

Ubiquitination, Ubiquitin-like Modifiers, and Deubiquitination in Viral Infection

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Ubiquitin is important for nearly every aspect of cellular physiology. All viruses rely extensively on host machinery for replication; therefore, it is not surprising that viruses connect to the ubiquitin pathway at many levels. Viral involvement with ubiquitin occurs either adventitiously because of the unavoidable usurpation of cellular processes, or for some specific purpose selected for by the virus to enhance viral replication. Here, we review current knowledge of how the ubiquitin pathway alters viral replication and how viruses influence the ubiquitin pathway to enhance their own replication.

Introduction

Ubiquitin is a small 76 amino acid protein widely expressed in eukaryotic cells. Viruses are connected to ubiquitin and ubiquitin-like modifiers in a variety of ways (Figure 1). Many viruses encode proteins that can modify the host's ubiquitin and ubiquitin-like machinery, often altering substrate specificity to favor replication. Viral proteins themselves can be directly modified by ubiquitin or ubiquitin-like proteins, and some viruses even encode their own ubiquitinating or deubiquitinating enzymes. Host cells utilize the ubiquitin-proteasome system to counteract viral infections through the generation of target structures recognized by T cells, whereas viruses can alter proteasome degradation machinery to interfere with class I major histocompatibility complex (MHC)-restricted antigen presentation and thus escape from T cell recognition. Viruses, in turn, depend on the ubiquitination and degradation of host surface receptors as countermeasures to escape from T cell recognition.

The ubiquitination pathway comprises E1, E2, and E3 enzymes, ultimately responsible for the conjugation of ubiquitin to protein substrates (for reviews see [Pickart, 2001](#); [Kerscher et al., 2006](#)). The E3 ligase usually determines substrate specificity, although the E2-conjugating enzyme can also play a role in substrate selection. The best understood function of the ubiquitin-conjugating system is to tag proteins for degradation by the proteasome. A classic example of viral-induced degradation of host proteins is the ability of human papilloma virus (HPV) to degrade the transcription factor p53 ([Scheffner et al., 1990](#)). This occurs by means of the E6 viral protein that activates an E3 ligase, E6AP, which then specifically targets p53 for ubiquitination. It is now known that ubiquitin and ubiquitin-like modifiers have a far greater impact on viral lifestyles. Ubiquitin and ubiquitin-like modifiers are involved in the innate and adaptive immune response (Figure 2), transcription, signal transduction, membrane trafficking, and more. Given the fact that viruses co-opt the biosynthetic and degradative apparatus of their host, extensive involvement of the ubiquitin-proteasome pathway is to be expected in almost every aspect of their life cycle. It is therefore a challenge to distinguish between adventitious modification of viral proteins by host ubiquitination machinery without immediate consequences for virus lifestyle and those events that are required for virus replication and viral gene transcription. For the viruses that encode their

own ubiquitin ligases or ubiquitin-specific proteases, it seems reasonable to infer functional relevance, but even in these cases, the identification of the exact role of such viral gene products remains a challenge. Nonetheless, the study of viral interactions with the ubiquitin system highlights the selective pressure to which these pathogens are exposed and the countermeasures required for them to ensure replicative success. Furthermore, these examples have helped clarify some of the inner workings of the ubiquitin-proteasome system itself and have brought to light other functions of ubiquitin, such as its role in the assembly of virus particles. In this review, we discuss several mechanisms viruses use to enhance their own replication, including modulation of the cell cycle by DNA viruses, interference with the innate and adaptive immune responses, and the requirement for ubiquitin in virion egress for many RNA viruses. We further discuss the many unknowns that remain, including the emerging and often undefined role of deubiquitinating enzymes, the uncertainties of ubiquitin-related modifiers ISG15 and SUMO, and the potential role of Urm-1 in viral replication.

Viral Proteins that Modify Host Ubiquitination: Targeting Cellular Proteins for Degradation

Some viruses modulate the cell cycle to enhance their own replication (Table 1). These viruses are generally DNA tumor viruses such as HPV, adenovirus, and simian virus 40 (SV40) (reviewed in [Levine, 2009](#)). A common mechanism shared by these viruses entails targeting cell cycle regulator proteins for degradation, often resulting in transformation. The HPV-16 and -18 E6 proteins co-opt the cellular E3 ligase E6AP to mediate ubiquitin-dependent proteasomal degradation of p53 (reviewed in [Beaudenon and Huibregtse, 2008](#); [Mammas et al., 2008](#)). Because p53 is a tumor suppressor protein, degradation of p53 by these HPV strains contributes to their oncogenicity by allowing uncontrolled cellular proliferation without inducing apoptosis. Other substrates besides p53 can be targeted for degradation via HPV E6, including MDM7 (murine double minute 7) protein, proteins containing a PDZ domain (a common structural motif found in signaling proteins), Bak (a protein involved in apoptosis), and E6TP1 (E6 targeted protein 1) (reviewed in [Beaudenon and Huibregtse, 2008](#)). For several substrates including the PDZ domain proteins and p53, HPV E6 can act as an E3 ligase in the

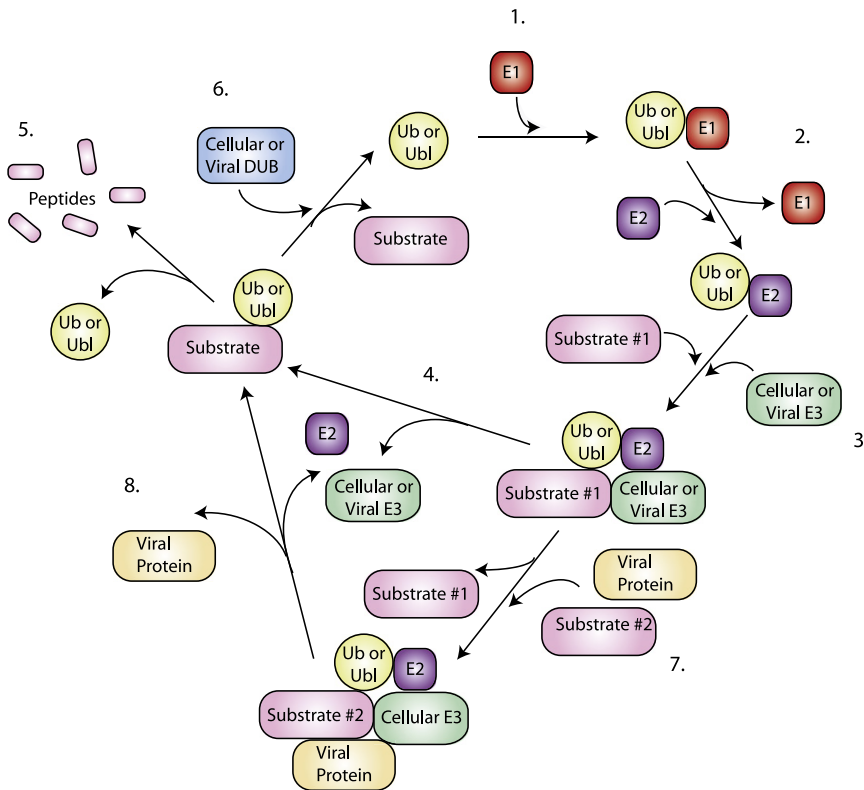


Figure 1. Schematic of Ubiquitin or Ubiquitin-Like Modifier Conjugation and Deconjugation during Viral Infection
Processed ubiquitin (Ub) or ubiquitin-like modifier (Ubl) is activated with ATP by an E1 ubiquitin-activating enzyme (1) and then transferred to an E2 ubiquitin-conjugating enzyme (2). The Ub/Ubl is then ligated to a substrate via the action of an E3 ubiquitin ligase (3). The E3 determines substrate specificity and can be either cellular or viral in origin. The ubiquitinated substrate is then released (4) and can be polyubiquitinated, often leading to proteasome-mediated degradation (5), or the Ub/Ubl can be removed via the action of a cellular or viral deubiquitinating enzyme (DUB) (6). Alternatively, in the course of viral infection, a viral protein can bind to a cellular ubiquitin ligase complex, altering substrate specificity (7). The substrate is released after ubiquitin conjugation (8).

absence of E6AP, but the mechanism is unknown. HPV infection also leads to degradation of the retinoblastoma protein pRb through the ubiquitin-proteasome pathway (reviewed in Mammas et al., 2008), mediated via the HPV E7-protein-induced generation of an E3 ligase complex consisting of the Cullin2

containing Cullin5, Elongins B and C, and the RING finger protein, Rbx1 (Huh et al., 2007). Like p53, pRb is a tumor suppressor and its inactivation contributes to transformation as well. Adenovirus proteins E1B55K and E4orf6 cause degradation of p53 (reviewed in Blanchette and Branton, 2009). E4orf6 induces the assembly of a E3 ligase complex similar to the HPV E7 complex,

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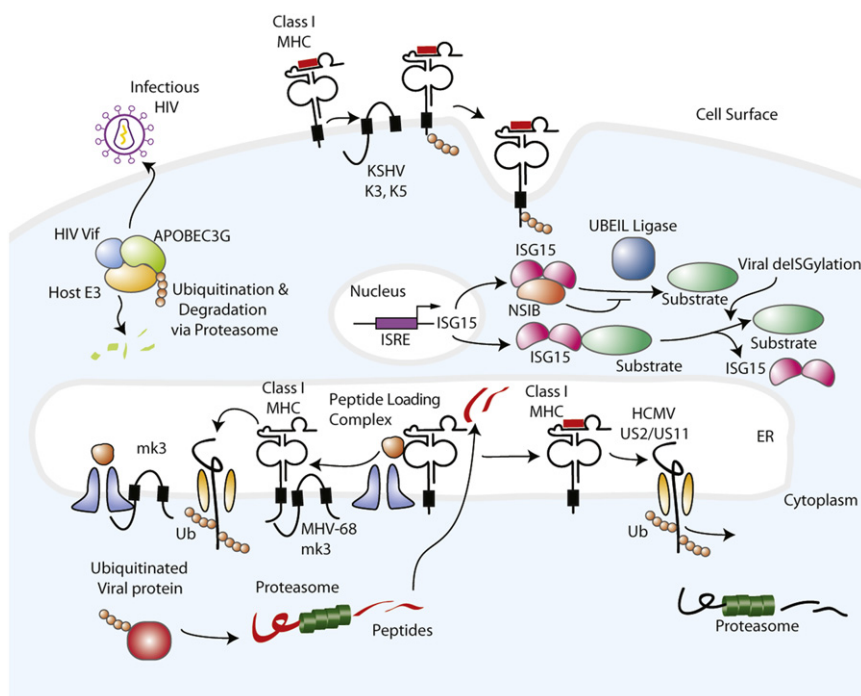


Figure 2. Viral Interference with the Host Immune Response
Class I MHC molecules are dislocated from the ER in response to HCMV viral proteins US2/US11 or MHV-68 mk3 protein. KSHV K3 and K5 proteins mediate ubiquitination and downregulation of class I MHC molecules at the cell surface. HIV Vif induces the formation of an E3 ubiquitin ligase complex, which binds to and ubiquitinates APOBEC3G, leading to its degradation and preventing its incorporation into HIV virions. Expression of antiviral ubiquitin-like modifier, ISG15, is induced upon infection of certain viruses, including influenza B. The NS1B protein of influenza B inhibits the UBE1L ligase and prevents conjugation of ISG15 to substrates. Some viruses also encode deubiquitinating enzymes that mediate removal of ISG15 from substrates. Abbreviations: ISRE, interferon sensitive responsive element; Ub, ubiquitin.

Table 1. Viral Proteins That Modify Host Ubiquitination

Viral Protein	Mechanism	Reference
HPV E6	generation of E3 ligase complex with E6AP to degrade p53	reviewed in Beaudenon and Huibregtse (2008); dMammas et al. (2008)
HPV E7	generation of E3 ligase complex to degrade p53	Huh et al. (2007)
Adenovirus E1B55K/E4orf6	generation of E3 ligase complex to degrade p53	reviewed in Blanchette and Branton (2009)
EBV EBNA-1 HSV-1 ICP0	interacts with USP7 (herpesvirus-associated USP) and prevents deubiquitination of p53 to enhance degradation	Everett et al. (1997); Holowaty and Frappier (2004)
HIV Vif	recruits formation of host E3 ubiquitin ligase complex to induce degradation of APOBEC3G	reviewed in Goila-Gaur and Strebel (2008); Huthoff and Towers (2008)
HIV Vpu	recruits formation of host E3 ubiquitin ligase complex to degrade CD4	reviewed in Nomaguchi et al. (2008)
Rubulavirus V	alters host E3 ligase substrate specificity to induce degradation of STAT-1, -2, -3	Ulane et al. (2003); Precious et al. (2005)
HPV E5	inhibits host E3 ligase to prevent degradation of EGFR	reviewed in Blanchette and Branton (2009)
HPV E2 Adenovirus E4orf6	interferes with the APC E3 ubiquitin ligase complex to inhibit degradation of cyclin B	Bellanger et al. (2005)
SV40 Large T Antigen	binds the SCF Ub ligase complex to inhibit the degradation of cyclin E	Welcker and Clurman (2005)
HCMV US2/US11	induces dislocation from ER into cytosol for ubiquitination and degradation of MHC class I molecules	Wiertz et al. (1996a, 1996b)
EBV LMP1/LMP2A	increases cellular DUB activity to stabilize β -catenin and associates with Nedd4 E3 ligase to ubiquitinate and degrade Lyn and Syk tyrosine kinases	Winberg et al. (2000); Ovaa et al. (2004)

targeting both p53 and the MRN complex can lead to transformation of the host cell.

Although degradation of cell cycle proteins by co-opting cellular E3 ubiquitin ligase complexes is the most common mechanism used by viruses to interfere with the cell cycle, some encode proteins that can inhibit cellular E3 ligases and prevent degradation of particular host proteins. HPV encodes the E5 protein, which inhibits the degradation of activated epidermal growth factor receptor (EGFR) (reviewed in Blanchette and Branton, 2009). E5 binds EGFR and interferes with receptor binding to c-Cbl (Zhang et al., 2005), an E3 ubiquitin ligase that downregulates activated phosphorylated receptor tyrosine kinases by ubiquitin-mediated degradation. The HPV E2 and adenovirus E4orf4 proteins can inhibit the cell cycle by interfering with the anaphase-promoting complex (APC), an E3 ligase required for progression through M phase (reviewed in Blanchette and Branton, 2009). Both viral E2 and E4orf4 ultimately inhibit the degradation of cyclin B and probably other substrates, leading to G2/M arrest (Bellanger et al., 2005). Viral E2 is itself controlled by an as yet unknown cellular ubiquitin ligase that ubiquitinates the viral protein (Bellanger et al., 2001). SV40 interferes with the cell cycle via its large T antigen, which binds to the SCF^{Fbw7} ubiquitin ligase complex and inhibits degradation of cell cycle proteins such as cyclin E, probably by acting as a competitor (Welcker and Clurman, 2005). The large T antigen may also induce the ubiquitination of new substrates not normally targeted by the SCF complex.

In addition to altering the cell cycle, viruses utilize the ubiquitin pathway to interfere with the immune response of the host cells. As depicted in Figure 2, viruses use a variety of methods to hijack

the host ubiquitin-proteasome pathway to enhance their own replication and prevent detection by the immune system. Virus infection rapidly induces an antiviral state in the host cell, as exemplified by the induction of an interferon response. The nuclear factor- κ B (NF- κ B) pathway is pivotal in activating both the innate and adaptive immune response. Furthermore, the NF- κ B transcription factor is itself regulated by the ubiquitin-proteasome pathway (reviewed in Bhoj and Chen, 2009). A variety of stimulators, including virus infection, induces the activation of NF- κ B through the degradation of the I κ B proteins, which normally sequesters NF- κ B in the cytoplasm. Upon ubiquitin-dependent proteasomal degradation of I κ B, NF- κ B is released, enters the nucleus, and activates a variety of genes involved in setting up an antiviral state in the cell. Activation of NF- κ B can also be controlled by A20, a dual enzyme with both deubiquitinating and ubiquitin ligase activity, and will be discussed in more detail later. The role of ubiquitin in activation of the innate and adaptive immune response has been extensively reviewed (Bhoj and Chen, 2009).

Many different host factors that control the innate and adaptive immune response pathways are often targeted by viral gene products to counteract it (Figure 2). Human immunodeficiency virus 1 (HIV-1) and most other lentiviruses encode several accessory proteins required for pathogenesis of the virus in vivo. One such accessory protein is viral infectivity factor (Vif), which acts as a substrate-recruiting subunit of a cellular E3 ligase complex (reviewed in Goila-Gaur and Strebel, 2008; Huthoff and Towers, 2008). Vif is required for viral replication in “nonpermissive” cells such as T cells and macrophages, but Vif is not required for replication in permissive cells such as epithelial cells. This is due to the

Table 2. Viral Proteins Directly Modified by Ubiquitin

Viral Protein	Mechanism	Reference
Retroviral (HIV) Gag	ubiquitination of Gag enhances recruitment of Tsg101 to the late domain (PTAP) for virion budding and release	Garrus et al. (2001); Demirov et al. (2002)
Ebola VP40	VP40 ubiquitinated by Nedd4 E3 ligase which is recruited to late domains (PPXY) for virion budding and release	Yasuda et al. (2003)
Retroviral Gag/Rhadinovirus M	PPXY motif bound by Nedd4 E3 ligase for virion budding and release	reviewed in Ingham et al. (2004)
EBV LMP2A	Nedd4 recruited to PPPY motifs in LMP2A for degradation	Ikeda et al. (2000)
HPV E7	ubiquitinated by the SOCS1 and SCF complex and deubiquitinated by USP11 to control degradation	Kamio et al. (2004); Oh et al. (2004); Lin et al. (2008)
HPV E2	ubiquitinated by unknown cellular ligase for degradation	Bellanger et al. (2001)

action of a cellular cytidine deaminase, APOBEC3G, present in nonpermissive cells, which acts as an intrinsic immune response modulator. APOBEC3G is normally encapsidated into budding virions and induces cytidine to uracil mutations in the single-stranded DNA of HIV-1 during reverse transcription. This hypermutation leads to a block in viral replication (Lecossier et al., 2003; Mangeat et al., 2003; Mariani et al., 2003; Zhang et al., 2003). However, when Vif is present, it prevents encapsidation of APOBEC3G into viral particles by inducing its ubiquitin-dependent degradation (reviewed in Goila-Gaur and Strebel, 2008; Huthoff and Towers, 2008). Vif recruits Elongins B and C, Cullin5, and the Rbx1 E3 ligase (Yu et al., 2003). The entire complex then polyubiquitinates APOBEC3G, leading to proteasome-mediated degradation (reviewed in Goila-Gaur and Strebel, 2008; Huthoff and Towers, 2008) and prevents its encapsidation into the retroviral virion (Figure 2).

Another accessory protein of HIV-1, Vpu, induces the downregulation of CD4 (reviewed in Nomaguchi et al., 2008), an entry receptor for the virus. It is likely that CD4 downregulation in the endoplasmic reticulum (ER) is required for proper trafficking and maturation of the viral glycoprotein, gp160, which would otherwise interact with CD4 and prevent its incorporation into the virion during egress. Vpu acts as an adaptor, interacting directly with CD4 in the ER and with the F box protein β TrCP, causing the transfer of ubiquitin via the Skp1-Cullin1 E3 ligase complex to CD4, which is then dislocated from the ER to the cytosol where it undergoes proteasomal-mediated degradation (reviewed in Nomaguchi et al., 2008). Vpu itself is not degraded during this process, but under certain circumstances Vpu can be ubiquitinated and degraded by an unknown E3 ligase complex (Estrabaud et al., 2007). The role of ubiquitin in controlling various aspects of receptor endocytosis has been reviewed extensively (see Miranda and Sorkin, 2007; Komada, 2008).

Similar to HIV-1 Vif, the rubulavirus V protein acts as an adaptor protein to alter the substrate specificity of a cellular E3 ligase (Ulane and Horvath, 2002). Rubulaviruses presumably seek to destroy STAT (signal transducer and activators of transcription) proteins by degradation as a mechanism to dampen the antiviral response. STAT-1 and -2 interact with IRF-9 (interferon regulatory factor-9), which is activated in response to interferon (IFN) binding to the IFN receptor. Viruses often activate the antiviral interferon response during infection, and thus degradation of STAT1/2 helps frustrate this response. Structurally, the mechanism for virus-induced degradation of the STAT proteins is very well understood.

The rubulavirus V protein binds to the ultraviolet damage-specific DNA-binding protein-1 (DDB1). Structural analysis has determined that DDB1 contains a three-propeller cluster that allows the V protein to bind between two of these propellers while the third recruits the E3 ligase Cullin4A (Li et al., 2006). The Rbx1 RING protein is then recruited and binds to an as yet unknown E2 conjugating enzyme. Rubulavirus V proteins can then either directly target STAT2 for ubiquitination (human parainfluenza virus type 2) or STAT2 can be used to recruit STAT1 (simian virus 5 and mumps virus) (Precious et al., 2005). Regardless, the result is polyubiquitination of STAT1/2 and degradation by the proteasome. Mumps V protein, along with Rbx1, directly targets STAT3 for ubiquitination and degradation (Ulane et al., 2003).

Degradation of target proteins is mediated not only by viral adaptor proteins that hijack cellular E3 ligases, but also by viral proteins that induce dislocation from the ER. Human cytomegalovirus (HCMV) downregulates MHC class I, presumably to decrease antigen presentation to CD8⁺ T cells, thus effectively avoiding immune recognition (Figure 2). HCMV US11 and US2 proteins induce dislocation of MHC class I from the ER into the cytosol where the class I molecules become polyubiquitinated and degraded by the proteasome (Wiertz et al., 1996a, 1996b). For US11, the Hrd1-Sel1L complex serves as the E3 ligase, a multisubunit complex that acquires its ubiquitin from a tail-anchored ER resident E2, Ubc6e. Class I MHC products are frequent targets of viral evasive maneuvers (see Figure 2 and van der Wal et al., 2002; Loureiro and Ploegh, 2006, for review).

Ubiquitin-Modified Viral Proteins: A Role in Virion Egress

Because a fraction of all newly synthesized proteins, regardless of whether derived from host or virus, fails to fold correctly, their disposal is essential and requires involvement of the ubiquitin-proteasome pathway. Accordingly, a fraction of newly synthesized viral proteins will undergo this fate as well. However, some viral functions require ubiquitin modification for purposes other than protein turnover (Table 2), as exemplified by the role of ubiquitin in virus budding. Some early observations include the detection of free ubiquitin in retroviral particles, such as HIV, and the inhibition by proteasome inhibitors of the budding of retroviruses and other RNA viruses, presumably because the store of free ubiquitin to be used for conjugation is tied up (Martin-Serrano, 2007).

The retroviral Gag protein contains multiple domains, including matrix, capsid, nucleocapsid, and p6, all of which play a role in the

assembly and budding process. The p6 domain is also known as the late budding domain (L-domain) and is thought to serve as an adaptor to recruit cellular proteins to mediate membrane fission, allowing release of the virion. Conserved motifs, such as PPXY or PTAP, are found in p6 and are thought to mediate its ability to recruit these proteins (Strack et al., 2000). The process of membrane fission that occurs during retroviral egress is similar to that of the multivesicular body (MVB) pathway (see Morita and Sundquist, 2004; Martin-Serrano, 2007, for reviews). During the formation of MVBs, the tumor susceptibility gene 101 (Tsg101) forms a multimolecular complex with ESCRT (endosomal sorting complex required for transport) proteins and binds to monoubiquitinated cargo proteins. This complex then sorts the cargo into the MVBs (Katzmann et al., 2001). During retrovirus budding, Tsg101, along with other ESCRTs, is recruited to and binds directly to the PTAP motif in HIV Gag, instead of the usual monoubiquitinated cargo (Katzmann et al., 2001). Ubiquitination of retroviral Gag proteins enhances Tsg101 binding. Tsg101 is a noncanonical ubiquitin E2 variant (UEV) and lacks the catalytic cysteine residue necessary for ubiquitin conjugation (Babst et al., 2000). Binding of Tsg101 to the PTAP motif in Gag occurs through the UEV domain of Tsg101. Tsg101 is required for HIV budding: its depletion with RNA interference (RNAi) or overexpression of its UEV domain both inhibit virion release (Garrus et al., 2001; Demirov et al., 2002).

The Nedd4 (neuronal precursor cell-expressed developmentally downregulated) protein is a WW domain-containing HECT E3 ubiquitin ligase required for budding of many viruses including retroviruses, Ebola, and rhabdoviruses (reviewed in Ingham et al., 2004). This E3 is recruited to PPXY motifs in late budding domains, including the Ebola VP40 protein, which is itself ubiquitinated (Yasuda et al., 2003). Ebola VP40 contains both a PPXY and PTAP motif. Like HIV-1 Gag, it binds Tsg101 through the PTAP motif and this interaction is required for virion budding and release. Nedd4 binds to the PPXY motifs in retroviral Gag proteins, including RSV, M-PMV, and MMLV. The PPXY motif in Gag of M-PMV, VP40 of Ebola, and M protein of rhabdoviruses (vesicular stomatitis virus [VSV] and rabies) controls virion budding and release.

Nedd4 may also function in the maintenance of EBV (Epstein-Barr virus) latency in B cells (reviewed in Ingham et al., 2004). The viral protein latent membrane protein 2A (LMP2A) contains two PPPY motifs that recruit Nedd4 family members. Signaling pathways initiated by the B cell receptor (BCR) activate the viral BZLF1 immediate-early gene, which then activates viral lytic genes. LMP2A binds, sequesters, and causes the degradation of the Lyn and Syk tyrosine kinases, preventing them from interacting with the BCR, and possibly preventing BCR signaling and reactivation from latency (Winberg et al., 2000). The Nedd4 E3 ligase ubiquitinates Lyn, Syk, and LMP2A itself and targets them for degradation (Ikeda et al., 2000). However, LMP2A with a mutated PPPY motif still blocks the induction of viral lytic genes, questioning the importance of the recruitment of Nedd4 through that motif.

Although viruses take advantage of ubiquitination to enhance their own replication, viral proteins can also be ubiquitinated by cellular ligases, leading to their degradation. HPV E7, in addition to modifying the ubiquitination of host proteins such as pRb as discussed above, is itself modified by both an E3 ubiquitin ligase

and a deubiquitinating enzyme. E7 is a short-lived protein degraded via the ubiquitin proteasomal pathway. The suppressor of cytokine signaling (SOCS1) protein is a member of the STAT signaling pathway and is induced upon exposure to IFN- γ . SOCS1 causes ubiquitination and degradation of E7, thus increasing pRb levels (Kamio et al., 2004). E7 interacts with the SCF complex, which ubiquitinates E7 with the help of the E2 enzyme UbcH7 (Oh et al., 2004). The two pathways of E7 degradation are complementary: one occurs in the cytoplasm (SOCS1) and the other in the nucleus (SCF). The action of these ligases is opposed by the USP11 deubiquitinating enzyme: it interacts with E7, removes ubiquitin, and extends the half-life of E7 (Lin et al., 2008).

Ubiquitin Ligases Encoded by Viruses: Dampening of the Immune Response

The list of viruses that encode ubiquitin ligases continues to grow (Table 3). Given the parsimonious use of genetic capacity available to viruses, it stands to reason that these activities were selected for as a means to increase viral fitness. Herpes simplex virus 1 (HSV-1) infected cell protein 0 (ICP0) is a E3 ubiquitin ligase that induces polyubiquitination and degradation of a variety of proteins, including the promyelocytic leukemia (PML) protein, Sp100 (another component of PML nuclear bodies) (Chelbi-Alix and de The, 1999; Boutell et al., 2002), cyclin D3 (Van Sant et al., 2001; Hagglund et al., 2002), p53, and the cellular deubiquitinating enzyme USP7. ICP0 has two E3 ligase sites: a RING domain and a herpesvirus ubiquitin ligase-1 (HUL-1) domain. The RING domain recruits the cellular E2 enzyme UbcH5a as well as USP7, which is then ubiquitinated and degraded. ICP0 also undergoes self-ubiquitination, but its interaction with USP7 can lead to the stabilization of ICP0 by reversal of autoubiquitination. In the course of infection, the end result of these two opposing forces is the stabilization of ICP0 by USP7.

ICP0 also likely plays a role in preventing activation of the antiviral response by suppressing interferon-stimulated gene (ISG) expression (Eidson et al., 2002). It was first discovered that HSV-1 induces an antiviral response in the absence of viral replication, suggesting that expression of a viral protein normally counteracts this response. Cells infected with an ICP0 null mutant virus replicate much less efficiently and express higher levels of ISGs than do cells infected with wild-type virus (Harle et al., 2002). ICP0 inhibits IRF-3- and IRF-7-mediated activation of the ISGs (Lin et al., 2004). This inhibition requires the RING domain of ICP0 and the PML protein, but the exact mechanism is unknown. ICP0 also mediates the degradation of DNA-PK, which stabilizes IRF-3, further inhibiting the activation of ISGs, required for the induction of the IFN response. However, the ability of an ICP null virus to replicate is not enhanced by the inhibition of the STAT-1 or IRF-3 pathways, suggesting that some other mechanism, such as PML degradation, may be responsible for this phenotype (Everett et al., 2008).

The Kaposi's sarcoma-associated herpesvirus (KSHV) proteins K3 and K5 and murine gammaherpesvirus 68 (MHV-68) K3 are E3 ubiquitin ligases that conjugate ubiquitin to MHC class I proteins, leading to their downregulation from the cell surface or the ER (Figure 2; Coscoy and Ganem, 2000; Ishido et al., 2000; Boname and Stevenson, 2001). K3 and K5 require cellular

Table 3. Ubiquitin Ligases and Deubiquitinating Enzymes Encoded by Viruses

Viral Protein	Mechanism	Reference
HSV-1 ICP0	viral RING-type E3 ligase induces ubiquitination and proteasomal degradation of PML, Sp100k, cyclin D3, p53, USP7, ICP0	Everett et al. (1997); Boutell et al. (2002)
KSHV K3 and K5	E3 ligase ubiquitination and proteasomal degradation of class I MHC, ICAM-1, B7	Coscoy and Ganem (2000, 2001)
MHV-68 K3	ubiquitination and downregulation from cell surface of MHC class I molecules	Boname and Stevenson (2001)
KSHV RTA	ubiquitin-mediated degradation of IRF-7 and inhibition of innate immune responses	Yu et al. (2005)
HSV-1 UL36	deubiquitination of unknown substrate	Kattenhorn et al. (2005)
MDV UL36	deubiquitination of unknown substrate, required for pathogenesis and T cell lymphoma formation	Jarosinski et al. (2007)
EBV BPLF1	deubiquitination of ribonucleotide reductase increases enzymatic activity	Whitehurst et al. (2009)
PRV	deubiquitination of unknown substrate, required for pathogenesis, virion egress, and neuroinvasion	Bottcher et al. (2008)
HCMV UL48/MCMV M48	deubiquitination of unknown substrate	Wang et al. (2006)
SARS-CoV PLpro	possibly protects viral replication complex from proteasomal degradation via deubiquitination	Lindner et al. (2005)
Adenovirus Protease Adenain	general decrease in ubiquitinated proteins especially in the nucleus	Balakirev et al. (2002)

components such as TAP and tapasin, proteins that form a complex with the class I MHC molecules (the peptide loading complex), for successful ubiquitination to occur. K5 also downregulates other molecules important for the stimulation of T cells, such as ICAM-1 and B7 (Coscoy and Ganem, 2001).

KSHV encodes yet another E3 ligase, the RTA (replication and transcription activator) protein (Yu et al., 2005), a transcription factor encoded by the ORF50 gene. The RTA protein is essential for activation of viral DNA replication upon initial infection of the virus. This activation occurs by transactivation of downstream lytic genes as well as the ORF50 gene as well. Furthermore, the RTA protein is also required for reactivation from latency. The RTA protein contains a cysteine-rich region that likely contains E3 ligase activity, as indicated by the fact that mutation of this region abrogates conjugation activity. RTA can not only polyubiquitinate itself but can also ubiquitinate several RTA repressors, including K-RBP (KSHV-RTA binding protein) and Hey1, a cellular transcriptional repressor, leading to their degradation by the proteasome (Yang et al., 2008; Gould et al., 2009). This ability of the RTA protein to induce the degradation of these transcriptional repressors is an important viral means of control and essential for the reactivation of lytic DNA replication. IRF-7 is also ubiquitinated by the RTA protein, which leads to its degradation and prevents the generation of IFN- α , compromising the innate immune response (Yu et al., 2005).

Ubiquitin-like Modifiers in Viral Infection

Several ubiquitin-like modifiers play a role in viral infection. These include SUMO and ISG15. Furthermore, recently discovered ubiquitin-like modifiers, such as Urm-1, may also prove to be involved in viral replication and will be also discussed.

SUMO

Because sumoylation is a predominantly nuclear event, it is the nuclear proteins encoded by DNA viruses that tend to be directly

sumoylated (Rodriguez et al., 2001). For herpes-, adeno-, and papillomaviruses, the viral proteins that are sumoylated generally have roles in viral transcription or replication; sumoylation often causes changes in their subcellular localization but it has remained a challenge to determine the exact role of sumoylation in viral infection. Some viral proteins can interact with SUMO or with Ubc9, the SUMO-conjugating enzyme, but are not themselves sumoylated, perhaps as a means of recruiting the sumoylation machinery to other viral or cellular proteins, in a manner analogous to recruitment of E6AP to p53 by HPV E6.

The first viral proteins found to be directly sumoylated was the immediate-early (IE) IE1 and IE2 proteins of HCMV (reviewed in Rosas-Acosta and Wilson, 2004). The IE proteins of EBV (BZLF1) and human herpes virus 6B (HHV-6B, IE-1) are also sumoylated. The IE proteins of herpesviruses are essential in initiating viral gene expression upon virus entry. They often act as transcriptional regulatory factors that help jump-start downstream early viral gene expression during viral infection (HCMV IE2), but IE proteins also regulate the cell cycle and apoptosis and can disrupt the PML nuclear bodies (HCMV IE1 and EBV BZLF1). There is currently no phenotype associated with a sumoylation-resistant HCMV IE1 protein (Lee et al., 2004). HCMV IE2 interacts with pRb, p53, as well as several cellular transcriptional activators. IE2 interacts with SUMO-1, SUMO-3, and Ubc9. Although a sumoylation-resistant IE2 shows no differences in subcellular localization or stability, it does not transactivate early viral promoters as well as wild-type IE2 (Hofmann et al., 2000). SUMO may thus alter the ability of IE2 to interact directly with those viral promoters or transcription factors required for efficient viral gene expression. The exact role of SUMO modification of the IE proteins in HCMV infection remains to be clarified. Sumoylation of the HHV6 IE1 protein may increase its stability, but whether its sumoylation affects transcriptional activity is not known. Similar to the other herpesviral IE proteins, the EBV

BZLF1 (Z) protein is a transcriptional activator and can disrupt the PML nuclear bodies. The role of sumoylation in the viral life cycle is unclear, because a sumoylation-deficient Z protein likewise disrupts nuclear bodies; however, sumoylation of Z does appear to decrease the ability of Z to transactivate certain promoters (Adamson, 2005). These examples once again illustrate the challenges in distinguishing between adventitious and purposeful ubiquitin and ubiquitin-like modifications by viruses.

The role of SUMO in adenovirus and papillomavirus replication is better understood than for herpesviruses (reviewed in Rosas-Acosta and Wilson, 2004). Adenovirus E1B55K, together with E4orf6 and cellular proteins, ubiquitinates and promotes proteasomal degradation of p53 as discussed earlier. E1B55K contains a consensus site for sumoylation responsible for its nuclear localization (Endtner et al., 2001). Indeed, a K104R sumoylation-resistant mutant shows altered localization and a reduced ability to interact with p53 and to transform cells (Endtner et al., 2001). Both the HPV and bovine papilloma virus (BPV) E1 proteins interact with Ubc9 (Yasugi and Howley, 1996) and BPV E1 can be sumoylated. An HPV E1 mutant unable to bind to Ubc9 fails to support viral replication (Yasugi and Howley, 1996) and demonstrates the importance of sumoylation for small DNA tumor viruses.

The ability to bind SUMO and/or Ubc9 is a property shared by numerous other viral proteins. The Bunyaviridae and Retroviridae families contain viral nucleocapsid proteins that interact with SUMO and/or Ubc9, but these are not themselves sumoylated. The Hantaan virus nucleocapsid (HTNV-NP) interacts with both Ubc9 and SUMO-1, possibly to relocate NP to the perinuclear region where viral replication and virion assembly occur (Kaukinen et al., 2003). Retroviral Gag proteins, required for virion assembly and budding, interact with Ubc9, as exemplified by Mason-Pfizer monkey virus (MPMV) (Weldon et al., 2003). Overexpression of Ubc9 causes both Ubc9 and Gag to localize to nuclear and perinuclear regions and therefore may help direct Gag to the site of virion assembly.

A further layer of complexity is provided by the existence of multiple isoforms of SUMO (SUMO-1, -2, -3), which appear to have different targeting abilities and only some of which have been explored in any detail in the context of virus infections. The distinct functions of these SUMO isoforms remain to be established, particularly for SUMO-2 and SUMO-3, for which even their cellular roles are not well defined. Examples of viral proteins that interact with specific isoforms of SUMO include vaccinia early protein (E3L) (which interacts with SUMO-1) and EBV nuclear antigen 3C (EBNA-3C) (which interacts with both SUMO-1 and SUMO-3, but not SUMO-2) (Lin et al., 2002). This is particularly surprising for EBNA-3C, because SUMO-2 and SUMO-3 are 97% identical but SUMO-1 and SUMO-3 are only 46% identical.

SUMO proteases (SENPs) remove the SUMO molecule from substrates, thereby reversing the effects of sumoylation. The Adenoviridae, Asfarviridae, and Poxviridae encode cysteine proteases related to the mammalian SENPs: they share conserved catalytic cysteine and histidine residues. These include the adenovirus protease (Li and Hochstrasser, 1999), the pS273R proteinase of African swine fever virus (ASFV) (Andres et al., 2001), and the I7 proteinase of poxviruses (Li and Hochstrasser, 1999). Thus far, desumoylating activity remains to be demonstrated for any of

these proteases and their role, if any, in viral replication is not known. By analogy with ubiquitination, however, where the importance of ubiquitin-specific proteases is now firmly established, it is a reasonable supposition that these activities would be important, all the more so when the host cell already comes equipped with its own complement of SENPs.

ISG15

ISG15 contains two ubiquitin-like domains. Upon viral infection, interferon is induced, leading to the induction of ISGs including ISG15, one of the most highly induced ISGs (reviewed in Sadler and Williams, 2008). ISG15 can be secreted from cells that produce it by an as yet unknown mechanism and therefore may act as a cytokine to modulate the immune response (D'Cunha et al., 1996). ISG15 has specific E1 (UBE1L), E2 (UBCH6 and UBCH8), and E3 (HERC5 and TRIM25) ligases. ISGylation is reversible via the action of ubiquitin-specific proteases (USPs), including USP18 (UBP43), USP2, USP4, USP13, and USP14 (reviewed in Sadler and Williams, 2008). However, unlike ubiquitination, ISGylation is not known to promote degradation, but instead acts as an antiviral effector. A complicating factor is the possibility of alternative modification of a given substrate protein either with ubiquitin or with ISG15, or modification with both ubiquitin and ISG15. Recombinant Sindbis viruses that drive the expression of a variety of ISGs was used to infect IFN- α / β receptor^{-/-} (IFN- α / β R^{-/-}) mice to identify attenuators of infection (Lenschow et al., 2005). Expression of ISG15 had an antiviral effect, protecting against Sindbis virus-induced death, and decreased viral replication. However, the antiviral state in the course of VSV and lymphocytic choriomeningitis virus (LCMV) infection in ISG15^{-/-} mice was similar to wild-type controls (Osiak et al., 2005).

Viruses can interfere with ISGylation. Influenza B virus strongly induces expression of ISG15, but the NS1 protein binds ISG15, inhibits activity of the UBE1L ligase, and prevents ISGylation (Yuan and Krug, 2001). Surprisingly, influenza A NS1 protein lacks this activity, but in this case, viral infection fails to induce expression of ISG15 (Yuan and Krug, 2001). Ovarian tumor (OTU)-domain proteases from nairoviruses of the Bunyaviridae family (Crimean-Congo haemorrhagic fever virus [CCHFV], large [L] protein) and arteriviruses (equine arteritis virus [EAV], nonstructural protein 2 [nsp2]) are deISGylases as well as deubiquitinases (Frias-Staheli et al., 2007). These OTU domain proteases are present in eukaryotes, bacteria, and viruses. The viral OTU domain proteases show much broader target specificity, unlike their host counterparts. Expression of these proteases reverses the antiviral effect of ISG15, increasing the host's susceptibility to Sindbis infection (Frias-Staheli et al., 2007). The viral OTU domain proteases inhibit NF- κ B-dependent signaling, likely through their deubiquitinating activity. Expression of the OTU domains of CCHFV-L and EAV-nsp2 inhibits the translocation of the p65 subunit of NF- κ B to the nucleus (Frias-Staheli et al., 2007). Severe acute respiratory syndrome-associated coronavirus (SARS-CoV) expresses a distinct protease, PLpro, which mediates deISGylation. Although ISG15 is its preferred substrate, PLpro is also capable of deubiquitination (Lindner et al., 2007).

ISG15 inhibits virus-mediated degradation of IRF-3, increasing IFN- β expression to enhance the innate immune response (reviewed in Sadler and Williams, 2008). The transcriptional

activity of IRF-3, essential for activation of type I IFN genes, is tightly regulated by protein degradation. Newcastle disease virus (NDV) infection or IFN treatment increases the conjugation of ISG15 to cellular proteins, including IRF-3 (Lu et al., 2006). This conjugation stabilizes IRF-3, prevents its ubiquitin-mediated degradation, increases activation of the IFN- β promoter, and facilitates IRF-3 translocation to the nucleus. After NDV infection of ISG15^{-/-} MEFs (mouse embryonic fibroblasts), the levels of IRF-3 decrease more drastically than in WT MEFs (Lu et al., 2006). ISG15 conjugation may also help direct modified proteins to the proteasome, because the proteasome-associated deubiquitinating enzyme (DUB), USP14, can recognize ISG15-modified substrates, or it may function to attract components of the ubiquitin conjugation machinery.

ISG15 plays a role in resistance to Ebola virus (also VSV and rabies virus) by blocking the activity of Nedd4 via ISGylation (reviewed in Sadler and Williams, 2008). ISG15 blocks the interaction of Nedd4 with its E2, preventing transfer of ubiquitin from the E2 to Nedd4 (Malakhova and Zhang, 2008). The ubiquitination of HIV-1 Gag and Tsg101 and Nedd4 are necessary for virion budding and release from the cell surface as discussed earlier. Type I IFN inhibits the assembly and release of HIV-1 virions. This occurs through the IFN-induced expression of ISG15, which inhibits the ubiquitination of Gag and Tsg101, preventing the interaction of the Gag L domain with Tsg101 (Okumura et al., 2006). However, ISG15 is not itself conjugated to Gag or Tsg101. Ubiquitination of Gag by the Nedd4 ubiquitin ligase is necessary for the release of virions. Overexpression of ISG15 effectively decreases the ability of Nedd4 to ubiquitinate the viral matrix proteins (VP40), thereby decreasing release of Ebola virus-like particles (VLPs) from cells (Okumura et al., 2008).

Other Ubiquitin-like Modifiers: Urm-1

The role of ubiquitin-like modifiers is no longer confined to the modification of proteins and lipids. A recent series of papers describes an unexpected role for ubiquitin-related molecule-1 (Urm-1) in transfer RNA (tRNA) modification (Schlieker et al., 2008; Leidel et al., 2009; Noma et al., 2009). Though only distantly related to ubiquitin, Urm-1 has the typical β -grasp fold that characterizes members of the ubiquitin family (Singh et al., 2005) and also possesses the typical diglycine motif at the C terminus of the mature polypeptide, common to ubiquitin and ubiquitin-like modifiers. The occurrence of proteins covalently modified by Urm-1 ("urmylation") has been reported (Furukawa et al., 2000), but thus far appears to be limited to a single protein of known identity, alkyl hydroperoxide reductase (Ahp1), in yeast (Goehring et al., 2003). Whether urmylation of proteins is a widespread modification, and if so, under which conditions (stress, growth conditions, and virus infection) remain open questions. Urm-1 serves as a sulfur carrier essential for thiolation of certain tRNAs [(UUU) Lys; (UUC) Glu; and (UUG) Gln], which carry a thiolated uridine residue in the wobble position of the anticodon (Schlieker et al., 2008; Leidel et al., 2009; Noma et al., 2009). Urm-1 can be isolated from mammalian cells as a C-terminal thiocarboxylate that is the immediate precursor for a sulfur transfer reaction involving a persulfidic intermediate (Schlieker et al., 2008). In the absence of Urm-1, yeast cells can no longer carry out the requisite thiolation reaction and become sensitive to a variety of stresses (Pedrioli et al., 2008). The mechanistic link between a failure to execute thiolation and the increased sensi-

tivity to stress observed in Urm-deficient cells is not yet understood. For example, it is not clear how tRNAs that fail to undergo thiolation affect translational efficiency, fidelity, or the half-lives of the tRNAs themselves. A number of plant viruses, including brome mosaic virus, possess tRNA-like structures at the 3' end of their genomes. These structures are sufficiently similar to tRNAs to serve as substrates for aminoacyl tRNA synthetases and other enzymes that act on tRNAs (Dreher, 2009). The mouse gammaherpesvirus MHV-68 encodes at least eight tRNA-like sequences that can be processed into mature uncharged tRNAs, the function of which in the context of the virus life cycle is obscure. Clearly, viruses have co-opted tRNAs and tRNA-like structures to initiate replication, as is well established for retroviruses (reviewed in Abbink and Berkhout, 2008), presumably for reasons that increase fitness, but we still lack an adequate mechanistic explanation. Given the relationship between Urm-1 and specific tRNA modifications, it is perhaps reasonable to suggest that aspects of translational control and fidelity, as well as virus replication itself, may be modified in a manner that involves Urm-1, although for the moment this remains pure speculation.

Deubiquitination in Viral Infection

There are currently seven known classes of DUBs that act to reverse ubiquitin and ubiquitin-like modifications: (1) the ubiquitin-specific protease (USP), (2) autophagin (ATG), (3) ubiquitin C-terminal hydrolase (UCH), (4) ovarian tumor (OTU) domain proteins, (5) Josephin-domain (JD) or Machado-Joseph disease (MJD) proteins, (6) ubiquitin-like protein-specific protease (ULP), and (7) JAMM (Jab1/MPN domain-associated metalloisopeptidase) domain proteins (see Nijman et al., 2005; Sulea et al., 2006, for review).

Several viral proteins are known to interact with cellular DUBs. One of the most well studied is USP7 or herpesvirus-associated ubiquitin-specific protease (HAUSP). ICP0 of HSV-1 and EBNA1 of EBV interact with USP7 (Everett et al., 1997; Holowaty and Frappier, 2004), which removes ubiquitin from p53, EBNA1, and ICP0, thereby preventing their degradation (Li et al., 2002; Canning et al., 2004). EBNA1 of EBV is required for multiple aspects of viral replication: maintenance of the viral genome, transcription and translation of the viral DNA, viral persistence, and transformation of cells. The exact role of HSV-1 ICP0 interaction with USP7 is not known, but as noted earlier, ICP0 is an E3 ligase that ubiquitinates USP7, causing both to be degraded. ICP0 is also a substrate for USP7 and is stabilized by interaction with this DUB. In the course of infection, the net result of these two opposing forces is the stabilization of ICP0 by USP7.

Upon EBV infection, several cellular DUBs are known to increase in activity including USP5, -7, -9, -13, -15, and -22 (Ovaa et al., 2004). Furthermore, the DUBs recruited by EBV may stabilize β -catenin, a component not only of cellular junctions, but also a key component of the *Wnt* signaling pathway that regulates growth and differentiation of cells. Furthermore, its dysregulation has been implicated in cancer development (reviewed in Reya and Clevers, 2005). This stabilization occurs in latently infected B cells and may involve viral proteins LMP1 and LMP2A, although a contradictory report suggests that LMP1 is not sufficient to induce activation of the *Wnt* pathway (Webb et al., 2008). Further investigation is needed to determine the exact role EBV plays in activation of the *Wnt* signaling

pathway, because EBV is associated with several cancers, including nasopharyngeal carcinoma and Burkitt's and Hodgkin's lymphomas.

Measles virus also induces the upregulation of a cellular deubiquitinating enzyme, A20 (Yokota et al., 2008). A20 is an OTU domain DUB that inhibits NF- κ B upstream by removing ubiquitin from TRAF6 and RIP1, two signaling molecules of the NF- κ B pathway. Furthermore, A20 also has ubiquitin ligase activity and can ubiquitinate RIP1, leading to proteasomal-mediated degradation. In certain cell types, measles virus infection dramatically upregulated A20. The expression of measles virus phosphoprotein (P protein) is necessary and sufficient to induce upregulation of A20, leading to the suppression of Toll-like receptor signaling (Yokota et al., 2008).

Virus-Encoded Deubiquitination Enzymes

Several viral proteins possess deubiquitinating activity (Table 3), including the adenovirus protease adenain SARS-CoV papain-like protease (PLpro) and herpesvirus large tegument protein. Deubiquitinating activity in herpesviruses was first discovered with activity-based HA-ubiquitin probes (HAUbVME) in HSV-1-infected cell lysates (Kattenhorn et al., 2005). The N-terminal fragment of UL36, the large tegument protein, is the source of this activity. UL36 homologs from α -, β -, and γ -herpesviruses all contain a putative cysteine box with a histidine box further downstream. UL36 lacks obvious similarity to any known DUB, and the crystal structure of murine cytomegalovirus (MCMV) M48 DUB domain has determined that the herpesviral DUBs represents a new family of deubiquitinating enzymes (Schlieker et al., 2007). DUB activity has been confirmed for several other herpesviruses including EBV, MCMV, and HCMV (Schlieker et al., 2005; Wang et al., 2006). Mutation of the putative active site cysteine in UL48 of HCMV produced lower virus yields but the activity is not essential for replication of HCMV in tissue culture (Wang et al., 2006). Further work with Marek's disease virus (MDV), an α -herpesvirus, has demonstrated a potential role for the herpesvirus DUB in pathogenesis in vivo. Mutation of the MDV DUB active site cysteine caused a stark reduction in the formation of T cell lymphomas in chickens (Jarosinski et al., 2007). Similar mutants were made for pseudorabies virus (PRV): mice infected with a DUB active site mutant virus survived twice as long and demonstrated a decrease in neuroinvasion compared to those infected with wild-type virus (Bottcher et al., 2008). The PRV DUB active site mutant demonstrates a defect in virion assembly and egress in vitro (Bottcher et al., 2008). The large tegument protein remains tightly bound to the nucleocapsid during transit to the nucleus, and could thus be important for entry of the virus, as well as for virion assembly and egress. As a key component of the virion, the tegument-encoded DUB might participate in either or both of these events. More recently, two other active DUBs (BSLF1 and BSLF2) were discovered to be encoded by EBV via computer modeling to search for the conserved C- and H-boxes of the known DUB families (Sompallae et al., 2008). Homologs of these proteins exist in some of the other herpesviruses; therefore, it is likely that more functional DUBs will be discovered in these viruses.

The crystal structure of the SARS-CoV PLpro enzyme demonstrates that it belongs to the USP family of DUBs; moreover, it has DUB activity in vitro (Lindner et al., 2005). PLpro is part of the repli-

case polyprotein and is involved in cleaving the nonstructural proteins (nsps), particularly nsps1, -2, and -3, to form the viral RNA replication complex. Whether the SARS-CoV PLpro functions as a DUB in the course of infection is not known, though it is probable. It might serve to protect the viral replication complex from ubiquitination and degradation by the proteasome.

The adenovirus protease adenain is found both in the nucleus and the cytosol. Upon activation in the nucleus, the adenain protease cleaves six viral capsid precursor proteins inside the viral capsids (Ruzindana-Umunyana et al., 2002). In the cytoplasm, adenain is thought to cleave cytoskeletal proteins, aiding in cell lysis and release of the virions. Adenain also contains deubiquitinating activity (Balakirev et al., 2002). During adenoviral infection, the levels of adenain correlate with a corresponding decrease in ubiquitinated proteins, particularly in the nucleus. The identity of the affected substrates is not known, a challenge common to most enzymes in this class, and the role of the adenain-associated DUB activity in the viral life cycle remains to be clarified.

Conclusion

Hijacking of the ubiquitin system by viruses continues to emerge as a central theme around virus replication. Not only is modification of ubiquitinated substrates during the course of viral infection a mainstream event, but examples of viruses that encode their own ubiquitin ligases as well as deubiquitinating enzymes are increasing rapidly. A continuing challenge that remains is the discovery of the substrates for these newly described viral enzymes. Enzyme-substrate interactions may demonstrate high affinity but may also be transient. The relatively recent advances in mutagenesis techniques that allow rapid production of virus mutants, also for the large DNA viruses, coupled with the ability to manipulate host protein expression, will help determine the function and substrates of these emerging viral and cellular ubiquitin modifiers.

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