Herpesvirus-encoded Deubiquitinating Proteases and Their Roles in Regulating Immune Signaling Pathways

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Viruses interact with the host ubiquitination system in a variety of ways. Viral proteins are often a substrate for ubiquitination, which leads to proteasomal degradation. Viruses also have functions to modify the cellular ubiquitination machinery. Recently, deubiquitinating protease (DUB) activity has been found in many viral proteins. In herpesviruses, the DUB domain is found within the large tegument protein, which is conserved in all members of the herpesvirus family. Although a limited number of viral and cellular targets have been identified to date, accumulating evidence shows that herpesviral DUBs may primarily target key cellular regulators of immune signaling pathways to promote viral replication. In this review, we summarize the recent findings on viral DUBs. In particular, we focus on the herpesviral DUBs and their targets, and discuss their potential roles in the regulation of immune signaling pathways.

Key Words: Ubiquitin, Deubiquitinating protease, Herpesvirus, Immune signaling

Introduction

Ubiquitin is a 76-amino acid molecule that is conjugated to a lysine residue of the target protein. Covalent modification of a protein by ubiquitin (ubiquitination) occurs through an enzymatic cascade comprised of a single ubiquitin-activating enzyme (E1), dozens of ubiquitin-conjugating enzymes (E2s), and hundreds of ubiquitin E3 ligases (E3s) (1). Ubiquitin is synthesized as a precursor, and its carboxy-terminal end is removed by specific ubiquitin carboxy-terminal hydrolases (UCHs). Ubiquitin is first activated by the E1 enzyme in an ATP-dependent manner. This process consists of the formation of ubiquitin-

adenylate compound and then the binding of ubiquitin to an E1 cysteine residue in a thioester linkage. Ubiquitin is then transferred to an E2 cysteine residue. E3 ligases carry out the transfer of ubiquitin to the substrate protein by one of two different mechanisms. The HECT-domain family of E3 receives ubiquitin from E2 to its active site cysteine residue, and subsequently transfers the ubiquitin to a lysine residue on the substrate. The other E3 family interacts with both E2 and the substrate tightly and helps transfer ubiquitin from E2 directly to the substrate. Ubiquitin modification can be reversed by ubiquitin-specific proteases (USPs), which catalyze the hydrolysis of the isopeptide bond between the C-terminal glycine residue of ubiquitin and a lysine residue in the substrate. Most of the deubiquitinating proteases

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(DUBs), which can have UCH or USP activity or both, are cysteine proteases characterized by a cysteine-histidine-aspartic acid catalytic triad (2).

The attachment of ubiquitin monomers and polymers to their target protein plays a central role in many cellular processes, such as immune response, cell cycle regulation, apoptosis, protein degradation, and signal transduction (3, 4). In particular, ubiquitination has received much attention for its role in the regulation of innate immune signaling pathways, including the nuclear factor-kappa B (NF-κB) and interferon (IFN) signaling. During most viral infections, pathogen-associated molecular patterns (PAMPs) are recognized by pathogen recognition receptors (PRRs) and this recognition activates canonical and non-canonical NF-κB signaling pathways as well as the type I IFN signaling pathway. Polyubiquitination through lysine 11-, 63- and 48-linkages, attachment of linear ubiquitin polymers, and deubiquitination by cellular DUBs are key components in the regulation of these signaling pathways (5, 6).

Given the important roles of the ubiquitination and deubiquitination systems in nearly all cellular processes, viral DUBs appear to play a key role at several steps of the viral replication cycle. Here, we review the recent findings on viral DUBs, with a focus on herpesviral DUBs and their targets. The possible role of herpesviral DUBs in the regulation of immune signaling pathways is discussed.

Virus-encoded deubiquitinating proteases

Viruses interact with the host ubiquitination system in a variety of ways. Many viruses encode proteins that can modify the ubiquitination machinery, often altering substrate specificity to favor viral replication. Viral proteins themselves can be directly modified by ubiquitin. Moreover, some viruses encode a ubiquitin E3 ligase or a DUB (7~9). Recently identified viral DUBs include the adenovirus protease adenain, severe acute respiratory syndrome-associated coronavirus (SARS-CoV) papain-like protease (PLpro), arterivirus nonstructural protein 2 (nsp2), bunya-virus L proteins, and the large tegument proteins encoded by herpesviruses. The adenovirus protease adenain is respon-

sible for a general decrease in the pool of ubiquitinated proteins in the infected cell (10). The cellular substrates of adenain have not been identified. SARS-CoV PLpro, which was proposed to counteract the host innate immune response, has been shown to possess DUB activity *in vitro* (11). The arterivirus nsp2 and bunyavirus L proteins are ovarian tumor (OTU) domain proteases, which are also found in bacteria and eukaryotes. The viral OTU domain proteases show much broader target specificity than their host counterparts, and are capable of inhibiting NF-κB-dependent signaling, probably through their DUB activity. Expression of the OTU domains of nsp2 and L proteins inhibits the translocation of the p65 subunit of NF-κB to the nucleus (12). Several members of the herpesvirus family also encode the DUB domain-containing tegument proteins that are involved in the regulation of cellular innate immune responses, as well as entry, assembly, and release of virus particles (see below).

Deubiquitinating proteases encoded by human herpesviruses

The herpesvirus family consists of large enveloped viruses harboring an icosahedral capsid and a double-stranded DNA genome. Many herpesviruses can establish persistent and latent infection in healthy hosts. Human herpesviruses cause a wide spectrum of disease during reactivation and, in some cases, upon primary infection. The herpesvirus DUB was first discovered as an N-terminal fragment of the 336-kDa UL36 tegument protein (also known as VP1/2) of herpes simplex virus type-1 (HSV-1) (13). This DUB domain is found to be conserved in the UL36 equivalents of other herpesviruses (14). Interestingly, the herpesviral DUBs bear no structural homology to known eukaryotic DUBs, although the key amino acid residues in the active site are highly conserved (8) (Fig. 1). Subsequently, DUB activity was discovered in other herpesviral proteins: the M48 protein of mouse cytomegalovirus (MCMV) (14), the UL48 protein of human cytomegalovirus (HCMV) (15), the UL36 proteins of Marek's disease virus (MDV) (16) and pseudorabies virus (PRV) (17), the BPLF-1, BSLF-1, and

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HSV-1	UL36	26	NQFAPD-LEPGGSVSCMRSSLSFLSLIFDVG	56 ... 153	TIGPLGMGLFRDGDTAYLFDPHGLPE	178
HSV-2	UL36	26	NQFATD-LEPGGSVSCMRSSLSFLSLLFDVG	56 ... 153	TIGPLGMGYFRDGDTAYLFDPHGLPA	178
VZV	ORF22	18	NQFDGA-LGPASGLSCLRTSLSFLHMTYAHG	48 ... 149	TVDSTGMAIYRQGNISFIFDPHGHGT	174
HCMV	UL48	9	HQGDIARFGARAGNQCVCNGIMFLHALHLGG	40 ... 141	TVNSLARAVVFTEDHMLVFDPHSSAE	166
HHV-6	U31	9	NQNDSK-YGPRAGKQCMSNSFSFLHTVYLNG	39 ... 137	TIGVLSRALFFLNNTLYLFDSHPTER	162
HHV-7	U31	9	NQNDPK-YGPRAGKQCMSNCFSFLHTVYLNG	39 ... 136	TIGVMARAIFIVDELFYLFDSHASDT	161
EBV	BPLF1	47	NQAHCK-FGRFAGIQCVSNCVLYLVKSFLAG	77 ... 174	CDIYAGAIIIETDGSFYLFDPHCQKD	199
KSHV	ORF64	15	HQANCI-FGEHAGSQCLSNCVMYLASSYYNS	45 ... 141	CQSKAGAIVVK-DKTYYMFDPHCIPN	165

Figure 1. Amino acid alignment of the conserved catalytic active site region of the DUB domains from human herpesviruses. Amino acids in the conserved region surrounding the active site cysteine residue in the DUBs of human herpesviruses are aligned. Black boxes indicate conserved residues, and grey boxes indicate similar residues. The cysteine, aspartic acid, and histidine residues in the catalytic triad are indicated by asterisks.

BXLF-1 proteins of Epstein-Barr virus (EBV) (18), and the ORF64 proteins of Kaposi's sarcoma-associated herpesvirus (KSHV) (19) and murine gamma-herpesvirus 68 (MHV68) (20).

1. HSV-1 UL36

HSV-1 is a representative member of the alpha-herpesvirus subfamily. The large tegument protein UL36 of HSV-1 is a multifunctional protein that plays crucial roles in virus entry (21), microtubule transport of capsids (22), release of the viral genome into the nucleus (23, 24), and virion maturation and egress (25, 26). In virus-infected cells, the N-terminal fragment of UL36 (called UL36^{USP}), which is produced by cleavage of the full-length UL36 protein, is detectable in the late phase of viral infection and exhibits DUB activity. UL36^{USP} cleaves both K48 and K63 polyubiquitin chains (13).

Ubiquitination has a crucial role in regulating the retinoic acid-inducible gene 1 (RIG-I) signaling pathway. Short viral double-stranded RNA or 5'-triphosphate RNA is selectively recognized by RIG-I. This induces ATP-dependent conformational changes in RIG-I that allow dimerization and association with mitochondrial antiviral signaling adaptor (MAVS), a downstream adaptor molecule. Two ubiquitin E3 ligases, tripartite motif-containing protein 25alpha (TRIM25α) and RING finger protein 135 (RNF135), catalyze K63-linked polyubiquitination of RIG-I, enhancing the binding of RIG-I to MAVS (27, 28). Following MAVS engagement, it recruits downstream signaling complexes

that lead to the activation of IFN-regulatory factors (IRFs) and NF-κB. One signaling complex is mediated by recruitment of tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3). K63-linked polyubiquitination of TRAF3 forms a scaffold for the assembly of a signaling complex composed of TANK-binding kinase 1 (TBK1) and IKKε, leading to phosphorylation of IRF3 and subsequent production of the type I IFNs (29∼33). Recently, Wang *et al*. reported that UL36 inhibits the production of IFN-β by removing polyubiquitin chains on TRAF3 (34) (Fig. 2). A virus encoding a mutant UL36, in which the active site cysteine residue is replaced by alanine (C40A), failed to deubiquitinate TRAF3, thereby inducing more IFN-β and IFN-stimulated gene (ISGs) expression than the wild-type virus. Therefore, the tegument UL36 protein appears to contribute to the inhibition of RIG-I-mediated antiviral responses.

2. HCMV UL48

In HCMV, which belongs to members of the beta-herpesvirus subfamily, the largest tegument protein UL48 is the homolog of HSV-1 UL36. The UL48 DUB is identified using a suicide substrate probe specific for ubiquitin-binding cysteine proteases in virus-infected cells (15). This DUB activity is mapped to the first 359 amino acids of the N-terminal region of UL48 (35). The bacterially purified UL48 DUB is shown to contain UCH activity that is specific for ubiquitin, but not for other ubiquitin-like proteins such as ISG15, SUMO, or Ufm1. Analysis of the
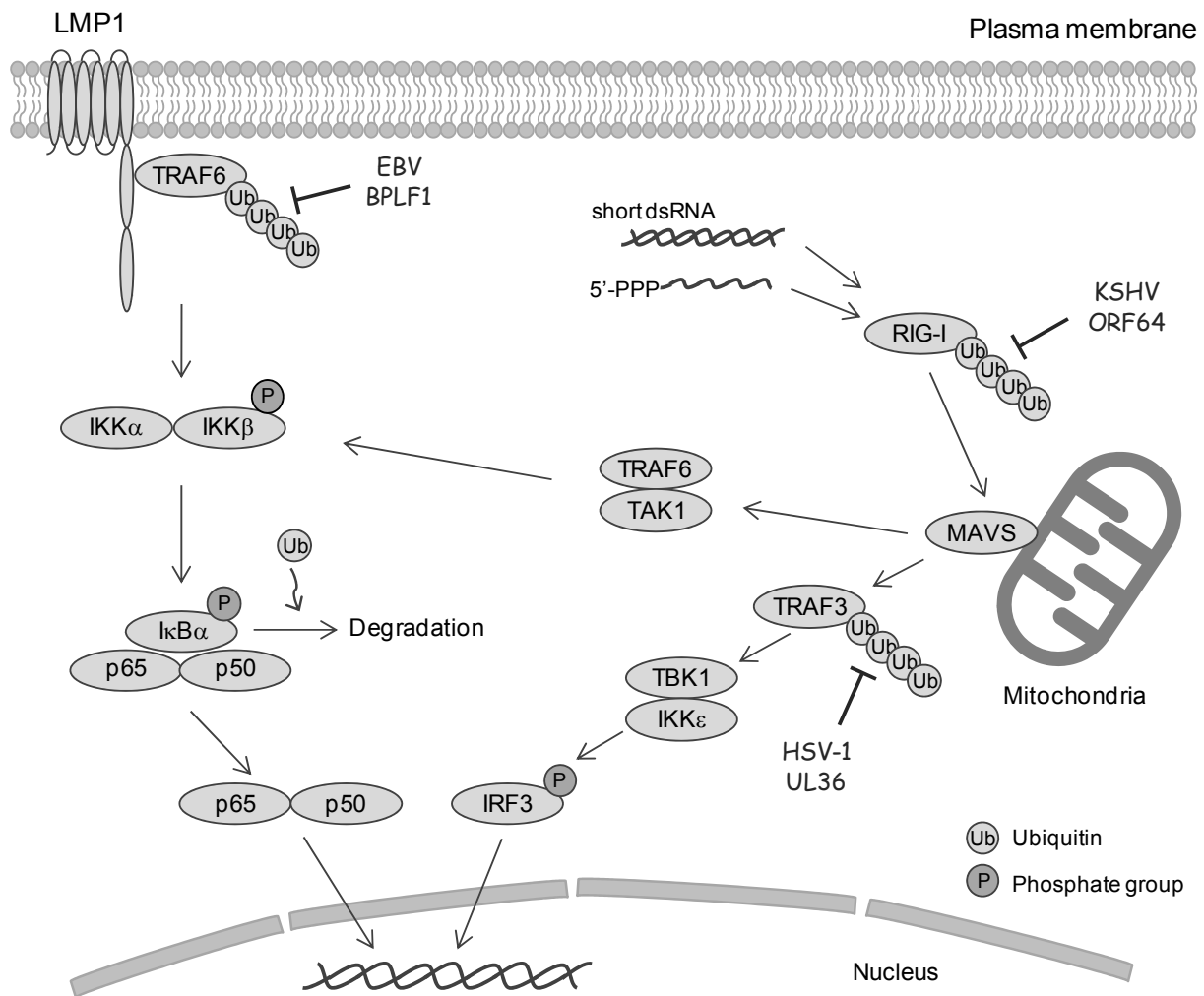
Figure 2. The NF-κB and IRF3 signaling pathways and the targets of herpesvirus DUBs. In EBV latent infection, NF-κB is activated by viral LMP1. TRAF6 associates with LMP1 and is constitutively polyubiquitinated. Activation of NF-κB confers cell survival and inhibits the spontaneous induction of lytic replication. Once lytic replication is induced, BPLF1 deubiquitinates and inactivates TRAF6 to block NF-κB signaling, promoting efficient viral genome replication. RIG-I is a cytosolic RNA sensor that recognizes viral RNA. Ubiquitination of RIG-I promotes its association with MAVS, and ubiquitination of TRAF3 mediates recruitment of the TBK1/IKKε complex, leading to activation of IRF3. KSHV ORF64 and HSV-1 UL36 deubiquitinate RIG-I and TRAF3, respectively, thus inhibiting the RIG-mediated activation of IRF3 and type I IFN production.

bacterially purified UL48 DUB and the full-length UL48 protein immunoprecipitated from virus-infected cells shows that the UL48 DUB cleaves both K48- and K63-linked ubiquitin dimers and oligomers. Thus, the UL48 DUB falls into a DUB family that contains both UCH and USP activity. Mutations in active site residues (C24 and H162) completely abolish DUB activity, and the virus containing the UL48 (C24S) gene is not lethal and shows moderately reduced growth compared to wild-type virus. This demonstrates that

the DUB activity of HCMV can influence viral replication in cultured cells (35). Interestingly, the UL48 DUB has a higher ubiquitin depolymerizing activity for K63-linked polymers than for UL48-linked polymers (35).

Since the substrate for the UL48 DUB has not been identified, the mechanism by which the HCMV DUB promotes viral replication remains undetermined. It may increase the stability of target proteins by inhibiting K48-linked polyubiquitination. Considering that UL48 DUB

efficiently reacts to K63-linked polyubiquitin chains, it is also possible that UL48 modulates cellular signaling pathways mediated by K63-linked polyubiquitin chains. It is worth noting that cellular DUBs that react to K63-linked ubiquitin chains have been shown to play important roles in the regulation of both innate and adaptive immune responses (36).

3. EBV BPLF1

In EBV, a member of the gamma-herpesvirus subfamily, BPLF1 is the homolog of the HSV-1 UL36. BPLF1 contains DUB activity within the first 205 amino acids of the N-terminal region, and mutation of the active site cysteine results in a complete loss of enzymatic activity (8, 14). Like the UL36 and UL48 DUBs, the BPLF1 DUB cleaves both K48 and K63 polyubiquitin chains (37). A functionally active BPLF1 fragment has been shown to block degradation of cytosolic and endoplasmic reticulum (ER) proteins by removal of ubiquitin from substrates (38).

So far, several viral and cellular substrates for BPLF1 have been identified. BPLF1 interacts with EBV ribonucleotide reductase (RR) and deubiquitinates its large subunit (RR1), leading to downregulation of RR activity (37). Although RR is the first viral target for herpesviral DUBs, the role of BPLF1-mediated inhibition of RR activity in viral infection is not clear. Proliferating cell nuclear antigen (PCNA) is found as a cellular BPLF1 target (39). In response to DNA damage and fork stalling, PCNA is monoubiquitinated and then initiates recruitment of specialized polymerases in the DNA damage tolerance pathway, known as trans lesion synthesis (TLS). PCNA also associates with EBV DNA during viral replication. BPLF1 targets and deubiquitinates PCNA, disrupting repair of damaged DNA by compromising the recruitment of TLS polymerase to stalled replication forks. Interestingly, BPLF1 and the homologs encoded by HSV-1, KSHV, and MHV68 have been shown to exhibit activity toward NEDD8 conjugates of Cullin ring ligases (40). This BPLF1 fragment containing deneddylase activity is produced by cleavage of the full-length protein by caspase-1 and is targeted to nuclear Cullins. The BPLF1 activity that removes NEDD8 from nuclear Cullins promotes productive virus infection (41).

TRAF6 has been identified as another cellular target of BPLF1 (42). TRAF6 is an important component for EBV latent membrane protein 1 (LMP1)-mediated NF-κB signaling (43). TRAF6 associates with LMP1 and is constitutively polyubiquitinated. TRAF6 polyubiquitination is important for recruitment of the IKK complex and subsequent NF-κB activation. In lymphocytes that are latently infected with EBV, LMP1 functionally mimics the TNF receptor superfamily member CD40, which is an activating receptor constitutively expressed on B-cells, constitutively activating the canonical NF-κB pathway (44). This LMP1-mediated activation of NF-κB is crucial for the survival of latently-infected B-cells and the inhibition of spontaneous induction of lytic replication. Changes in the host cell microenvironment or other unknown triggers can downregulate NF-κB activity and disrupt the balance between the latent cycle and the lytic cycle of EBV. Recently, Saito *et al.* demonstrated that once lytic replication is induced, BPLF1 deubiquitinates TRAF6 to block NF-κB signaling, and that this process is important in promoting efficient viral genome replication (42) (Fig. 2).

4. KSHV ORF64

The ORF64-encoded protein of KSHV, a member of the gamma-herpesvirus subfamily, has been identified as a lytic protein that is present in the tegument region and appears to act as a scaffold protein during tegumentation (19). ORF64 can also catalyze cleavage of both K48 and K63 polyubiquitin chains. Gonzalez *et al.* reported that knockdown of ORF64 in KSHV-infected HEK 293 cells results in decreased levels of ORF57 lytic transcripts, as well as decreased expression of the lytic protein vIL6 (19). Thus, ORF64 expression appears to enhance the KSHV lytic cycle through deubiquitination.

RIG-I has been identified as a cellular target of ORF64 (45). Polyubiquitination of RIG-I by TRIM25α and RNF135 enhances the association of RIG-I with MAVS as well as subsequent activation of IRF3. ORF64 suppresses the RIG-I-mediated IFN signaling by reducing RIG-I ubiquitination,

which is crucial for signal activation (Fig. 2). A negative cellular regulator of RIG-I signaling is the RNF125 ubiquitin E3 ligase, which catalyzes K48-linked polyubiquitination of RIG-I, leading to its proteasomal degradation (46). Therefore, both ORF64 and RNF125 have a similar negative effect on the RIG-I-mediated IRF3 activation. Increased levels of KSHV persistency are observed in RIG-I-deficient or -depleted cells (45). Therefore, the ORF64-mediated inhibition of RIG-I-dependent IRF3 activation should promote persistent KSHV infection.

5. Deubiquitinating proteases from other animal herpesviruses

Two other members of the alpha-herpesvirus subfamily, PRV and MDV, are shown to have DUB activity in their homologs of HSV-1 UL36. PRV UL36 is the essential tegument protein (47), which plays a role in capsid transport and virion assembly (48~50) and formation of mature virions (47). Recently, Zaichick *et al.* reported that PRV UL36 associates with the dynein/dynactin microtubule motor complex, and that this interaction enhances capsid transport along microtubules, neuroinvasion, and pathogenesis. (49). MDV is a lymphomagenic virus that causes Marek's disease in chickens. The DUB activity of MDV UL36 promotes MDV replication and pathogenesis in chickens. Mutation of the MDV UL36 DUB causes a reduction in the formation of T-cell lymphomas (16).

MHV68 is a natural rodent pathogen belonging to the gamma-herpesvirus subfamily. The large tegument protein encoded by MHV68 ORF64 is a functional deubiquitinating enzyme (51). MHV68 encoding an enzymatically inactive ORF64 protein is cleared faster than wild-type and revertant viruses in an *in vivo* mouse infection model (20), suggesting that it is required for persistent infection. The targets for the DUBs of PRV, MDV, and MHV68 have not been identified yet.

Conclusion

The herpesvirus-encoded DUBs appear to play a key role in several steps of the viral replication cycle. Although a limited number of viral and cellular targets have been identified so far, evidence accumulates that herpesviral DUBs may primarily target key cellular regulators in immune signaling pathways. KSHV ORF64 and HSV-1 UL36 target RIG-I and TRAF3, respectively, and down-regulate their ubiquitination levels, leading to the inhibition of IRF3 activation. Furthermore, EBV BPLF1 is shown to target to TRAF6 and inhibit its ubiquitination; this down-regulates the activation of the NF-κB pathway when latently-infected B-cells enter the lytic cycle. It is unclear if other herpesviral DUBs target the same IRF3 and NF-κB signaling pathways during lytic infection. Interestingly, we observed that a mutant HCMV encoding UL48 (C24S) inhibits TNFα-induced NF-κB activation less efficiently than wild-type virus (Kwon and Ahn, unpublished data). Therefore, it is likely that the DUB-mediated regulation of NF-κB signaling is indeed conserved in several herpesviruses. Certainly, more substrates should be identified to reveal the function of herpesviral DUBs in the regulation of immune signaling pathways.

For the DUBs encoded by HSV-1 and EBV, the DUB domain-containing N-terminal region is cleaved to the small fragment, which is then delivered into the nucleus to perform their nuclear functions (13, 42). So far, this process has not been reported in beta-herpesviruses. Whether similar processing and nuclear translocation happens in beta-herpesviruses needs further investigation.

It is unclear why the DUB domain is conserved in the large tegument proteins that surround the capsid in herpesviruses. It is plausible that DUB activity is required for the events immediately after virus entry, since the viral tegument proteins are exposed in the cytoplasm as soon as virus enters the cell. Their activities regulating cellular immune responses may be necessary for efficient activation of viral immediate-early gene expression. Given that the DUB-containing large tegument proteins tightly surround the capsid, DUBs may play a role in in virion assembly and maturation. More analysis of the viral replication cycle using the DUB activity-defective mutant viruses will be necessary to reveal the functions of these evolutionarily conserved herpesviral DUBs in the viral replication cycle.

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