of HC molecules and trigger its ubiquitination? Access of the HC lumenal domain to the cytosol would invoke a "partial dislocation" model that has been proposed for HCMV US2 and US11 (Furman *et al*., 2003; Shamu *et al*., 1999): the HC luminal domain must begin to emerge in the cytosolic face of the ER so ubiquitination can take place. This would mean that the trigger for dislocation would reside upstream from tail ubiquitination. An alternative explanation would be that the class I MHC tail lysine mutant HC molecules are dislocated as a "bystander effect" of dislocation of wild type molecules by mK3, simply because they are in the proximity of the dislocation machinery that has been recruited by the viral protein for the wild type HC clientele.

The HCMV US2 and US11 and MHV-68 mK3 immunoevasins all target nascent class I MHC HCs for degradation by inducing their dislocation from the ER membrane. The mechanisms used by US2, US11, and mK3, however, when analyzed in detail, are strikingly different. For instance, the stage of class I MHC HC biosynthesis that is targeted by each viral protein is distinct: US11 is the most promiscuous, targeting multiple class I MHC assembly intermediates, whereas US2 targets only properly folded class I MHC complexes and mK3 targets predominantly incompletely assembled HC while in association with the peptide-loading complex. The main difference resides in the fact that mK3 encompasses ubiquitination activity (by means of its RING domain), substrate selection (by means of its association with the peptide-loading complex), and recruitment of ER membrane and cytosolic factors necessary for dislocation (like Derlin-1 and p97) all in one polypeptide. HCMV US2 and US11 do not possess E3 ligase activity and possess no obvious sequence similarity with known genes/proteins that would hint at their function. However, at least US11, and presumably also US2, are still able to induce assembly of a dislocation complex (Fig. 7) that encompasses all the necessary ERAD activities.
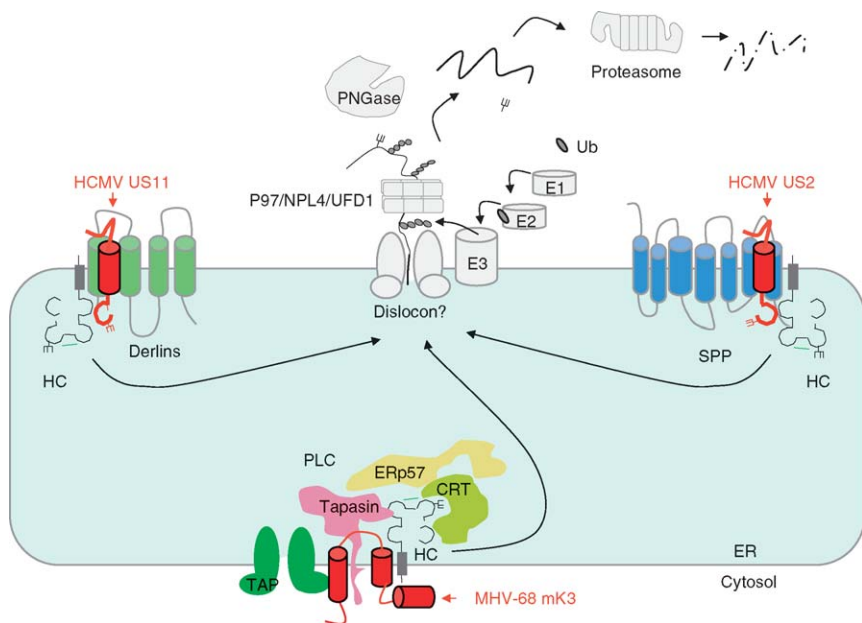
Figure 7 Three viral immunoevasins that co-opt distinct ERAD pathways. The HCMV US11 immunoevasin delivers class I MHC HC molecules to Derlins for dislocation from the ER membrane and degradation, whereas HCMV US2 uses a pathway that is dependent on signal peptide peptidase. The MHV-68 mK3 immunoevasin is an E3 ligase that uses the PLC as a platform to target murine class I MHC molecules for ubiquitination and degradation. Although the three pathways are superficially similar, the substrate selection and targeting steps at the ER membrane are very distinct. Ub, ubiquitin; E1, Ub-activating enzyme; E2, Ub-conjugating enzyme; E3, Ub-ligase enzyme; PNGase, peptide-*N*-glycanase; SPP, signal peptide peptidase; TAP, transporter associated with antigen presentation; CRT, calreticulin.

The knowledge that the TMD of US11 is essential for its function (Lilley *et al*., 2003), led to the discovery of Derlins (Lilley and Ploegh, 2004) and of multiprotein complexes at the ER membrane that function in US11-mediated dislocation of HCs and US11-independent dislocation of a subset of ERAD substrates (Lilley and Ploegh, 2005a; Oda *et al*., 2006; Ye *et al*., 2005). US2 uses its cytosolic tail to recruit SPP and its TMD for an additional step (so far unknown) also critical for HC dislocation (Loureiro *et al*., 2006), presumably resulting in recruitment of US2-specific-components of the ER dislocation machinery. The mechanism by which US2 operates will hopefully be clarified as we continue characterizing the structural and host cofactor requirements for its function in dislocation. The mechanistic details of dislocation catalyzed

by the HCMV US2 and US11 and the MHV-68 mK3 immunoevasins must be quite diverse and their study will most certainly keep providing new insights into ER dislocation. Furthermore, it is nothing short of remarkable how sequence and structurally unrelated herpesvirus proteins have converged into (although only superficially) similar mechanisms to dislocate newly synthesized class I MHC molecules from the ER membrane.

31. Pathogen Interference with Class II MHC Antigen Presentation

Class II MHC-restricted $CD4^+$ T cells are crucial for lymphocyte activation, antibody responses, and coordination of the immune response and rely on activation by the professional APCs. Immunoevasins aimed at interfering with class II MHC antigen presentation are not expected to block presentation of exogenous antigens to $CD4^+$ T cells, unless the APC is infected by the virus (Yewdell and Hill, 2002). Class II MHC expression can, however, be modulated by IFN and receptor signaling through the CIITA transcription factor (Boss and Jensen, 2003). There are relatively few examples of viruses and bacteria that directly infect APCs and affect IFN-induced class II MHC expression or directly interfere with class II MHC-restricted antigen presentation (Hmama et al., 1998; Hussain et al., 1999; Miller et al., 1998; Rinaldo, 1994; Schuller et al., 1998; Srisatjaluk et al., 2002; Zhong et al., 1999). It is likely that more examples will be found as this important question is being revisited. For viruses that do not infect professional APCs, the route to avoiding helper T cell and antibody-mediated responses is to interfere with activation of $CD4^+$ T cells by APCs. In this section, we present an overview of some of the mechanisms used mostly by viral pathogens to actively subvert class II MHC antigen presentation (Fig. 8). The degree to which other pathogens, like bacteria and parasites, actively interfere with class II MHC antigen presentation, by encoding "immunoevasins" rather than passively, due to their residence in endocytic compartments, is difficult to discern.

32. Inhibition of Recognition at the Surface of the APC

Epstein–Barr virus is an example of a virus that can infect and establish latency in B lymphocytes and is associated with a number of malignancies. The product of the BZLF2 EBV gene, gp42, is a bifunctional protein. First, it functions as the coreceptor for viral entry into B cells by binding to the HLA-DR product. Second, gp42 is generated through proteolytic cleavage in the ER and matures into a secreted form that binds class II MHC molecules at the cell surface. gp42 bound to class II MHC molecules at the cell surface prevents
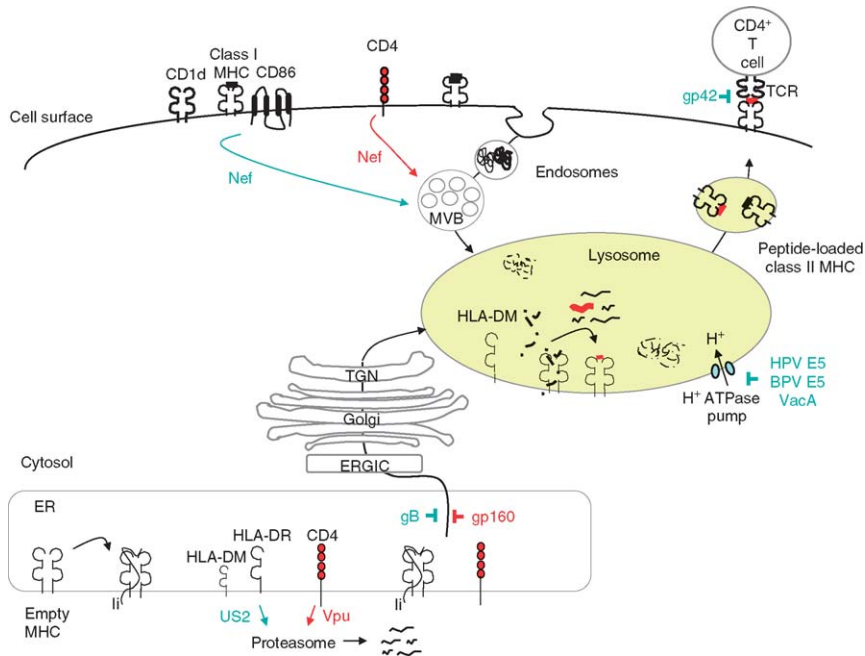
Figure 8 Pathogen interference with class II MHC antigen presentation. TCR, T cell receptor; PLC, peptide-loading complex. Nef, HIV-1 Nef; gp42, EBV glycoprotein 42; BPV E5, bovine papillomavirus protein E5; HPV E5, human papillomavirus protein E5; VacA, *H. pylori* VacA toxin; US2, HCMV US2; Vpu, HIV-1 Vpu; gp160, HIV-1 glycoprotein 160; MVB, multivesicular body; Ii, invariant chain. The immunoevasins and pathways depicted in red take place in the effector CD4$^+$ T cell, whereas those in blue occur in the antigen-presenting cell.

TCR-(peptide-loaded class II MHC) interactions and CD4$^+$ T cell activation (Li *et al.*, 1997; Ressing *et al.*, 2003, 2005; Spriggs *et al.*, 1996).

33. Class II MHC Downregulation from the Surface of the APC

The HIV-1 Nef protein downregulates class II MHC molecules from the cell surface by restructuring the endocytic pathway such that invariant chain (Ii) degradation is impaired and immature class II MHC complexes (αβIi) are granted increased access to the cell surface (Stumptner-Cuvelette *et al.*, 2001). Nef induces a reduction of surface levels of peptide-loaded class II MHC as well as a strong accumulation of surface-displayed immature class II MHC complexes, still containing (and thereby blocked by) intact invariant chain (Ii).

Nef expression results in accumulation of both class II MHC and invariant chain (Ii) in multivesicular bodies (MVBs) (Stumptner-Cuvelette et al., 2003). MVBs are a specialized type of endosome that constitutes a major pathway of delivery of transmembrane proteins for lysosomal degradation (Hurley and Emr, 2006). Sequestering in MVBs suggests a reduced capacity of immature class II MHC complexes to reach lysosomes, either due to a defect in class II MHC sorting to the lysosomes or due to slower internalization of immature complexes. The mechanism is still unclear (Stumptner-Cuvelette et al., 2003).

The HCMV US2 immunoevasin was proposed to downregulate class II MHC from the cell surface, presumably by targeting HLA-DRα and HLA-DMα for degradation by the proteasome, thus inhibiting antigen presentation to CD4$^+$ T cells (Chevalier et al., 2002; Hegde and Johnson, 2003; Tomazin et al., 1999). This effect of US2, however, was only seen in cell lines in which induction of class II MHC was induced by stable transfection with the class II MHC trans-activator (CIITA), but not in human DCs or several other cell lines, which express class II MHC endogenously (Rehm et al., 2002). Presumably the relative expression levels of class II MHC and/or US2 could account for the observed differences, and in fact, in CIITA-transfected cells, even US3 was seen to downregulate class II MHC molecules (Hegde et al., 2002). HSV-1 can downregulate surface expression of class II MHC complexes in B cells and inhibits the ability of B cells to stimulate CD4$^+$ T cells. HSV-1 inhibits synthesis of Ii and also encodes an envelope glycoprotein B (gB) that binds both HLA-DR and HLA-DM (Neumann et al., 2003; Sievers et al., 2002). By binding to HLA-DR, gB affects trafficking of the molecule in the secretory pathway and by binding HLA-DM it sequesters this peptide editor and thus prevents peptide loading of class II MHC molecules that may have escaped (Neumann et al., 2003).

34. CD4 Downregulation from the Surface of the CD4$^+$ T Cell

The CD4 protein serves as the primary cellular receptor for HIV at the surface of CD4$^+$ cells. However, its presence inhibits virus budding and interferes with incorporation of the gp120 protein into the budding virion, not to mention its crucial role in eliciting of a CD4$^+$ T cell response (Keppler et al., 2006; Lama et al., 1999; Ross et al., 1999). Not surprisingly, three HIV-1 proteins, Nef, Vpu, and gp160 dramatically reduce the steady state levels of CD4 on the cell surface (Mangasarian et al., 1999; Piguet et al., 1999a,b). One of the many mechanisms used by Nef involves acceleration of the constitutive endocytosis of CD4. In T cells, CD4 is stabilized at the cell surface by p56Lck, a Src-family tyrosine kinase, which binds a dileucine motif in the CD4 cytoplasmic tail, preventing CD4 from being recruited into clathrin-coated pits

(Aiken *et al.*, 1994; Jin *et al.*, 2005). Nef can displace Lck by directly binding a dileucine sorting motif in the CD4 tail, overcoming the normal Lck phosphorylation-dependent route of CD4 downregulation. Nef itself contains a C-terminal dileucine motif that recruits a subunit of the tetrameric adaptor protein complex-2 (AP-2), a component of clathrin-coated pits at the cell membrane. Thus, Nef connects CD4 to AP-2 on clathrin-coated pits, triggering rapid CD4 endocytosis (Jin *et al.*, 2004b, 2005; Mangasarian *et al.*, 1999; Piguet *et al.*, 1998, 1999a). Nef can bind not only the AP-2 components of clathrin-coated pits, but also the regulatory V1H subunit of the vacuolar proton (H^+) ATPase. V1H (also called NBP1 or Nef binding protein-1) binds AP-2 in clathrin-coated vesicles. By binding V1H, Nef strengthens its weak direct interaction with AP-2 (Geyer *et al.*, 2002; Mandic *et al.*, 2001). Again, by binding both CD4 (using the aforementioned dileucine motif in the cytoplasmic tail) and V1H, Nef directs internalization of CD4 from the cell surface.

The endocytosed CD4 accumulates in early endosomes from where it is sorted to lysosomes for degradation. Adaptor protein (AP) complexes mediate transport of proteins to numerous compartments within the cell. Whereas AP-2 initiates early endocytic vesicle formation at the cell membrane, AP-1 is involved in vesicle formation at the TGN and vesicle targeting to early endosomes and AP-3 participates in vesicle formation at the TGN and targeting to late endocytic/lysosomal compartments (Bonifacino and Traub, 2003). The "coat" on vesicles in the endocytic pathway is composed of AP complexes and another set of coat proteins, clathrin in clathrin-coated vesicles, and COP proteins, in COP-coated vesicles. The COP proteins are involved in vesicle trafficking early in the secretory pathway between the ER and the Golgi (McMahon and Mills, 2004). Trafficking in the endocytic pathway can be targeted by HIV Nef by direct binding to AP complexes or by interference with the recruitment and release cycles of AP complexes from vesicle membranes.

AP complexes cycle from the cytosol to vesicles in a process dependent on the GTPase cycle of ADP-ribosylation factor-1 (ARF1). Nef can bind to and stabilize the small GTPase ARF1 on the endosomal membrane, preventing AP complexes from being released, affecting trafficking of host molecules (including CD4) along the endocytic pathway. Nef can also mediate the formation of a ternary complex composed of Nef, ARF1, and a component of the COP-I coat, βCOP. COP-I-coated vesicles are mostly subject to retrograde transport within the Golgi and between the Golgi and the ER, but some are involved in transport from early to late endosomes (McMahon and Mills, 2004), and thus association of Nef with ARF1 and βCOP mediates targeting of CD4 for lysosomal degradation (Faure *et al.*, 2004). Nef has a preference for AP-1 and AP-3 complexes *in vitro* (Janvier *et al.*, 2003b), suggesting that the Nef modus operandi is mostly

at the level of endosomal membranes. Downregulation from the cell surface by binding AP-2 does not seem to be a major route for CD4 downregulation (Rose *et al.*, 2005). Which one of these strategies—downregulation from the cell surface or intracellular retention—or what combinations of these strategies and adaptor protein complexes are used may allow the HIV-1 Nef protein to downregulate cell surface expression of CD4 perhaps in different cell types (Mangasarian and Trono, 1997).

Vpu targets newly synthesized CD4 molecules in the ER for proteasomal degradation (Kerkau *et al.*, 1997) by recruiting the cytosolic F-box protein β-TrCP, the receptor component of the SCF$^{β-TrCP}$ E3 ligase, to the ER membrane (Margottin *et al.*, 1998). A motif in the Vpu C-terminus binds a WD repeat on β-TrCP, directing SCF$^{β-TrCP}$ E3 ligase activity to catalyze the ubiquitination of lysine residues on the CD4 tail, which serves as the trigger for CD4 degradation (Margottin *et al.*, 1998; Schubert *et al.*, 1998). gp160 retains newly synthesized CD4 molecules in the ER (Crise and Rose, 1992; Kimura *et al.*, 1994). In the absence of CD4, HIV gp160 is posttranslationally cleaved into its gp120 and gp41 subunits at the level of the ER-to-Golgi compartment (ERGIC). In the presence of CD4, gp160 forms a complex with CD4 that mediates ER retention of both molecules and downregulation of CD4 from the cell surface. In the context of an HIV-infected cell, coexpression of Vpu liberates gp160 from the complex, ensuring gp160 translocation to the ERGIC and ensuing maturation, and simultaneously accelerating CD4 turnover (Crise and Rose, 1992; Rose *et al.*, 2005).

HIV downregulates class I MHC, class II MHC, CD4, and CD1d, a class I MHC-like molecule that presents lipid antigens (Le Gall *et al.*, 1998; Piguet *et al.*, 1999b), from the cell surface by virtue of its Nef, Vpu, and gp160 proteins, reflecting its ability to manipulate various aspects of the immune response (Joseph *et al.*, 2005; Piguet *et al.*, 1999b). Furthermore, each of these mechanisms may be more far-reaching than downregulation of each individual receptor. For instance, by directly associating with the proton (H$^+$) ATPase, which is required for the acidification of lysosomes, Nef not only downregulates CD4 but may also interfere with the pH of class II MHC-positive compartments, affecting antigen processing by lysosomal proteases and class II MHC antigen presentation. This strategy is used by other pathogens: the E5 protein from both human and bovine papillomavirus (Andresson *et al.*, 1995) and the VacA toxin secreted by *Helicobacter pylori* (Molinari *et al.*, 1998) actively manipulate acidification in the endocytic pathway, resulting in impaired CD4$^+$ T cell-mediated responses (Brodsky *et al.*, 1999). By binding to AP complexes or to the small GTPase ARF1, Nef may interfere with trafficking and alter the fate of numerous host molecules in the endocytic pathway (Janvier *et al.*, 2003a), affecting aspects that range from viral replication

to modulation of the immune system (Lama and Ware, 2000; Sol-Foulon *et al.*, 2002; Swigut *et al.*, 2001).

35. Pathogen Manipulation of the Ubiquitin-Proteasome System

Intracellular pathogens exploit the ubiquitin-proteasome system mostly to destroy or avoid destruction of specific cellular proteins. This serves to create a more hospitable environment for themselves in the host cell, or to prevent destruction of their own proteins, and so to ensure replication or avoid detection by the immune system. Many events at the initial stage of infection, entry into the cell, are controlled by signaling pathways—for instance, those involved in cytoskeletal rearrangements or in receptor engagement—that rely heavily on the Ub-proteasome system. The same is true for signaling cascades involved in cell survival, differentiation, and proliferation. Not surprisingly, pathogens have devoted much energy and coding capacity to interfere with cell signaling events through interference with the Ub-proteasome system. An illustrative example is that of tumor viruses and degradation of cellular tumor suppressor proteins, like the retinoblastoma protein or p53, often resulting in malignant transformation (Shackelford and Pagano, 2005). There have been a number of recent reviews on viral interference with signaling pathways, so we will not describe this aspect in detail here. For comprehensive reviews, the reader is referred to Banks *et al.* (2003) and Shackelford and Pagano (2004, 2005).

In this chapter, we will focus on a few examples of viral and bacterial interference with the Ub-proteasome system that illustrate how the latter is crucial for aspects of pathogen life cycles that are as distinct as integration into host chromosomes, exit from the host cell, or RNA interference, not to mention control of the immune response. As mentioned in the first section of this chapter, proteasome-mediated degradation of cytoplasmic proteins as well as the proteolytic events in the endolysosomal system are important for class I and class II MHC presentation and cross-presentation. Mounting of an immune response involves complicated signaling cascades like NF-κB and JAK/STAT signaling, which are heavily dependent on the Ub-proteasome system, and that can be manipulated from early events of cell-surface receptor-mediated signaling, for example, to later events of ubiquitination and deubiquitination of downstream targets. Certainly, because of the central role of the Ub-proteasome system in many different aspects of cellular physiology, interference with this pathway will have pleiotropic effects, and which of the observed effects predominates may be difficult to discern.

It is not our intention to make the following a comprehensive list. We rather propose to provide an overview of the possibilities of manipulation of this system that have been described for pathogens (Fig. 9). We discuss in more
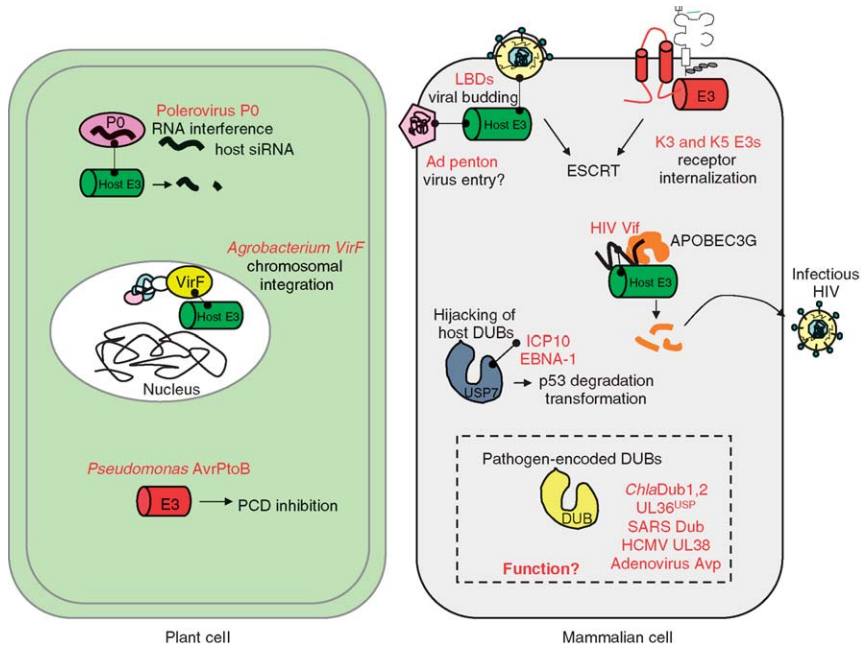
Figure 9 Pathogen interference with the Ub-proteasome system. Pathogens interfere with the Ub-proteasome system not only to manipulate antigen presentation and other aspects of the immune system, but for processes as distinct as chromosomal integration, virus budding, RNA interference, and many others. They may rely on hijacking of host E3 ligase activities or encode their own. Pathogens can manipulate host DUBs as well as encode DUB activities. The function of pathogen-encoded DUBs remains a mistery.

detail pathogen-encoded modulators of the ubiquitin-proteasome system, ranging from pathogen-encoded proteolysis-resistant peptides, to ubiquitin ligases and DUB, with obvious implications for the viral or bacterial life cycle or with consequences for the control of host immune responses. We elaborate on novel pathogen-encoded DUBs and their putative functions.

36. Interference with Proteasomal Proteolysis

The classic example of a viral protein that interferes with proteasomal processing is the EBNA-1. The EBNA-1 protein contains an internal repeat exclusively composed of glycines and alanines, the Gly-Ala repeat, which not only interferes with its own proteasomal proteolysis (Levitskaya et al., 1995), but also reduces its rate of translation, blocking viral DRIPs formation and inhibiting the presentation

of EBNA-1 class I MHC-restricted T cell epitopes (Yin *et al*., 2003). A single residue change in the mouse leukemia virus (MuLV)-derived CTL epitope (from *K*SPWFTTL to *R*SPWFTTL) can eliminate the proteolytic cleavage site required for its presentation (Ossendorp *et al*., 1996). As discussed earlier, phosphorylation of the HCMV IE-1 by the HCMV pp65 tegument protein interferes with production of IE-1-derived peptides (Gilbert *et al*., 1996). The HIV-1 transcriptional activator (Tat) protein manipulates 26S proteasome function by directly interacting with the LMP7 and MECL1 subunits of the proteasome and competing with the 11S proteasome for binding to the 20S proteasome (Andre *et al*., 1998; Apcher *et al*., 2003), leading to inhibition of proteolytic activity. Tat also acts at the transcriptional level by modifying proteasome composition by upregulating the LMP7 and MECL1 subunits and downregulating the LMP2 subunit, leading to an increased presentation of cryptic and subdominant CTL epitopes (Gavioli *et al*., 2004). By preventing display of viral peptides, these viral proteins interfere with cytotoxic T cell recognition.

37. Control of Infection

Salmonella enterica serovar typhimurium is an important bacterial pathogen, the causative agent of food poisoning and typhoid fever. *S. typhimurium* temporally regulates the initial phase of bacterial internalization and host cell recovery after invasion through two type III secretion system (TTSS)-delivered substrates, SptP and SopE with different proteasomal half lives (Kubori and Galan, 2003). SopE is a bacterial guanine nucleotide exchange factor (GEF) delivered by a TTSS that mimics a host cell GEF for the small Rho GTPases Cdc42 and Rac1, involved in actin remodeling and formation of membrane extensions required for bacterial engulfment. SptP, delivered by another TTSS, is a Rho GTPase activating protein (GAP) factor for Cdc42 and Rac1, which accelerates GTP hydrolysis. SpT plays a role once bacterial internalization has taken place, inactivating the Rho GTPases, inhibiting actin polimerization, and assuring closure of the plasma membrane and recovery of the normal cellular architecture (Pizarro-Cerda and Cossart, 2006). *Salmonella* initially delivers equal amounts of SopE and SptB to the host cell. However, 15–20 min after infection SopE is rapidly degraded, whereas SptB degradation occurs only slowly after 3 h, thereby efficiently timing the actin remodeling events that lead to the initial bacterial engulfment and the later host cell recovery. Both processes are proteasome-dependent and catalyzed by the N-terminus of the bacterial proteins, but the mechanism and the host factors involved are currently unknown (Kubori and Galan, 2003).

The HIV-1 Vif protein targets the RNA editing protein APOBEC3G for degradation by a cellular E3 ligase to allow production of infectious viral

progeny (Yu *et al*., 2003). The *apo*lipoprotein *B* mRNA *e*diting enzyme, *c*atalytic polypeptide-like *3G* (APOBEC3G) and related cytidine deaminases are involved in mRNA editing and in immunoglobulin gene class switching and hypermutation but are also potent antiretroviral enzymes. The cytosolic APO-BEC3G is incorporated into budding virions, where on infection of new target cells, its cytidine deaminase activity induces G to A hypermutation on the minus-strand viral DNA, resulting in abortive infection (Bishop *et al*., 2004b; Zhang *et al*., 2003). Vif hijacks the elongin-C-elongin-B-Cullin-5-E3 (ECS) complex. The ECS5 E3s recognize substrate receptor proteins containing a BC-box. Vif possesses a BC-box motif, through which it binds elongin C (Luo *et al*., 2005). Vif hijacks the E3 ligase complex and by bridging APOBEC3G and elongin C targets APOBEC3G for ubiquitination and degradation (Yu *et al*., 2003). This ultimately allows production of infectious virus progeny (Bieniasz, 2004; Bishop *et al*., 2004a; Harris and Liddament, 2004).

38. Virus Budding

The endosomal sorting complexes required for transport (ESCRT) are highly conserved from yeast to mammals and consist of the ESCRT I, II, and III complexes. ESCRT complexes are composed of vacuolar protein sorting (VPS) proteins (in yeast), which are recruited from the cytoplasm to promote sorting of ubiquitinated proteins to MVBs (Katzmann *et al*., 2002). MVBs are formed by invagination of the late endosome membrane, which generates internal vesicles into which proteins destined to the lysosomes are sorted; they are critical for receptor downregulation and other normal and pathological cell processes (Hierro *et al*., 2004; Hurley and Emr, 2006; Kostelansky *et al*., 2006), as well as for virus budding.

The tumor susceptibility gene TSG101, the mammalian homologue of yeast VPS23, is essential for sorting of ubiquitinated proteins to MVBs (Babst *et al*., 2000). TSG101 recruits *h*epatocyte growth factor–*r*egulated tyrosine kinase *s*ubstrate (HRS), the mammalian homologue of yeast VPS27, to the endosomal membrane. HRS nucleates recruitment of the ESCRT-1 complex, which in turn recruits ESCRT 2 and -3, leading to formation of the inner membranes of the MVB. TSG101 is a noncanonical Ub E2 variant (UEV) protein—it does not possess Ub-conjugating activity, only an N-terminal UEV domain that binds ubiquitin. Binding of the TSG101 UEV domain to a P(S/T)AP tetrapeptide motif on HRS recruits HRS to the endosomal membrane, triggering assembly of ESCRT complexes and genesis of the MVB (Clague and Urbe, 2003; Garrus *et al*., 2001; Pornillos *et al*., 2002, 2003). Many viruses such as HIV-1 and Ebola recruit this Ub-dependent sorting machinery to the viral release sites at the plasma membrane to promote virus budding from host cells

(Li and Wild, 2005; Liu, 2004), by making use of conserved P(S/T)AP, PPXY, or FPIV motifs, also called late budding domains (Bieniasz, 2006).

Retroviruses like HIV use the P(S/T)AP motif on the L-domain contained in their GAG protein to mimic the TSG101-recruiting activity of the HRS protein (Klinger and Schubert, 2005; Martin-Serrano *et al*., 2003; Sorin and Kalpana, 2006; Stuchell *et al*., 2004). Other viruses can also recruit *ne*uronal precursor cell-expressed *d*evelopmentally *downregulat*ed 4 (NEDD4) and NEDD-4-like HECT E3 ligases to facilitate viral budding (Bieniasz, 2006; Harty *et al*., 2001; Sakurai *et al*., 2004; Yasuda *et al*., 2003). NEDD4 E3s ligases are a diversified group of orthologues of yeast Rsp5p and are involved in a wide range of processes such as receptor internalization and degradation (presumably through the endolysosomal pathway), maintenance of EBV latency, and regulation of cytokine signaling. NEDD4 E3s have a catalytic C-terminal HECT domain, two or more central N-terminal WW domains, and an N-terminal C2 domain. The N-terminal C2 domain seems to be responsible for membrane association and cellular localization of the protein. The WW domain is a protein–protein interaction module of about 35 amino acids with two crucial tryptophan (W) residues spaced 20–22 amino acids apart, which appears to mediate substrate selection. It binds mostly proline-rich motifs, such as the PPXY motif present in many cellular proteins, targeting them for degradation (Ingham *et al*., 2004, 2005). Viral proteins with the PPXY motif can therefore bind these WW domains on Nedd4 E3s, but the nature of these interactions and how they facilitate virus budding is so far unknown. Some viruses, like Ebola, have two different late budding motifs, PPXY and P(S/T)AP, and the Ebola late domain-containing VP40 matrix protein can recruit both TSG101 and NEDD4 for effective budding (Licata *et al*., 2003; Liu, 2004; Yasuda *et al*., 2003). The nonenveloped adenovirus possesses a PPXY motif on its penton base protein, which is essential for virus internalization that can interact with several NEDD4 HECT E3 ligases (Galinier *et al*., 2002). Whether or not this interaction or E3 ligase activity is required for adenovirus entry is currently unknown.

39. Bacterial Chromosome Integration

Agrobacterium tumefaciens exploits a host cell SCF E3 ligase for integration of its T-DNA by encoding an F-box protein (Tzfira *et al*., 2004b). *Agrobacterium* is a common phytopathogenic bacterium that induces "crown gall" disease in plants by transfer and integration of a segment of its tumor-inducing (Ti) plasmid DNA into the plant genome. This process relies also on delivery of several virulence (Vir) proteins into the host cell, such as the bacterial VirE2 protein, which is thought to package and protect the transported T-DNA

molecule, and, together with the host plant VIP1 protein, assist its nuclear import (Tzfira *et al*., 2004a). However, disassembly of this VirE2/T-DNA/VIP1 complex must occur before integration and involves intranuclear proteolysis of VirE2 and VIP1 induced by the VirF protein, an F-box-domain–containing protein. The bacterial VirF F-box hijacks the SCF E3 plant homologue. The VirF-containing SCF^{VirF} then leads to degradation of VIP1 and VirE2, and integration of the *Agrobacterium* T-DNA (Tzfira *et al*., 2004b). We consider it unlikely that this possibility has been exploited only by plant pathogens.

40. ISGylation and deISGylation

The host innate response triggered by type I interferon (IFN-α and -β) innate response is crucial in early immunity against viruses, bacteria, and some parasites, acting to limit pathogen infection limiting replication of the pathogen and constraining cellular permissiveness to infection (Smith *et al*., 2005). Type I interferon receptor signaling occurs through the JAK/STAT pathway and leads to transcription of several IFN-stimulated genes (ISGs), of which the gene encoding the Ub-like modifier ISG15 is one of the most strongly induced (Farrell *et al*., 1979). *ISG15* and protein modification by *ISG15* (ISGylation) are induced by viral and bacterial infection or other stresses, suggesting important roles for the ISG15 system in innate immune responses (Liu *et al*., 2005; Ritchie and Zhang, 2004). ISG15 is conjugated onto several signaling molecules with immunomodulatory functions, like JAK/STAT proteins (Giannakopoulos *et al*., 2005; Malakhov *et al*., 2003). The ISGylation cascade is initiated by ISG15 activation by an E1-like enzyme, UBE1L, transfer to the ISG-conjugating UBCH8 enzyme (Zhao *et al*., 2004), and incorporation into UBCH8-compatible Ub E3 ligases (Dastur *et al*., 2006; Zou and Zhang, 2006). A deISGylating enzyme, ubiquitin-binding protein 43 (UBP43), also called USP18, specifically removes ISG15 from ISGylated substrates (Malakhov *et al*., 2002), and is regulated by ubiquitination by the SCF^{Skp2} E3 (Tokarz *et al*., 2004). USP18 is unlikely to be the sole enzyme capable of acting on ISG15 conjugates.

The role of ISG15 in orchestration of the innate antiviral response sets the ground for pathogen interference. Although the mechanism is unclear, viral replication in UBP43 knockout mice is impaired, a phenotype that was initially attributed to inhibition of deISGylation and deregulation of STAT signaling (Ritchie *et al*., 2002). There are conflicting views on this (Dao and Zhang, 2005; Kim *et al*., 2006b; Knobeloch *et al*., 2005) and UBP43 may in fact inhibit type I IFN signaling irrespectively of its ISG15 isopeptidase activity, by binding to the IFN receptor and blocking the interaction between JAK and the IFN receptor (Malakhova *et al*., 2006). IFN-mediated inhibition of HIV replication and budding is dependent on ISG15 (Kunzi and Pitha, 1996; Pitha, 1994; Poli *et al*., 1989). Expression of ISG15 inhibits ubiquitination of the HIV-1 Gag protein and TSG101, and disrupts the interaction of the Gag late

budding domain with TSG101 (Okumura *et al.*, 2006). Either of these ISG15 effects and/or additional mechanisms of action could lead to failure to recruit ESCRT complexes and inhibition of HIV budding. ISG15 is strongly induced by infection with influenza B virus. The exact cellular function and targets of ISG15 are not known, but the NS1 protein of influenza B viruses (NS1B) blocks its conjugation to target proteins: the NS1B N‑terminus binds ISG15, inhibiting activation of ISG15 by its E1 enzyme, UBE1L. Influenza A viruses also manipulate cellular ISGylation processes, through an even less well characterized mechanism: the influenza A virus NS1A protein does not directly bind the ISG15 protein, but little or no ISG15 protein is produced during infection (Yuan and Krug, 2001; Yuan *et al.*, 2002). Whether this reflects ISGylation, deISGylation, and/or UBP43 ubiquitination‑mediated control of the IFN innate immune response is so far unknown.

41. Control of Inflammation

Suppression of NF‑κB signaling is a common theme for many viral as well as bacterial pathogens and can be certainly achieved through modulation of Ub‑dependent events in the NF‑κB cascade (Bowie *et al.*, 2004; Hiscott *et al.*, 2001; Mason *et al.*, 2004). The human enteric flora may influence intestinal epithelium inflammatory tolerance by inhibiting the NF‑κB pathway, which can be achieved by blocking any one of the many Ub‑dependent steps that control it. *Shigella flexneri*, which causes severe diarrhea in humans, injects TTSS‑effector proteins into host cells to induce their entry into epithelial cells or trigger apoptosis in macrophages. The OspG effector is a serine/threonine kinase that binds various E2s, including UbcH5, a component of the SCF$^{\beta\text{-TrCP}}$ E3 complex. OspG binding to UbcH5 inhibits the SCF$^{\beta\text{-TrCP}}$ complex and thereby phospho‑IkB degradation, blocking NF‑κB signaling in response to the bacterial infection. The Cullin subunit of the SCF$^{\beta\text{-TrCP}}$ complex is itself regulated by NEDD8 attachment (Pan *et al.*, 2004). Certain enteric bacteria can lead to rapid deneddylation of Cullin‑1 and consequent repression of the NF‑κB pathway (Collier‑Hyams *et al.*, 2005), but the bacterial activities responsible are still to be determined. In any case, because NF‑κB is crucial for both the initial innate inflammatory response and for coordination of the adaptive immune response, dampening of inflammation may endow *Shigella* and other enteric bacteria with the ability to invade and later colonize the gastrointestinal epithelium (Kim *et al.*, 2005).

42. Posttranscriptional Gene Silencing

RNA interference (or posttranscriptional gene silencing) in plants and invertebrate animals is important for many regulatory processes and a primitive form of antiviral immunity that is nucleic acid based. Consequently, plant

viruses have evolved proteins, the so-called silencing suppressors, which directly bind to and inactivate the plant microRNAs (Ding *et al.*, 2004; Zamore, 2004). Poleroviruses are small positive-strand RNA viruses that cause leafroll phenotypes in many plant species. Poleroviruses encode a silencing suppressor P0, which is a viral F-box protein (Barry and Fruh, 2006). P0 has a minimal conserved F-box motif that can bind the plant Skp-1 homologue and form a functional complex with the plant Cullin-1 homologue. The P0 F-box is required for polerovirus infectivity and its silencing suppressor function (Pazhouhandeh *et al.*, 2006). P0 presumably binds the microRNAs and targets them for Cullin-4A-dependent degradation. This constitutes a remarkable finding, as degradation of RNA (and not protein) by an E3 ligase had not been documented before.

This primitive form of antiviral immunity, although widely used as a laboratory tool was only recently shown to occur in the context of a natural viral infection in jawed vertebrates (Browne *et al.*, 2005). HIV-1 encodes viral small interfering RNA (siRNA) precursors that provoke RNA silencing in human cells, so not surprisingly, the HIV-1 Tat protein also contains a silencing suppressor function (Bennasser *et al.*, 2005), that prevents Dicer from processing the precursor double-stranded RNAs into siRNAs. Suppression of RNA silencing by other RNA viruses is likely to occur. It will be interesting to see whether the mechanism used by the plant poleroviruses is conserved and whether manipulation of the Ub-proteasome system for suppressing RNA interference is more widely used by mammalian RNA viruses.

43. Downregulation of Cell Surface Receptors by Pathogen-Encoded E3s

As mentioned earlier, several γ-herpesviruses and poxviruses encode PHD/LAP E3s, the K3 homologues, that include the Kaposi's-sarcoma-associated herpesvirus (KSHV) kK3 and kK5 (also called modulator of immune recognition MIR-1 and MIR-2, respectively), the MHV-68 mK3, and the rabbit myxoma virus M153R (Coscoy and Ganem, 2003). We discussed mK3 which catalyzes ubiquitination and proteasomal degradation of nascent murine class I MHC HCs after dislocation from the ER membrane (Lybarger *et al.*, 2005; Wang *et al.*, 2005). KSHV kK3 and kK5 and myxoma virus M153R catalyze ubiquitination of cell surface class I MHC HC, thus providing the trigger for their internalization from the cell membrane, as well as for sorting through MVB formation to lysosomal degradation (Duncan *et al.*, 2006). In addition to downregulation of class I MHC molecules, some of these K3 homologues target the lymphocyte costimulatory molecules CD86 (B7.2) and intercellular adhesion molecule ICAM-1, CD1d, and CD4 (Coscoy and Ganem, 2003;

Coscoy *et al*., 2001; Lehner *et al*., 2005; Mansouri *et al*., 2003), as well as the Fas/CD95 death receptor (Collin *et al*., 2005; Guerin *et al*., 2002). By targeting not only class I MHC molecules, the viral K3 homologues are adding an extra layer of protection predicted to be important for viral escape after reactivation from latency (Lehner *et al*., 2005). The K3 homologues are most likely making use of old ideas: the mechanisms used by the viral Ub ligases have probably been "borrowed" from their mammalian counterparts, the MARCH proteins, and will certainly continue to yield insights into the Ub-proteasome system.

44. Programmed Cell Death in Plants

A bacterial E3 ligase was recently shown to inactivate another type of immune response in plants (Janjusevic *et al*., 2006). Antipathogen responses in plants, although not as sophisticated as those afforded by the vertebrate immune system, are nevertheless quite efficient. One mechanism, immunity-induced programmed cell death (PCD), is a response that sacrifices a limited portion of the plant to limit spread of the infection. The *Pseudomonas syringae* bacterium, which causes disease in tomato and *Arabidopsis*, delivers its AvrPtoB protein into plant cells through a type III secretion system. AvrPtoB can inhibit PCD in susceptible hosts, allowing *Pseudomonas* to cause systemic infection and disease. The AvrPtoB C-terminus encodes a U-box E3 ligase activity necessary for the pathogenic role of AvrPtoB, since mutation of the putative E2-recruitment sites abolishes the anti-PCD and virulence activities of the AvrPtoB protein (Janjusevic *et al*., 2006). Determining the host targets of the *Pseudomonas* E3 will be crucial in clarifying its mechanism of action and might even explain plant susceptibility to infection. Such experiments may also assist in the identification of similar targets in mammalian species infected with comparable Gram-negative microbes. Furthermore, it is rather striking that another pathogen uses a mimic of a host E3 ligase to encode an immunomodulatory function. There are bound to be others.

45. Cytokine Responses

Mumps virus and other Paramyxoviridae family members hijack the Cullin-4A-SCF$^{\beta\text{-TrCP}}$ E3 ligase to suppress IFN- as well as IL-6-mediated signaling (Ulane *et al*., 2003), important for control of inflammation and apoptosis during inflammation (Hodge *et al*., 2005). IFN and IL-6 signaling activates their cognate STAT factors and transcription of genes involved in IFN and cytokine signaling. Some of these paramyxoviruses use their V protein to bind DDB1, the Cullin-4A-SCF$^{\beta\text{-TrCP}}$ E3 substrate adaptor that recruits STAT proteins to the E3 complex, targeting STATs for proteasome-mediated

degradation (Li *et al*., 2006b; Ulane and Horvath, 2002; Ulane *et al*., 2005). Many other viruses target STAT transcription factors for proteasomal degradation (Garcin *et al*., 2002; Lin *et al*., 2005; Ramaswamy *et al*., 2004; Zimmermann *et al*., 2005). Recruitment of Cullin E3s is likely to be a more widely used viral strategy of interfering with IFN and cytokine signaling.

The interleukin-2 (IL-2)-inducible deubiquitinating enzyme DUB-2 is induced by IL-2 stimulation and may regulate IL-2 signaling. The IL-2-inducible DUB-2 is constitutively expressed in cells transformed by human T cell leukemia virus-1 (HTLV-1). Like other cytokines, IL-2 is an important modulator of apoptosis in T cells that acts through the STAT pathway. Although the mechanism is not at all clear, DUB-2 activity prolongs IL-2-stimulated phosphorylation and transcriptional activity of STAT5, inhibiting T cell apoptosis in the aftermath of the immune response (Migone *et al*., 2001; Shackelford and Pagano, 2004) and possibly contributing to cell immortalization. Cytokine-inducible DUBs could interfere with cytokine signaling, thereby playing an active role in modulation of the immune response. The IL-1-inducible DUB-1 is specifically induced by interleukin 3 (IL-3), GM-CSF, and IL-5, which suggests a role in responses mediated by these cytokines (D'Andrea and Pellman, 1998) and might constitute a target for pathogen modulation of the immune response through interference with the Ub-proteasome system. Although a review of this rapidly expanding field is beyond the scope of this chapter, even this simple example suffices to demonstrate how both Ub addition and removal control immune physiology, and consequently are likely targets for interference by pathogens.

46. Pathogen-Encoded DUBs

Bacteria of the *Yersinia* genus are the causal agents of plague, septicemia, and gastrointestinal syndromes. Enteropathogenic *Yersinia* species are extracellular multiplying Gram-negative bacteria that make use of type III secretion systems to inject virulence factors into host cells. The *Yersinia* YopJ virulence factor encodes a protein reported to be a cysteine protease that can cleave Ub and SUMO. The YopJ DUB inhibits NF-κB and mitogen-activated protein kinase (MAPK) pathways, a function ascribed previously to its somewhat promiscuous deubiquitination of critical cellular proteins, such as TRAF2, TRAF6, and IkB. The MAPK/JUN pathway is involved in transcriptional control of several cytokine genes. The YopJ activity induces macrophage death and blocks their ability to activate these inflammatory pathways (Orth, 2002; Orth *et al*., 2000; Zhou *et al*., 2005). However, recent reports show that the true function of YopJ is that of a serine/threonine acetyltransferase (Mukherjee *et al*., 2006). The way by which the functions assigned to YopJ have changed as the field advances demonstrates the complexity of assigning a function to proteins that lack obvious

mammalian counterparts. Both *Salmonella* and the plant pathogen *Xanthomonas* secrete proteins with homology to YopJ, but their putative DUB activity or function in the host have not been assessed yet (Gurlebeck *et al*., 2006; Hardt and Galan, 1997).

A novel viral USP or deubiquitinating enzyme, UL36^{USP}, was recently identified in herpes simplex virus-1 (HSV-1) by labeling with an Ub-derived probe (Kattenhorn *et al*., 2005). The UL36^{USP} is located at the N-terminus of the UL36, the large tegument protein of HSV-1, an α-herpesvirus. Despite the overall low sequence homology—at the exception of almost only the amino acid residues composing the catalytic triad—the UL36^{USP} activity is well conserved in all members of the Herpesviridae family, as the homologous proteins in murine cytomegalovirus (a β-herpesvirus) and EBV (a γ-herpesvirus) also exhibit DUB activity *in vitro* (Schlieker *et al*., 2005). One of the two severe acute respiratory syndrome (SARS) Coronavirus proteases responsible for cleavage of the replicase polyprotein, the SARS-CoV papain-like protease PLpro, is also a DUB (Barretto *et al*., 2005; Lindner *et al*., 2005), predicted to be structurally similar to human USP7 (Hu *et al*., 2002). The SARS-CoV Plpro protease activity is involved in the processing of the viral polyprotein, thereby contributing to replication of the viral RNA genome. The function of the SARS virus deubiquitinating activity, which extends to ISG15-removal activity (Lindner *et al*., 2005), is unknown at this point. The adenovirus proteinase (Avp) and the human cytomegalovirus UL48 protein also encode DUB activities, but the viral and/or cellular targets remain unidentified (Balakirev *et al*., 2002; Wang *et al*., 2006b). *Chlamydia trachomatis,* an obligate intracellular bacterium that causes a variety of diseases in humans has two genes, *Chla*Dub1 and *Chla*Dub2, whose products encode deubiquitinating and deNEDDylating activities (Misaghi *et al*., 2006). Unlike *C. pneumoniae* whose genome is devoid of *Chla*Dub genes, *C. trachomatis* is able to block NF-κB signaling and thus the inflammatory response, as well as host cell apoptosis. Both processes could be modulated by the *C. trachomatis* DUBs.

These unexpected DUB activities encoded by pathogens suggest a novel strategy of modulation of host defense by manipulating the cellular ubiquitination machinery. Although the experimental evidence is at best tenuous, one can speculate that many of these other pathogen-encoded DUBs may be important for modulation of the Ub-proteasome system during the pathogen life cycle and/or in the context of immune evasion. The fact that these DUB activities are conserved suggests functional importance. They may be delivered to the host cell at the time of infection (for instance, by a bacterial type III secretion system or by a viral tegument protein) or may be transcribed early during infection. The pathogen-encoded DUB could interfere with the levels of Ub- or Ubl-conjugated proteins, thereby altering cellular processes

ranging from signaling pathways, protein degradation, antigen presentation, vesicular trafficking, and many others.

47. Conclusions and Future Directions

We have elaborated on several examples of how the host immune system can be manipulated by a pathogen-encoded E3 or pathogen hijacking of a host E3. Similarly—and although the sample pool is rather small at the moment—there is no reason to believe that encoding their own DUBs and/or hijacking of host DUBs has not "occurred" to pathogens. An attractive possibility is that some of these activities are aimed at manipulating the host immune system. Possible targets would include pathways involved in "housekeeping" processes like life and death of the cells of the immune system, and extend to, for example, interference with antigen presentation. These DUB activities could manipulate membrane trafficking in ways that would prevent MHC molecules from reaching the cell surface, or prevent proteasomal degradation of pathogen-derived proteins. This initial window of deubiquitinating activity could help viruses escape detection. Bacterial pathogens could benefit from targeting of transcription factors and cytokine signaling networks that are crucial for coordination of macrophage effector functions. Other immune responses that could, in principle, be controlled by DUBs are IFN and NF-NF-κB signaling, and the post-transcriptional gene silencing "immune response" at least in plants. The possibilities are endless. Exploitation of the Ub-proteasome system by pathogens to enhance their own survival is beginning to emerge as a central theme. The development of the appropriate genetic and biochemical tools will help place this subject in the realm of essential host–pathogen interactions.

Acknowledgments

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