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Minireview

Deubiquitination in virus infection

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Abstract

Post-translational modification of proteins and peptides by ubiquitin, a highly evolutionarily conserved 76 residue protein, and ubiquitin-like modifiers has emerged as a major regulatory mechanism in various cellular activities. Eukaryotic viruses are known to modulate protein ubiquitination to their advantage in various ways. At the same time, the evidence for the importance of deubiquitination as a viral target also is growing. This review centers on known viral interactions with protein deubiquitination, on viral enzymes for which deubiquitinating activities were recently demonstrated, and on the roles of viral ubiquitin-like sequences.

Keywords: β-Catenin; Deubiquitinating enzymes; Epstein–Barr nuclear antigen 1; Herpes simplex virus regulatory protein ICP0; Ubiquitin; Ubiquitin-specific protease 7

Introduction

Post-translational modification of proteins and peptides by ubiquitin (Ub), a highly evolutionarily conserved 76 residue protein, and ubiquitin-like modifiers (Ubls) (Fig. 1) has emerged as a major regulatory mechanism in various cellular activities including signal transduction, transcription, membrane protein trafficking, nuclear transport, autophagy, and immune responses (d'Azzo et al., 2005; Haglund and Dikic, 2005; Welchman et al., 2005). Protein modifications by Ub and Ubls, such as Nedd8, ISG15, and SUMO, modulate protein–protein interactions (Kerscher et al., 2006), while ubiquitin-like Atg8 homologs become lipidated and attached to cellular membranes (Tanida et al., 2004). Ub and most Ubls are produced as precursor proteins, and only carboxy-terminal processing after recognition sequence motifs by deubiquitinating enzymes (DUBs) generates the active modifiers (Amerik and Hochstrasser, 2004). Conjugations of Ub and Ubls to their targets relies on analogous enzymatic cascades comprising the sequential action of three enzymes (Passmore and Barford, 2004): a modifier activating enzyme (E1), one of several modifier carrier enzymes (E2s), and a member of the large and diverse group of modifier-target ligases (E3s), which chiefly determine target specificity. The enzymatic trio transfers the carboxy terminal glycine of Ub to the epsilon-NH2 group of an internal lysine residue of the target protein, or less often to its terminal amino group. In contrast to the known Ubl modifications, Ub can further be assembled into polymeric chains (polyubiquitination). One out of five internal lysine residues at position 6, 11, 29, 48, and 63 of Ub, but mostly lysine-48 or lysine-63, is used for the attachment of additional Ub units.

While attachment of at least four lysine-48 linked Ub molecules usually promotes the degradation of a protein by the Ub-proteasome system (UPS), the most important machinery for the degradation of cytoplasmic and nuclear proteins, chain formation via other lysine residues, or conjugation of individual Ub molecules mediates largely non-proteolytic functions of Ub (Ciechanover, 2006). Ub and Ubl modifications are reversed through the isopeptidase activities of DUBs (Fig. 1), with most studied DUBs deconjugating only a small number of targets (Nijman et al., 2005). In fact, deubiquitination, a term used here inclusive of Ub and Ubl deconjugation, is an emerging regulatory process in signaling pathways, chromatin structure, endocytosis, and apoptosis (Nijman et al., 2005) and is important for physiological activities including neuronal function, development, and immunity (Evans, 2005).

Viruses of eukaryotes are known to take advantage of protein ubiquitination in various ways. Entry or release of different

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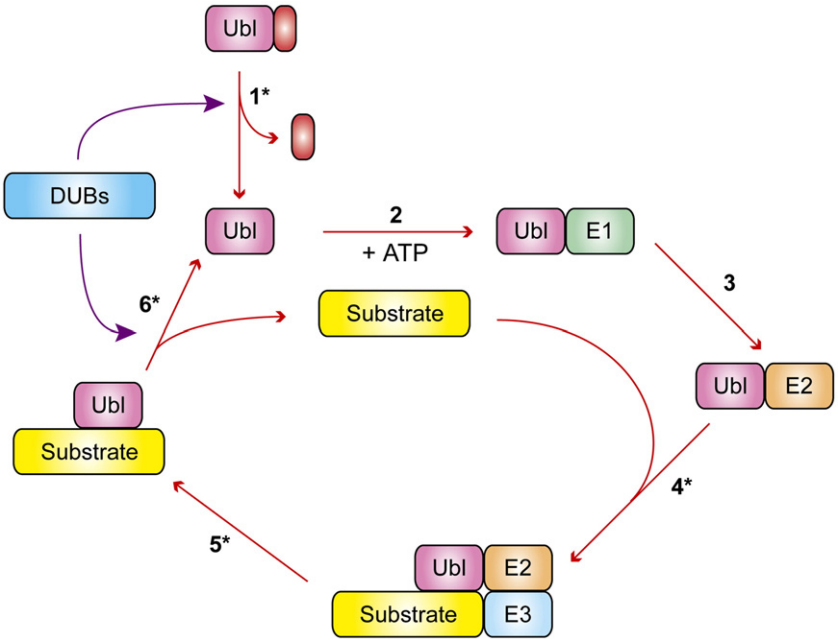

Fig. 1. General schematic for Ubl conjugation and deconjugation and points of viral interference. Proteolytic maturation of Ubl (including Ub) precursor proteins by DUBs (step 1) exposes a new C-terminus in the modifier, which is then activated by E1 in an ATP-dependent manner (step 2) and next transferred to the E2 (step 3). An E3 generally recognizes the target protein (step 4) and facilitates the ligation of the Ubl to it (step 5), altering its protein interaction repertory and thereby its function. Polyubiquitination as a particular example of Ubl modification of a protein can lead to its proteasomal degradation. Ubl deconjugation by DUBs reversely regulates target protein function and, additionally, replenishes the pool of free Ubl (step 6). Points of viral interference are marked (*). Viruses are known to intervene with the Ub conjugation pathway (at steps 4 and 5, see text), but also target Ubl deconjugation (step 6), inclusively referred to as deubiquitination, which is the principal theme of this review. Viruses may also interfere with Ubl precursor processing (step 1), as suggested by the ability of some viral DUBs to process the ISG15 precursor protein (see text).

viruses, for example, was shown to depend on the proteasome or on certain cellular E3s (Banks et al., 2003; Bieniasz, 2006; Ros and Kempf, 2004; Yu and Lai, 2005). What is more, many viruses manipulate protein ubiquitination in order to overcome host cell defense mechanisms, including apoptosis, the type 1 interferon (IFN) response, and major histocompatibility complex (MHC) class 1 antigen presentation. To this end, numerous viruses encode proteins that redirect cellular E3s of the UPS to proteins with antiviral activity (Fig. 1, step 4), including for example the tumor suppressor protein p53 (induction of apoptosis) and the signal transducers and activators of transcription (IFN response). Alternatively, some viruses express their own E3s (Fig. 1, step5), which commit cellular defense proteins, such as p53 or MHC class 1 molecules, to degradation. Such viral strategies were reviewed recently by Shackelford and Pagano (2005), Gao and Luo (2006), and Barry and Früh (2006) and are not considered here in detail.

Different from ubiquitination, only few examples for the targeting of protein deubiquitination (Fig. 1, step 6) by viruses have been described to date. They include the potential recruitment of DUBs for the stabilization of β-catenin in Epstein–Barr virus (EBV)-infected B cells (Ovaa et al., 2004; Shackelford et al., 2003), and the specific targeting of the cellular DUB ubiquitin-specific protease 7 (USP7) by the Epstein–Barr nuclear antigen 1 (EBNA1) and the herpes simplex virus type 1 (HSV-1) regulatory protein ICP0 (Everett et al., 1997; Holowaty and Frappier, 2004). Despite the compelling biochemical evidence for the specificity of these two interactions, the importance of viral targeting of USP7 remains vague. The possibility that modulation of deubiquitination is, nevertheless, a more common viral strategy has gained support by the recent in vitro demonstration of deubiquitinating activities for three viral enzymes: the adenovirus protease adenain (Balakirev et al., 2002), the papain-like protease (PLpro) of severe acute respiratory syndrome coronavirus (SARS-CoV) (Barretto et al., 2005; Lindner et al., 2005), and a protease domain contained in the N-terminal fragment of the large tegument protein UL36 ($UL36^{USP}$) from several herpesviruses, namely, HSV-1, EBV, and mouse and human cytomegalovirus (MCMV and HCMV) (Kattenhorn et al., 2005; Schlieker et al., 2005; Wang et al., 2006). However, the roles of these deubiquitinating activities during virus infection remain elusive. Here, the known viral interactions with protein deubiquitination are reviewed, and potential roles of viral DUBs are considered.

Deubiquitinating enzymes

The majority of the DUBs from all kingdoms of life (excluding archaea), including the known and predicted viral enzymes, represent cysteine protease homologs, with the remainder forming a separate family of metalloproteases (Rawlings et al., 2004). At least six structural classes (families) of cysteine protease DUBs have been identified: the ubiquitin-specific protease (USP), autophagin (ATG), ubiquitin C-terminal hydrolase (UCH), ovarian tumor-related protease (OTU), Josephin-domain protease (JD), and ubiquitin-like

protein-specific protease (ULP) families (Amerik and Hochstrasser, 2004; Nijman et al., 2005; Sulea et al., 2006). They all feature structural variations of the papain fold and display canonical papain-like spatial arrangement of catalytic centers. Many are further characterized by additional variable amino- and/or carboxy-terminal sequences, for which a growing number of structures and functions, including localization, substrate recognition, and/or activation of catalytic activity, are being reported (Nijman et al., 2005; Reyes-Turcu et al., 2006; Sulea et al., 2006).

Despite recent advances in the understanding of DUB structure–function relationships (Amerik and Hochstrasser, 2004; Nijman et al., 2005; Sulea et al., 2006), inference of biological importance for uncharacterized DUBs from sequence information alone remains challenging. The human genome encodes around 90 cysteine protease DUBs and 12 metalloprotease DUBs (Rawlings et al., 2004). The number of catalytically active human cysteine protease DUBs, inclusive of putative alternative splice isoforms, amounts to over 100. In order to further the functional characterization of DUBs on a large scale, Ploegh and co-workers synthesized specific probes for the proteomic profiling of cysteine protease DUB activities (Borodovsky et al., 2002). They used an intein-based chemical ligation method to modify the free C-terminus of a hemagglutinin (HA)-tagged Ub with a series of thiol-reactive groups, such as vinylmethylsulfone (VME) or bromoethylamine. After incubation of cellular lysates with some of these active site-directed suicide substrates and subsequent anti-HA immunoprecipitation, tandem mass spectrometry facilitated the simultaneous identification of multiple DUBs and some associated proteins (Borodovsky et al., 2002). HAUbVME exhibited the broadest reactivity, and, in the following, was used to detect active cysteine protease DUBs and associated proteins in EBV-infected B cells (Ovaa et al., 2004; Shackelford et al., 2003), and human papillomavirus (HPV)-infected cervical carcinoma cells as well as HPV E6/E7 immortalized keratinocytes (Rolén et al., 2006).

β-Catenin stabilization in Epstein–Barr virus infection

In a study by Ovaa et al. (2004), lymphoblastoid transformation of freshly isolated B cells by in vitro EBV infection led to an increase in the activities of six cellular DUBs, identified as UCH37, UCH-L3, UCH-L1, USP7, USP9X, and USP15, at different times postinfection. Interestingly, USP9X was previously shown to interact with the bifunctional armadillo repeat protein β-catenin in vitro as well as in cultured epithelial cells and was able to stabilize β-catenin in these cells (Taya et al., 1999). β-Catenin is further known to be stabilized in latency type III EBV-transformed B cells (see below).

β-Catenin is a component of cell-cell adherens junctions. At the same time, a low-level cytoplasmic pool of this protein functions in canonical Wnt signaling (reviewed by Brembeck et al., 2006). In the absence of Wnt signaling, cytoplasmic β-catenin is continuously phosphorylated by casein kinase followed by glycogen synthase kinase 3-β, which together with the two scaffold proteins axin and the adenomatosis polyposis coli tumor-suppressor protein constitute the so-called degradation complex. These consecutive phosphorylations events initiate the polyubiquitination of β-catenin by a Ub ligase complex, known as Skp1/cullin/F box protein$^{\beta\text{-TrCP}}$ ($SCF^{\beta\text{-TrCP}}$) (Liu et al., 2001, and references therein), and its rapid proteasomal degradation. Binding of the Wnt ligand to its cell surface receptors is thought to trigger a series of phosphorylation events that result amongst others in the degradation of axin, causing disassembly of the above-mentioned degradation complex and consequently the stabilization of β-catenin which then accumulates in the nucleus. Here, β-catenin acts as a coactivator of Wnt target genes that regulate cellular proliferation and differentiation during animal development and tissue homeostasis (Städeli et al., 2006).

β-Catenin stabilization in latency type III EBV-transformed B cells occurs by mechanisms that involve EBV latent membrane proteins 1 (LMP1) and 2A (LMP2A) (Hayward et al., 2006). This does, however, not necessarily entail its nuclear accumulation, indicating that effects of EBV induced β-catenin stabilization are dependent on the cellular context and in lymphoid cells may be distinct from Wnt activation (Morrison et al., 2004). Lately, Pagano and coworkers identified LMP1 as a transcriptional down-regulator of another Ub ligase complex that targets β-catenin for proteasomal degradation and is called seven in absentia homolog-1 (Jang et al., 2005). In any event, the observed up-regulation of DUBs in EBV transformed B cells by Ovaa et al. (2004), together with the almost simultaneous demonstration that β-catenin in the type III latently infected B cell line Sav III exists in a complex with active, yet unidentified DUBs by Shackelford et al. (2003), strengthens the possibility that DUBs contribute to EBV induced β-catenin stabilization.

Viral targeting of ubiquitin-specific protease 7

USP7 is an evolutionary conserved mammalian DUB of the USP family, which was originally identified by its ability to bind to two different herpesviral proteins, namely, EBNA1 of EBV, and the HSV-1 regulatory protein ICP0 for which it has also been coined herpes virus-associated ubiquitin-specific protease (HAUSP) (Everett et al., 1997; Holowaty and Frappier, 2004). USP7 displays debranching activity against lysine-48 linked polyubiquitin chains and was shown to play an important part in the dynamic regulation of nuclear p53 turnover (Brooks and Gu, 2006; Cheon and Baek, 2006).

Ubiquitin-specific protease 7 and the p53-MDM2 pathway

In unstressed cells, p53 is constitutively polyubiquitinated, which leads to its proteasomal degradation, keeping p53 levels low (Gomez-Lazaro et al., 2004). p53 levels dramatically increase upon various types of stress, including viral infection, triggering either growth arrest or apoptosis. Virus mediated gain of p53 ubiquitinating activity as a means of apoptosis avoidance was already mentioned above. Expression of USP7, the target protein of EBNA1 and ICP0, however, effectively promotes increased levels of p53 by antagonizing proteasomal

degradation of p53 through deubiquitination (Li et al., 2004). As may be expected, partial reduction of USP7 levels by RNA interference (RNAi) was shown to destabilize p53 (Li et al., 2004). With the collaboration of the adapter protein Daxx (death domain associated protein) (Tang et al., 2006), USP7 also deubiquitinates the mouse double minute 2 (MDM2) oncogene, one of several E3s that mediate p53 proteasomal degradation. This counteracts autoubiquitination of MDM2 and its proteasomal degradation (Li et al., 2004). In fact, a genetic knockout of USP7 caused depletion of MDM2 and, despite the aforementioned rescue effect of USP7 on p53, effectively stabilized p53 (Cummins et al., 2004; Li et al., 2004). Brooks and Gu (2006) have recently proposed that the predominant role of MDM2 during cellular stress is to ubiquitinate p53, and to keep its levels in check in order to maintain growth arrest while avoiding default execution of the apoptotic program and thereby to afford cell survival in the event of successful cellular repair and overcoming of the original challenge. In view of these activities, USP7 appears to be well-positioned to relay signals that regulate the p53-MDM2 pathway (Fig. 2). But it also becomes clear that the possible reduction of p53 levels through viral inhibition of USP7 would likely need to be well-balanced in order not to achieve the opposite. Recent structure–functional studies on the EBV protein EBNA1 have brought to light how this protein may accomplish this and are outlined in the following. The role of the ICP0-USP7 interaction in p53 metabolism during HSV-1 infection has as yet proven complex to delineated (see below).

Epstein–Barr nuclear antigen 1

For a long time, EBNA1 has been known as a regulator of both transcription and replication of the EBV genome, as well as being required for the segregation of EBV genomes with chromosomes during mitosis (Frappier, 2004; Wang and Sugden, 2005). EBNA1 is essential for viral persistence, promotes cellular immortalization and is found consistently expressed in EBV-associated human malignancies including Burkitt's lymphoma, Hodgkin's lymphoma, and nasopharyngeal carcinoma. Functional studies by Saridakis et al. (2005) have indicated that an interaction of transfected EBNA1 with USP7 fosters cellular degradation of p53, presumably by preventing p53 deubiquitination through USP7, and confers apoptosis resistance to UV-irradiated cells. Structural analyses from the laboratories of Shi and Frappier have recently provided insight into the molecular mechanism by which EBNA1 modulates p53 turnover and likely contributes to the anti-apoptotic and survival factor function of EBNA1 in vivo. Crystal structures for short EBNA1, p53, and MDM2 peptides, respectively, in complex with the N-terminal tumor necrosis factor-receptor associated factor (TRAF)-like domain of USP7 show that these three proteins use a consensus tetrapeptide recognition sequence to engage in structurally conserved contacts with the same surface groove of the TRAF-like domain of USP7 (Hu et al., 2006; Saridakis et al., 2005; Sheng et al., 2006). The buried contact surface areas and extent of directed interactions, as well as the measured binding affinities of corresponding peptides to the USP7 TRAF-like domain increase in the order of p53 < MDM2 < EBNA1 (Hu et al., 2006; Sheng et al., 2006). It appears that the competitive binding to USP7 of EBNA1 versus p53 and MDM2 prevents deubiquitination of the lowest affinity binder (p53) sufficiently to reduce cellular p53 levels, while it permits deubiquitination of the intermediate binder (MDM2) to continue, avoiding MDM2 depletion and p53 stabilization, as observed in the USP7 knockout (Fig. 2). Otherwise, i.e., in case MDM2 levels also

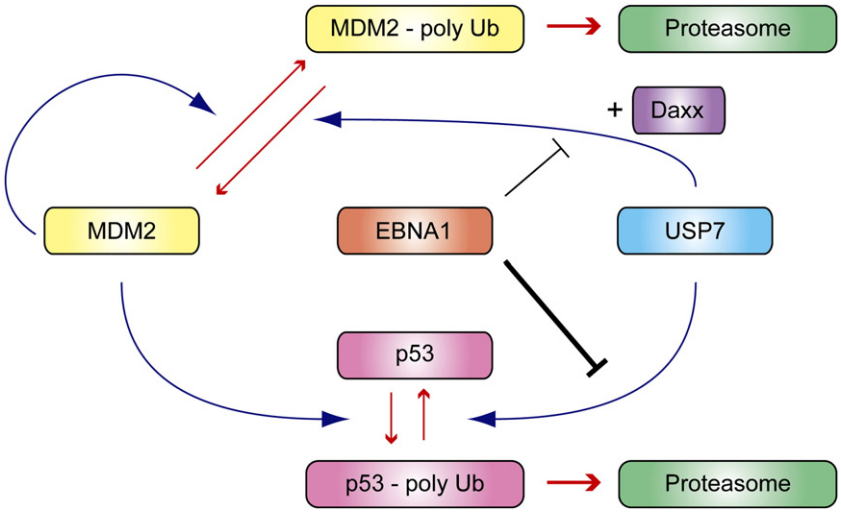

Fig. 2. Model for regulation of the p53-MDM2 pathway by USP7 and interference by EBNA1. The E3 enzyme MDM2 catalyses both autoubiquitination and ubiquitination of p53 leading to proteasomal degradation in both cases. Deubiquitination by USP7 stabilizes p53 and MDM2, with the adaptor protein Daxx directing USP7 to MDM2. Partial reduction of USP7 activity by RNAi destabilizes p53 through reduced deubiquitination. Contrarily, genetic knockout of USP7 increasingly commits MDM2 to proteasomal degradation thus leading to an important reduction in p53 ubiquitination and, indirectly, effective p53 stabilization. The EBV protein EBNA1, MDM2, and p53 compete for same binding site on USP7, with affinities decreasing in this order. Hence, inhibition of USP7 by EBNA1 may exhibit selectivity and contribute twofold to p53 destabilization. By blocking the deubiquitination of p53 more efficiently than the deubiquitination of MDM2, it may allow for sufficient levels of MDM2 that maintain p53 ubiquitination.

dramatically drop in the presence of EBNA1, one of the remaining E3s for p53 (Brooks and Gu, 2006) may play an important role in keeping p53 levels low in EBV-infected cells.

Herpes simplex virus regulatory protein ICP0

The immediate–early HSV-1 gene product ICP0 is required for efficient initiation of lytic infection by stimulating the reactivation of quiescent viral genomes (Hagglund and Roizman, 2004). ICP0 functions as an E3 whose putative in vivo targets include p53. Early in HSV-1 infection, ICP0 associates with nuclear domain 10 (ND10), nuclear substructures that are found juxtaposed to the genomes of many DNA viruses including herpesviruses and adenoviruses (Everett, 2006). ND10 have been implicated in DNA repair, the IFN response, and the regulation of p53 activity (Everett, 2006; Takahashi et al., 2004) and might be preferred sites of transcription and replication of DNA viral genomes (Ching et al., 2005). ICP0 induces the proteasomal degradation of the ND10 organizing promyelocytic leukemia (PML) protein and promotes rapid dispersal of ND10 (Everett, 2006). This has been proposed to alleviate PML protein mediated anti-HSV-1 effects of IFN (Chee et al., 2003). Recent results by Everett et al. (2006), obtained by the use of RNAi to reduce PML levels, indeed argue for the contribution of PML to a cellular antiviral repression mechanism that is countered by ICP0.

Everett and coworkers (1997) had previously demonstrated that ICP0 increases the proportion of USP7 localized to ND10. Contrary to EBNA1, p53, and MDM2, the binding of ICP0 to USP7 was mapped to a domain of unknown structure, located C-terminally to the catalytic core domain of USP7 (Holowaty et al., 2003). Possible effects of ICP0 on p53 metabolism were shown not to depend on the interaction of ICP0 with USP7 but on the particular cell type under investigation (Boutell and Everett, 2004). Instead, the significance of ICP0 binding to USP7 possibly lies in the ability of USP7 to counteract auto-ubiquitination of ICP0 and to protect it from proteasomal degradation (Canning et al., 2004). Although USP7 is conversely ubiquitinated and marked for proteasomal degradation by ICP0, the biological net effect of the reciprocal activities between ICP0 and USP7 is thought to be the stabilization of ICP0 early during HSV-1 lytic infection or reactivation from latency, when ICP0 levels are low (Boutell et al., 2005). Interestingly, the aforementioned USP7-MDM2 adaptor protein Daxx (Tang et al., 2006) is a major ND10 component (Everett et al., 2006), raising the question whether it also has an adaptor function for USP7 in this nuclear compartment.

Viral deubiquitinating activities

Deubiquitinating activities for viral enzymes have directly been demonstrated for the adenovirus protease adenain, SARS-CoV PLpro, and herpesviral UL36^{USP}. Structural aspects of these specificities have been reviewed recently by Sulea et al. (2006). In the following, potential roles for these confirmed viral DUBs are considered against the background of their established functions and properties.

Adenovirus protease adenain

During adenovirus infection, adenain is first made in an essentially inactive form of 23 kDa which localizes to both the cytoplasm and the nucleus (reviewed by Mangel et al., 2003). In the nucleus, binding to viral DNA partially activates the enzyme inside nascent virions, allowing it to cleave an eleven amino acid peptide, named pVIc, from the precursor of the viral DNA binding capsid protein VI (pVI). pVIc binding fully activates adenain, and the peptidic cofactor becomes eventually disulphide-linked to the enzyme. Activated adenain is thought to subsequently complete the proteolytic maturation of altogether six virus capsid precursor proteins inside the virion. The stepwise activation of adenain by viral DNA and pVIc prevents precursor protein cleavage before virion assembly and the generation of immature capsids. Maturation of the capsid proteins is important for their ability to promote low-pH activated endosomal lysis and cytoplasmic entry of viral capsids during the next infection cycle (Cotten and Weber, 1995).

Cytoplasmic adenain is believed to contribute to cell lysis and release of virions by the cleavage of cytoskeletal proteins, including cytokeratin 18 and actin. In contrast to nuclear adenain, the cytoplasmic form has no access to either of the two capsid bound viral cofactors, viral DNA and pVIc. Instead, the C-terminal sequence of actin, which is highly homologous to pVIc, efficiently replaces pVIc acting as a cellular cofactor (Brown and Mangel, 2004). Within the extracellular virus, adenain resides in an oxidized and dormant state, but becomes activated again up on infection and re-entry into a reducing cellular environment. Adenain eventually participates in the final steps of the viral uncoating program, i.e., dissociation of the viral DNA from the capsid at the nuclear pore complex, by the digestion of pVI (Greber et al., 1996).

Using a biotinylated form of the specific DUB inhibitor Ub-aldehyde as a probe, Balakirev et al. (2002) retrieved adenain from a lysate of adenovirus-infected HeLa cells. They showed that the enzyme accounted for a time-dependent increase in global and, especially, nuclear deubiquitinating activity in late phase adenovirus-infected cells compared to mock-infected cells, as judged by the decline of the Ub conjugate pool analyzed by Western blotting with anti-Ub antibodies (Balakirev et al., 2002). Additionally, the authors overexpressed in the same cell line hexahistidine-tagged Ub together with either adenain or an autocleavable fusion of the enzyme to its activating peptide cofactor pVIc, followed by enrichment of Ub conjugates from cell lysates over a metal affinity resin. Anti-Ub Western blotting of the resulting fractions demonstrated, again, an overall reduction in Ub conjugates with both versions of the transfected enzyme. Enzymatic in vitro assays with recombinant purified adenain and substrate proteins, and chemically synthesized pVIc peptide showed that adenain, indeed, exhibits debranching activity against lysine-48 linked polyubiquitin and could also process the ISG15 precursor protein, but not a fusion of the yeast SUMO homolog Smt3 to the green fluorescent protein. This specificity, seemingly, conflicts with the structural classification of adenain as a member of the ULP family of deSUMOylating enzymes (Balakirev et al., 2002) but agrees

well with the molecular binding site features of the enzyme (reviewed by Sulea et al., 2006).

The described prevalence of adenain-dependent deubiquitination of nuclear proteins in infected cells (Balakirev et al., 2002) is in accordance with the presence of adenain in this subcellular compartment, where it is involved in virion maturation. At the same time, however, activation of nuclear adenain is thought to occur only inside the nascent virion particle, with about 70 adenain-pVIc complexes remaining incorporated per particle (Mangel et al., 2003). While this may suggest that virion resident adenain deubiquitinates capsid proteins, no change in the overall pattern of the anti-Ub staining in comparison to nuclei of mock-infected cells was observed (Balakirev et al., 2002), indicating that the overall decline in nuclear Ub conjugates, for the most part, reflected deconjugation of cellular proteins. This could mean that activated adenain partially escapes from nascent virions and acts on proteins in the surrounding nucleoplasm (the possibility that nuclear adenain is activated in a virion independent manner is considered below).

As already mentioned, besides HSV-1, adenoviruses belong to the DNA viruses whose genomes associate with ND10. Interestingly, ND10 in turn are associated with nuclear aggresomes, sites that recruit chaperones, Ub, and proteasomes and that may be specialized in protein degradation (reviewed by Wileman, 2006). Moreover, ND10 have been proposed to present passageways for proteins, including viral proteins that are destined for proteasomal degradation in the proximity of ND10 (Bailey and O'Hare, 2005; Hay, 2005). In this light, it is tempting to speculate that deubiquitination by adenain safeguards the delivery of viral proteins to nascent capsids by protecting them from proteasomal degradation at close-by aggresomes, much as cellular USP7 is thought to stabilize ICP0 of HSV-1 (Boutell et al., 2005).

It is also noteworthy that the plasmid mediated overexpression of adenain in HeLa cells, both with and without its peptidic cofactor, resulted in an overall loss of cellular Ub conjugates (Balakirev et al., 2002). The strong dependence of adenain catalytic activity on cofactor complex formation and the ability of the C-terminal sequence of the traditionally cytoplasmic protein actin to act as a cofactor (Brown and Mangel, 2004) may suggest that predominantly cytoplasmic adenain activity was detected in this experiment. Alternatively, a notable fraction of adenain may have entered the nucleus together with actin derived peptide cofactor. Actin itself has also been identified in the nucleus as a component of protein complexes active in various aspects of gene transcription (Grummt, 2006; Miralles and Visa, 2006). Actin may therefore also be involved in a virion independent stimulation of adenain activity in the nucleus.

Severe acute respiratory syndrome coronavirus papain-like protease

After entry of the coronaviral single-stranded positive-sense RNA genome into the cytoplasm, the viral replicase gene is translated directly from it. Two coronaviral proteases, 3C-like protease (3CLpro) and PLpro, are part of the replicase polyprotein, the precursor of the altogether sixteen non-structural proteins (nsps) that form the viral RNA replication complex (Snijder et al., 2003; Thiel et al., 2003). 3CLpro, on the one hand, is contained in nsp5, and after autocleavage, releases all downstream replicase subunits. PLpro, on the other hand, originally referred to a domain of around 24 kDa within nsp3, whose boundaries are defined by homology to the papain-fold (Herold et al., 1999). PLpro processes the amino-proximal nsps (Harcourt et al., 2004). In accordance with previous structural bioinformatics prediction (Sulea et al., 2005), the crystal structure of SARS-CoV PLpro recently established the enzyme's membership in the USP family of DUBs (Ratia et al., 2006). Additionally, it revealed an unexpected Ub-like domain at the N-terminus of the PLpro catalytic core domain. Ub-like domains are defined by a common β-grasp three-dimensional structure (Kiel and Serrano, 2006). Within the multidomain nsp3, PLpro is further preceded by an acidic sequence forming the N-terminus of nsp3, a macro-domain with ADP-ribose-1″-phosphatase activity, which is potentially involved in viral RNA modification (Saikatendu et al., 2005), and a so-called SARS unique domain (Snijder et al., 2003). PLpro is followed by a hydrophobic domain with putative transmembrane regions (Harcourt et al., 2004). PLpro cotranslationally liberates nsp1 to 3 in this order (Harcourt et al., 2004). All three cleavage products become part of the replication complex, which is found bound to double-membrane vesicles that are characterized by autophagosome markers (Prentice et al., 2004). A more recent ultrastructural study, however, points to the endoplasmic reticulum as the direct origin of the membranes associated with SARS-CoV replication complex, including nsp3 (Snijder et al., 2006).

SARS-CoV PLpro, which now refers to the enzyme's catalytic core domain plus the N-terminal Ub-like domain, was recombinantly expressed and purified (Barretto et al., 2005; Lindner et al., 2005). As predicted based on the similarity of its catalytic core domain to the corresponding domain of USP7 (Sulea et al., 2005), SARS-CoV PLpro displays DUB activity. Specifically, it debranches lysine-48 polyubiquitin chains, very efficiently hydrolyzes the general DUB substrate Ub-7-amino-4-methylcoumarin (Ub-AMC) (Barretto et al., 2005; Lindner et al., 2005), and exhibits ISG15 precursor processing activity (Lindner et al., 2005). Although this proves the enzyme's proficiency as a DUB in vitro, it is not clear whether SARS-CoV PLpro can gain access to potential deubiquitination targets, other than replicase polyprotein sequences themselves, during its synthesis as part of the replicase polyprotein, cotranslational autoprocessing, and incorporation into the membrane bound replication complex. It is noteworthy that the synthesis of both negative- and plus-strand coronavirus RNA requires ongoing viral protein production (Kim et al., 1995; Perlman et al., 1986; Sawicki and Sawicki, 1986), and it is conceivable that concomitant deubiquitination by SARS-CoV PLpro protects replicase subunits against proteasomal degradation.

Herpesvirus UL36^{USP}

Prompted by the known interaction of ICP0 with cellular USP7, Ploegh and coworkers made use of the active-site

directed probe HAUbVME, mentioned above, to monitor DUB activity in lysates of primary fibroblasts infected with HSV-1 (Kattenhorn et al., 2005). A major HAUb-adduct corresponding to an ~47-kDa protein occurred late in infection and persisted. The protein was identified as N-terminal fragment of the essential large (3164 amino acid residues) tegument protein UL36 (also called VP1/2 or ICP1/2). Further labeling attempts with similar Ubl probes, and in vitro enzymatic assays using a 533 residues recombinant N-terminal fragment of UL36, established that the enzyme, baptized UL36^{USP}, is specific for Ub, but exhibits relatively low catalytic efficiency. Recombinant HSV-1 UL36^{USP} disassembled lysine-48 but not lysine-63 polyubiquitin chains which may implicate the enzyme in protein stabilization (Kattenhorn et al., 2005).

Sequence alignment of UL36 homologs from α-, β-, and γ-herpesvirus genomes identified a putative cysteine box around the HAUbVME reactive cysteine residue, and a histidine box located around 130 amino acids further downstream (Kattenhorn et al., 2005). Although none of the UL36^{USP} sequences shows similarity to any known DUB, the conservation of cysteine box and histidine box motifs is characteristic of cysteine protease DUB families (Amerik and Hochstrasser, 2004). In addition to HSV-1 (α-subfamily), analogous labeling experiments confirmed the presence of DUB activity and specificity for Ub in recombinant UL36^{USP} from EBV (γ-subfamily), and MCMV and HCMV (β-subfamily) (Schlieker et al., 2005; Wang et al., 2006). The sequence of the recombinant UL36^{USP} variant examined for EBV extended only little beyond the histidine box motif, demonstrating that an N-terminal UL36 fragment of just under 22 kDa carries the UL36^{USP} specificity (Schlieker et al., 2005).

Wang et al. (2006) detected HAUbVME labeling of full-length UL36 in HCMV, simian CMV (SCMV) and HSV-1-infected fibroblasts. They also reported the detection of the 47-kDa fragment of UL36 for HSV-1 previously described by Kattenhorn et al. (2005) but ascribed its occurrence to uncontrolled proteolysis (Wang et al., 2006). The authors went ahead to confirm catalytic activity in isolated wild-type and mutant HCMV extracellular particles. Mutation of either the putative catalytic cysteine or histidine residue abolished catalytic activity, identifying UL36^{USP} as the sole source of DUB activity in the virion. For additional mutants of several conserved cysteine box and histidine box residues, the spread and development of cytopathic effects as well as virus yields in virus-infected cells were wild-type like. Only the respective cysteine and histidine mutants gave notably lower virus yields and delayed the development of cytopathic effects. However, the cysteine mutation caused no apparent changes during cell infection as judged by electron microscopy. Overall, this indicates that UL36^{USP} is important for optimal HCMV replication but is not essential in cell culture (Wang et al., 2006).

So far, we can only speculate on possible roles for UL36^{USP} considering the known implications of the large tegument protein UL36, which according to earlier studies shows equal cytoplasmic and nuclear distribution (McNabb and Courtney, 1992). UL36 was demonstrated to interact with a region of the viral α sequence, which is required for cleavage and packaging of the viral genome in the nucleus (Chou and Roizman, 1989) into icosahedral capsids (see Pomeranz et al., 2005, for a review of the α-herpesvirus life cycle). Studies of α-herpesvirus-infected cells using microscopic techniques coupled with protein labeling methods have led to two different theories that aim at explaining how nucleocapsids exit the nucleus, obtain the viral tegument, and acquire an envelop. They imply different pathways for the capsid attachment of UL36, which is thought to form the innermost, capsid-proximal layer of the tegument. According to the more prevalent of the two theories (reviewed by Mettenleiter, 2004; Mettenleiter et al., 2006), viral capsids reach the cytoplasm by successive envelopment and de-envelopment at the inner and outer membrane of the nuclear envelope, respectively. Primary attachment of UL36 in the cytoplasm then affords further assembly of inner tegument proteins. The inner tegument subsequently coalesces with the outer tegument, which assembles independently at future sites of budding into the exocytic pathway, likely into the trans-Golgi network (Mettenleiter, 2006). The alternative theory (Leuzinger et al., 2005; Wild et al., 2005) assumes a dual pathway for nuclear egress of capsids. Capsids may either undergo nuclear envelopment followed by intraluminal transport to the Golgi, or they may leave the nucleus directly through dilated nuclear pores and afterwards bud from the cytoplasm into compartments of the exocytic pathway. Here, the deposition of tegument is thought to occur during the budding at the inner nuclear membrane or at cytoplasmic membranes (Leuzinger et al., 2005; Wild et al., 2005), hence presumably involving nuclear or cytoplasmic UL36, respectively. It has however to be noted that UL36 has not yet been detected as a component of virions inside the lumen of the nuclear envelope (Mettenleiter and Minson, 2006). Wherever herpesvirus tegumentation occurs, tegument proteins are known to engage in many complex protein–protein interactions (reviewed by Mettenleiter, 2006). Yeast two-hybrid analyses suggest that UL36 uses a domain downstream of UL36^{USP} to directly bind to UL37, another inner tegument protein (Klupp et al., 2002; Vittone et al., 2005). Fusion of Golgi derived vesicles with the plasma membrane eventually releases infectious virions from the cell.

After infection by fusion of the viral envelope with the cell membrane, UL36 initially stays associated with the capsid. UL36 is in fact emerging as a main candidate among herpesviral proteins that may dock incoming capsids to the dynein motor during transport along microtubules (MTs) to the nuclear pores (Granzow et al., 2005) and during retrograd axonal transport (Antinone et al., 2006; Luxton et al., 2005). In this regard, it is interesting to note that the minus end-directed transport of misfolded proteins to the microtubule-organizing center during aggresome formation was shown to require the interaction of the dynein motor with the cellular DUB ataxin-3 (Burnett and Pittman, 2005). As UL36^{USP}, ataxin-3 debranches lysine-48 polyubiquitin chains, but possible targets of this activity during retrograd transport are unknown. Last of all, a temperature sensitive mutation in HSV-1 UL36 impeded the release of viral DNA from the capsid into the nucleus at the nuclear pore (Batterson et al., 1983).

Taken together, during herpesvirus infection UL36 reportedly localizes to the nucleus, the cytoplasm, and the lumen of

the nuclear envelope as well as the exocytic pathway where it is eventually part of the infectious virion, whose yield is reduced by mutational inactivation of UL36^{USP}. It remains speculative at this point whether deubiquitination of viral or cellular proteins by its UL36^{USP} domain plays part in any of the processes that have been associated with the presence of UL36 in these locations, i.e., nucleocapsid formation, tegumentation, budding, viral egress from and entry into cells, MT-dependent capsid transport, and nuclear entry of the viral genome.

Viral ubiquitin and ubiquitin-like sequences

The genomes of some viruses encode Ub or Ub-like sequences. The baculovirus *Autographa californica* nucleopolyhedrovirus, for example, expresses a viral Ub precursor protein during the late phase of infection (Guarino, 1990). Although non-essential for replication in cell culture, the gene is required for optimal virus production (Reilly and Guarino, 1996). The baculoviral Ub is functional in protein conjugation in vitro but inhibits the formation of more extended lysine-48 linked polyubiquitin chains necessary for proteasomal targeting (Haas et al., 1996). The authors speculated that this protects otherwise short-lived viral proteins from proteasomal degradation.

Viruses also express Ub or Ub-like sequences as part of multidomain polyproteins. Intriguingly, Ub and Ubls including NEDD8, SUMO, and Atg8 homologs represent the most frequent inserts in the polyprotein of several strains of bovine viral diarrhea virus (BVDV), where their presence is associated with a viral cytopathogenic phenotype (Baroth et al., 2000; Meyers et al., 1998; Qi et al., 1998; Tautz et al., 1993). In fact, these inserts function as polyprotein processing signals that allow cellular proteases, presumably DUBs, to process the polyprotein at positions corresponding to the precursors processing sites of the respective cellular Ub or Ubl sequences. In the case of an Atg8 sequence insert in the BVDV isolate JaCP, the processing enzyme was indeed identified as a specific cellular DUB, namely, autophagin-1 (ATG4B) (Fricke et al., 2004).

Compared to Ub and Ubls, the already mentioned N-terminal domain of SARS-CoV PLpro (Ratia et al., 2006) for example represents a different but not less common type of Ub-like domain (Kiel and Serrano, 2006). Here, the lack of a C-terminal recognition sequence motif precludes proteolytic processing by DUBs and subsequent conjugation to other molecules. Nevertheless, such intrinsic Ub-like domains also serve in mediating protein–protein interactions in a variety of multidomain proteins (see Kiel and Serrano, 2006, and references therein). These include players of the UPS, such as specialized Ub receptors (Elsasser and Finley, 2005), and last but not least DUBs (Nijman et al., 2005; Zhu and Sulea, unpublished data). The domain arrangement in SARS-CoV PLpro is in fact reminiscent of USP14 and its homologs, where the Ub-like domain likewise precedes the catalytic core domain. USP14 and its yeast homolog Ubp6 both bind to the regulatory subunit of the proteasome via their Ub-like domain which greatly stimulates their catalytic activities (Hu et al., 2005; Schmidt et al., 2005). Comparison of the crystal structures for the free and Ub bound catalytic core domain of USP14 suggests that the enzyme is activated by conformational translocation of two enzyme surface loops, which block access of the Ub C-terminus to the active site in the free enzyme (Hu et al., 2005). Proteasome binding may promote this activation step. USP14 was further shown to debranch lysine-48 linked polyubiquitin chains from the distal end (Hu et al., 2005). USP14/Ubp6 is thought to prevent the translocation of Ub from incoming substrates into the inner core particle of the proteasome and to contribute to the homeostasis of the cellular pool of free Ub (Schmidt et al., 2005). It is tempting to speculate that the Ub-like domain of SARS-CoV PLpro may similarly anchor the enzyme to a larger protein complex. There is, however, no indication from the available crystal structure of the free enzyme, like for USP14, that its catalytic activity may require activation (Ratia et al., 2006).

Conclusions

The binding of ICP0 and EBNA1 to different domains of the cellular DUB USP7 contributes to the maintenance of a productive life cycle for HSV-1 and the establishment of latency for EBV, respectively, representing the only well established examples of viral interference with deubiquitination so far. Interestingly, however, infection by EBV, HCMV, and HPV modulates the activities of several cellular DUBs (Ovaa et al., 2004; Rolén et al., 2006; Wang et al., 2006) the significance of which remains to be established. DUBs, such as USP9X, for instance, might be involved in β-catenin stabilization in latency type III EBV-transformed B cells.

It will further be important to establish the roles of viral deubiquitinating activities. Like USP7, the three recently confirmed viral DUBs, adenain, SARS-CoV PLpro, and herpesvirus UL36^{USP}, all exhibit lysine-48 linked polyubiquitin debranching activities. It is conceivable that viruses avail themselves of this activity in order to stabilize viral gene products or host cell proteins whose proteasomal degradation promotes anti-viral responses such as IκB (Evans, 2005). The ability of adenain and SARS-CoV PLpro to additionally cleave the ISG15 precursor protein has led to the speculation that these enzymes mimic cellular USP18 (Sulea et al., 2006), a DUB with delSGylating activity and a negative regulator of the IFN response (Dao and Zhang, 2005). Recent data by Zhang and coworkers (Malakhova et al., 2006) revealed, however, that USP18 attenuates JAK-STAT signaling, and thereby the type 1 IFN response, in a non-enzymatic manner, i.e., by directly competing with JAK1 for binding to the IFNAR2 subunit of the type 1 IFN receptor. As for SARS-CoV PLpro, the importance of the deubiquitinating activity of USP18 remains to be determined.

Table 1 summarizes the demonstrated and potential roles in viral infection of both cellular and viral DUBs discussed thus far. The review of the possible subcellular sites of action for the three confirmed viral DUBs presented here suggests that their deubiquitinating activities could, similar to the polyprotein processing activities of adenain and SARS-CoV PLpro, also contribute to more basic viral needs such as viral genome replication and packaging, or viral egress and entry. Similar to adenain (Greber et al., 1996), UL36^{USP} may for example exhibit digestive activity during the release of viral DNA from

Table 1
Demonstrated and potential roles of DUBs in virus infection

DUB	Roles	References
	Demonstrated roles [a]	
Adenovirus protease adenain	Release of its own activating peptidic cofactor, maturation of capsid precursor proteins, promotion of cell lysis by cleavage of cytokeratin 18 and actin, support of viral uncoating, and DNA release by capsid protein digestion at the nuclear pore	Mangel et al. (2003)
SARS-CoV PLpro	Processing of nsp1 to 3 from the viral replicase polyprotein	Harcourt et al. (2004)
	Potential roles [b]	
USP9X	Stabilization of β-catenin in EBV-infected B cells	Shackelford et al. (2003)
USP7	Impaired stabilization of p53 in the presence of EBNA1 of EBV leading to apoptosis avoidance	Hu et al. (2006); Saridakis et al. (2005); Sheng et al. (2006);
	Stabilization of ICP0 of HSV-1 supporting reactivation of quiescent viral genomes and lytic infection	Boutell et al. (2005); Canning et al. (2004)
Adenovirus protease adenain	Stabilization of virion proteins, negative regulation of the IFN response [c]	Balakirev et al. (2002)
SARS-CoV PLpro	Stabilization of viral polyprotein sequences, negative regulation of the IFN response [c]	Lindner et al. (2005); Barretto et al. (2005)
Herpesvirus UL36^{USP}	Stabilization of viral proteins	Kattenhorn et al. (2005)

[a] Among the viral enzymes with deubiquitinating activity, proteolytic roles during virus infection have only been demonstrated for the adenovirus protease adenain and SARS-CoV PLpro. In both cases, however, they involve hydrolysis of polypeptides at regular peptide bonds by these enzymes and not isopeptide bond cleavage.
[b] DUBs have been proposed to benefit viral infection by stabilizing viral gene products or, selectively, cellular proteins by protecting them from proteasomal degradation.
[c] The delSGylating activities of adenain and SARS-CoV PLpro may mimic USP18, a demonstrated negative regulator of the interferon response (Dao and Zhang, 2005). Yet, an isopeptidase independent mechanism for this function of USP18 was discovered recently (Malakhova et al., 2006), and the significance of its de-ISGylating activity remains unclear.

the capsid, explaining the prevention of this step in a temperature sensitive mutant of HSV-1 UL36 (Batterson et al., 1983).

The example of UL36^{USP} of HCMV has shown that infection of cell cultures with mutant viruses does not necessarily cause a tangible phenotype. For adenain and SARS-CoV PLpro it may prove difficult to dissect their essential functions in polyprotein cleavage from the significance of their DUB activities. For SARS-CoV, the actual importance of the nsp1/2, nsp2/3, and nsp3/4 cleavages carried out by the PLpro is still not clear. Nevertheless, in cell culture infection nsp1 appears to suppress host gene expression by promoting host mRNA degradation (Kamitani et al., 2006), and nsp2, although dispensable, was shown to be required for optimal virus replication (Graham et al., 2005). In order to, however, more comprehensively study potential roles of cellular and viral DUBs in virus pathogenicity, e.g., through the modulation of the host antiviral immune responses, animal models of viral infection are needed (cf. Arrode and Davrinche, 2003; Cantin et al., 1999; Cinatl et al., 2005; Jogler et al., 2006).

The acquisition of Ub and Ubl sequences by strains of BVDV as polyprotein processing signals is intriguing and suggests a possible scenario for the evolutionary origin of viral DUBs. In bovine cells, the essential processing of the Atg8 insert in the BVDV isolate JaCP was demonstrated to be carried out by the DUB ATG4B. This processing step also occurred when an Atg8 containing BVDV polyprotein sequence was recombinantly expressed in avian, fish, and insect cells, as well as in a rabbit reticulocyte lysate (Fricke et al., 2004). The utilization of a Ub or Ubl sequence as polyprotein processing signal may present an advantageous viral strategy because a newly infected host cell is very likely to already express a DUB that can perform the processing reaction. It is tempting to speculate that Ub or Ubl sequence containing viruses originally have happened to insert sequences of cellular DUBs into their genomes as functional protein processing enzymes, thereby becoming independent of the cellular enzymes. As a fitness advantage, this could have enhanced the viral host cell spectrum.

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