Herpes virus deneddylyases interrupt the cullin-RING ligase neddylation cycle by inhibiting the binding of CAND1

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The conserved N-terminal domains of the major tegument proteins of herpes viridae encode cysteine proteases with potent ubiquitin and NEDD8-specific deconjugase activity. Here we show that the Epstein–Barr virus-encoded member of this enzyme family, BPLF1, is targeted to cullin-RING ubiquitin ligases (CRLs) via the interaction of the conserved helix-2 with helix-23 of the C-terminal domain (CTD) of cullins, at a site involved in electrostatic interaction with helix-8 of the CRL regulator CAND1. Mutation of the solvent-exposed Asp86 and Asp90 of helix-2 to Arg does not affect the enzymatic activity of BPLF1 but abolishes cullin binding and prevents CRL inactivation. The binding of the catalytically active BPLF1 to cullins inhibits the recruitment of CAND1 to the deneddylated CRLs and promotes the selective degradation of cullins by the proteasome. Cullin proteolysis is rescued by the overexpression of CAND1 or its CTD-binding N-terminal domain. These findings illustrate a new strategy for viral modulation of CRL activity where the combined effects of cullin deneddylylation and their targeting for proteasomal degradation drive stable inactivation of the ligases.

Keywords: Epstein–Barr virus, BPLF1, NEDD8, cullin-RING ligases, CAND1

Introduction

The cullin-RING ubiquitin ligases (CRLs) are the largest family of multi-subunit ubiquitin E3 ligases in eukaryotes that ubiquitinate protein substrates in numerous cellular pathways (Petroski and Deshaies, 2005). CRLs are modular complexes composed of a cullin scaffold, a RING finger protein (RBX1 or RBX2) that recruits a ubiquitin-charged E2 enzyme, and a substrate adaptor that determines the specificity of the complex. The founding member of the CRL family, the SCF (SKP1/Cul1/F-box protein) ubiquitin ligase, recognizes its targets via an adapter module composed of SKP1 and one of 68 F-box proteins in humans (Jin et al., 2004). Six additional cullin (2, 3, 4A, 4B, 5, and 7)-RING complexes interact with distinct sets of adapter modules, forming a great variety of unique CRLs (Petroski and Deshaies, 2005). Central to the assembly of an active CRL is the modification of a conserved lysine residue in the cullin with NEDD8 (Zheng et al., 2002b; Wolf et al., 2003; Parry and Estelle, 2004), which induces a conformational rearrangement of the C-terminal RBX1-binding domain that promotes the processivity of ubiquitin transfer (Duda et al., 2008; Saha and Deshaies, 2008). Neddylation is reversed by the activity of an eight-subunit deneddylylase known as the COP9 signalosome complex (CSN), which inactivates the CRL (Wolf et al., 2003). In addition, the heat-repeat protein CAND1 regulates the assembly of active CRLs by binding to unneddylated adaptor-free cullin-RBX1 complexes (Zheng et al., 2002a; Goldenberg et al., 2004). Although both the CSN and CAND1 function as negative regulators of CRLs in vitro, genetic data indicate that a catalytically active CSN is required for CRL function in vivo (Cope and Deshaies, 2003; Bosu and Kipreos, 2008); while CAND1 mutants in plants (Hotton and Callis, 2008) and C. elegans (Bosu et al., 2010) display defects that are consistent with positive regulation of at least a subset of CRLs (Bosu et al., 2010). This apparent paradox has been rationalized through the idea that CRLs must undergo cycles of neddylation and deneddylation in order to be fully functional in cells (Deshaies et al., 2010), but the details of this neddylation cycle remain unknown.

CRLs are widely exploited by viruses to promote the degradation of specific cellular proteins (Barry and Fruh, 2006). Eminent examples of viral highjacking strategies are the capture of Elongin B/C-Cul2/5-SCS-box CRLs by the Vif protein of human immunodeficiency virus (HIV) (Yu et al., 2003), the E4orf6 and E1B-55 of adenoviruses (Luo et al., 2007) and BZLF1 protein of Epstein–Barr virus (EBV) (Sato et al., 2009), which promotes the degradation of the RNA-editing enzyme APOBEC3G and of p53, respectively. Cul1 and Cul4A-based CRLs are also captured by viral proteins, which weakens the host innate defense through targeting of
NF-κB (Qu et al., 2004; Chang et al., 2009) and the interferon activated transcription factors STAT-1, -2 or -3 (Garcin et al., 2002; Ulane and Horvath, 2002; Lin et al., 2005; Precious et al., 2005). A different strategy is illustrated by the cysteine proteases encoded in the N-terminal domain of human and animal herpesviruses (Gastaldello et al., 2010). The EBV-encoded member of this enzyme family, BPLF1, plays an essential role in the productive virus cycle by promoting the establishment of an S-phase-like environment that is required for efficient replication of the viral genome. A key feature of this effect is the stabilization of several CRL substrates involved in the regulation of DNA replication and cell cycle progression, including the licensing factor CDT1 that supports the efficient virus replication in the absence of BPLF1. BPLF1 and other herpesvirus-encoded homologs exhibit strong ubiquitin and NEDD8 specific deconjugase activity in vitro but the phenotype of cells expressing BPLF1 closely resembles that of cells treated with the NE1 inhibitor MLN4924 that selectively blocks the neddylation cascade (Soucy et al., 2009), implying that the neddylylation activity is functionally dominant in vivo.

Here we report that the capacity of BPLF1 to interrupt the CRL neddylation cycle is explained by targeting the enzyme to the CRL complex via the interaction of the conserved acidic N-terminus of helix-2 with the basic helix-23 in the RBX1-binding domain of cullins that also forms electrostatic bonds with the acidic helix-8 of CAND1. Binding of a catalytically active BPLF1 promotes the degradation of deneddylated cullins by the proteasome, which is inhibited in a dose-dependent manner by the overexpression of CAND1. Thus, stable inactivation of the ligase is achieved through the combined effect of cullin neddylation and inhibition of CAND1 mediated rescue from proteosomal destruction.

**Results**

**Herpesvirus cysteine proteases interact with the RBX1-binding domain of cullins**

In order to gain insights on the mechanism of CRL regulation we first asked whether BPLF1 and its homologs bind to human cullins. Immunoprecipitation assays were performed in HeLa cells co-transfected with Flag-tagged versions of the N-terminal catalytic domains of EBV-BPLF1, henceforth indicated as BPLF1, KHSV-Orf64, HSV1-Orf36, or MCMV-M48 and Myc-tagged cullin-1, -2, -3, -4A, -4B, or -5. We observed robust reciprocal co-immunoprecipitations of BPLF1 with all cullins (Figure 1A) and of Cul1 and Cul4A with different BPLF1 homologs (Figure 1B), indicating that conserved protein domains are involved in the interaction. To explore this possibility, we took advantage of the published crystal structures of CRL complexes (Schulman et al., 2000; Zheng et al., 2002b) to identify structural domains in Cul4A that are predicted to maintain a stable conformation upon the expression as GST fusions in bacteria (Supplementary Figure S1A and B). Purified GST-tagged Cul4A (amino acid 1-759, A), the substrate-adaptor binding domain (B), cullin helical repeats-2 and -3 (C), the RBX1-binding domain (D) and the entire C-terminal domain (CTD) including the neddylation domain (E) (see Supplementary Figure S1C) were used as baits in pulldown assays performed with lysates of HeLa cells transfected with Flag-BPLF1. Flag-BPLF1 interacted with the full-length Cul4A and with the RBX1-binding domain alone or together with the neddylation domain, whereas no interaction was observed with the substrate-adaptor-binding and central domains, or with a GST-tagged proteasome subunit (S) that was included as a pulldown control (Figure 2A). In view of the high degree of conservation of the RBX1-binding domain (Supplementary Figure S7), this finding is consistent with the capacity of BPLF1 to interact with all cullins. To further assess whether binding is direct or dependent on accessory proteins present in the cell lysates, an enzymatically active HIS-BPLF1 expressed in bacteria (Supplementary Figure S1D) was used in *in vitro* binding assays together with GST-Cul4A and the Cul4A domains. Direct interaction of BPLF1 with Cul4A and the RBX1-binding domain was confirmed by reciprocal pulldown of the GST- and HIS-tagged-binding partners (Figure 2B).

To investigate whether a conserved domain in BPLF1 is involved in binding to cullins, we took advantage of the 30% sequence conservation in the N-terminal domains of all viral homologs to generate good quality structural models using as template the published crystal structure of MCMV-M48 (Supplementary Table S1). CLUSTAL-W generated multiple sequence alignments was then used to search for candidate cullin-binding domains based on the presence of solvent-exposed regions of homology whose engagement in binding would not sterically interfere with the catalytic site (Supplementary Figure S2A). These requirements were fulfilled by the N-terminal helix-2 that is well conserved, solvent exposed, and located opposite to the catalytic groove (Supplementary Figure S2A and B). The N-terminal part of helix-2 exposes an acidic surface that could mediate electrostatic interactions with the CTD of cullins. In BPLF1, the charged residues include the fully conserved Asp86, the solvent-exposed Asp90, and surrounding Glu85 and Glu91 residues (Supplementary Figure S2B).

In order to assess whether helix-2 may mediate the interaction with cullins, an HIS-tagged fragment encompassing amino acid 81-107 of BPLF1 (HIS-h2) was expressed in bacteria and the purified polypeptide was used in in vitro binding and pulldown assays together with GST-Cul4A and Cul4A domains. As illustrated by the reciprocal pulldown shown in Figure 2C, the pattern of Cul4A interaction was comparable to that observed with the full-length BPLF1, indicating direct binding to the cullin CTD.

Binding to cullins is required for BPLF1 activity

In order to assess whether binding to cullins is required for BPLF1 activity, the solvent-exposed Asp86 and Asp90 residues were exchanged for either Gly (h2-G86-G90) or Arg (h2-R86-R90) by site-directed mutagenesis. This reduced the efficiency of cullin pulldown by >90% (Figure 3A and Supplementary Figure S3A), suggesting that the acidic residues are critical components of the binding surface. This conclusion was further supported by competition assays where pre-incubation of

Figure 2 Mapping of the binding site of BPLF on the cullin CTD and identification of the binding domain. (A) GST pulldown illustrating the interaction of BPLF1 with the RBX1-binding domain of Cul4A. Lysates of HeLa cells transfected with Flag-BPLF1 were incubated with equimolar amounts of purified GST-Cul4A and the Cul4A domains. Western blots were probed with anti-GST and anti-Flag antibodies. A GST-tagged S12 subunit of the proteasome (S) was included as the control. (B) Binding assay illustrating the direct interaction of BPLF1 with the RBX1-binding domain of Cul4A. Purified HIS-BPLF1 and GST-Cul4A or GST-Cul4A domains were mixed in equimolar amounts. GST proteins were purified from half of the mixture with glutathione GST beads (GST pulldown) and HIS-BPLF1 was purified from the other half with nickel affinity beads (HIS pulldown). Western blots were probed with anti-GST and anti-HIS antibodies. The enzymatic activity of purified HIS-BPLF1 was confirmed by labeling with the NEDD8-VS probe (Supplementary Figure S3). Input corresponds to the 10% of the volume used for pulldown. (C) Western blots illustrating the direct interaction of BPLF1 helix-2 with the RBX1-binding domain of Cul4A. Purified HIS-BPLF1-h2 and GST-Cul4A or GST-Cul4A domains were mixed in equimolar amounts and immunoprecipitations were performed from half of the mixture with either anti-GST or anti-HIS antibodies. GST alone and GST-S12 (S) were included as controls.
Inactivation of CRLs by herpes virus deneddylases

Figure 3 Acidic residues in BPLF1 helix-2 are involved in binding to cullins. (A) Mutation of Asp86 and Asp90 of BPLF1 helix-2 prevents binding to Cul4A. In vitro binding assay performed with equimolar amounts of purified GST-Cul4A and HIS-BPLF1 helix-2 (h2-WT) or mutated versions where Asp86 and Asp90 were substituted with Gly (h2-G86-G90) or Arg (h2-R86-R90). (B) In vitro binding assay illustrating the failure of mutated helix-2 to compete the binding of BPLF1 to Cul4A. Binding assays were performed as described in the figure. GST-Cul4A-D was pre-incubated with 0.3:1, 1:1, and 3:1 molar excess HIS-h2-WT or HIS-h2-R86-R90 before the addition of HIS-BPLF1. (C) Mutation of acidic residues in helix-2 prevents the binding of BPLF1 with cullins in transfected cells. Co-immunoprecipitation was performed in lysates of HeLa cells transfected with Myc-Cul1 or Myc-Cul4A and Flag-BPLF1, -BPLF1-G86-G90, or -BPLF1-R86-R90. Western blots were probed with the anti-Myc and anti-Flag antibodies. (D) The enzymatic activity of BPLF1 and mutants expressed in HeLa cells was assessed by labeling with the functional probes ubiquitin-VS (Ub) and NEDD8-VS (N8). Binding was visualized in western blot probed with the anti-Flag antibodies as a band shift of ~10 kDa, corresponding to the size of the probe.

GST-Cul4A with increasing amounts of purified wild-type helix-2 (h2-WT) inhibited the binding of BPLF1 in a dose-dependent manner (Figure 3B), whereas the h2-R86-R90 and h2-G86-G90 (data not shown) mutants failed to do so even at the highest concentrations tested.

We then asked whether binding to cullins is required for BPLF1 activity in vivo as illustrated by the accumulation of CRL substrates and induction of DNA re-replication. To this end, Asp86 and Asp90 were mutated to either Gly or Arg in Flag-BPLF1 and immunoprecipitation experiments were performed in HeLa cells co-transfected with Myc-Cul1 or Myc-Cul4A and Flag-BPLF1, Flag-BPLF1-G86-G90, or Flag-BPLF1-R86-R90 (Figure 3C). Both mutants showed virtually complete loss of binding to cullins. In order to exclude artifacts of misfolding, the enzymatic activity of the mutants was assayed in lysates of transfected HeLa cells by labeling with the Ub-VS and NEDD8-VS functional probes that specifically bind to the catalytic Cys residue (Borodovsky et al., 2001). Both mutants retained the capacity to interact with Ub and NEDD8 (Figure 3D). However, while the activity of the R86-R90 mutant was virtually unaffected, the activity of the G86-G90 mutant was significantly decreased against both probes (Figure 3D and Supplementary Figure S3B), suggesting that the lack of binding may be at least partially due to misfolding.

This is in line with the energy minimizations analysis and molecular dynamic simulation that predict important conformational changes in the G86-G90 mutant, while the secondary structure and overall folding are preserved in the R86-R90 mutant (Supplementary Figure S4A). Thus, BPLF1-R86-R90 is a bona fide catalytically active binding mutant.

We then tested the effect of this mutant on the levels of expression of a panel of known CRL substrates and other short-lived cellular proteins that are not primary targets of CRLs. Both the catalytic mutant BPLF1-C61A and the cullin-binding mutant BPLF1-R86-R90 failed to induce the accumulation of Cdc25A, CDT1, p21, and p27 that were efficiently stabilized by the wild-type BPLF1 to a level comparable to that induced by blockade of the proteasome by treatment with MG132, while non-CRL substrates were not significantly affected (Figure 4A). Similar results were obtained in HeLa cells transfected with NEDP1 that also fails to proficiently interact with cullins (Gastaldello et al., 2010). The failure to stabilize CRL substrates was paralleled by failure of BPLF1-R86-R90 to interfere with DNA replication and cell cycle progression as assayed by propidium iodide (PI) staining 48 h after transfection (Figure 4B).

Thus, binding to cullins appears to be critical for the capacity of BPLF1 to inactivate CRLs.
The BPLF1 cullin-binding mutant fails to induce the accumulation of CRL substrates. Western blot illustrating the selective accumulation of CRL substrates (Cdc25A, CDT1, p27, and p21) in HeLa cells expressing the wild-type BPLF1 but not in cells expressing a catalytic mutant (BPLF1-C61A) or cullin-binding mutant (BPLF-R86-R90). CRL substrates failed to accumulate also in HeLa cells expressing the deneddylyase NEDP1 that does not bind to cullins. The accumulation of CRL substrates induced by the wide-type BPLF1 was comparable to that induced by treatment with the proteasome inhibitor MG132. Catalytically inactive and cullin-binding mutant BPLF1 failed to induce the DNA re-replication. PI staining of HeLa cells transfected with BPLF1, the catalytic or cullin-binding mutants or NEDP1. Only the wild-type BPLF1 induced the accumulation of cells with ≥4 N DNA content within 40 h after transfection.

**BPLF1 competes with CAND1 for binding to the cullin CTD**

In the next set of experiments we asked whether the binding of BPLF1 might interfere with the assembly of CRLs. To this end, endogenous Cul1 and Cul4A were immunoprecipitated from HeLa cells mock transfected (Ctrl) or transfected with wild-type, catalytic mutant (C61A), binding mutant (R86-R90) BPLF1 or NEDP1, and the presence of neddylylated species and co-precipitated substrate adaptors, SKP1 and DDB1, RBX1, the CSN subunits CSN1, CSN5, and CSN6 and CAND1 were assayed in western blots. Since we observed a significant decrease in endogenous Cul1 and Cul4A in cells expressing the wild-type BPLF1 (Supplementary Figure S5), the amount of immunoprecipitated material loaded in each lane adjusted in order to achieve cullin bands of comparable intensity. The expression of the wild-type BPLF1 was associated with the disappearance of the neddylylated species detected by the NEDD8-specific antibody, but did not affect the amount of co-precipitated substrate adaptors and RBX1 (Figure 5A). In line with previous reports suggesting that neddylylation may be required for recruitment of the CSN (Bennett et al., 2010), the amount of co-precipitated CSN1, CSN5, and CSN6 was strongly reduced. Unexpectedly, and at odds with the preferential binding of CAND1 to deneddylylated cullins (Liu et al., 2002; Zheng et al., 2002a), the amount of co-precipitated CAND1 was also strongly decreased. As expected, wild-type BPLF1 and the catalytic mutant were efficiently co-precipitated, whereas only trace amounts of the BPLF1-binding mutant and NEDP1 were detected in the complexes. Reciprocal immunoprecipitations performed with the anti-Flag antibody from cell lysates made in the presence of 1 mM orthophenanthroline, which minimized the loss of neddylylated species (Bennett et al., 2010), confirmed that the cullins immunoprecipitated by wild-type BPLF1 are deneddylylated, whereas a substantial fraction of deneddylylated cullins is found in complex with the catalytic mutant BPLF1 (Figure 5B) and CAND1 is not present in the complexes. This finding supports the essential role of deneddylylation in the capacity of BPLF1 to alter the composition of the CRL complex. The binding of BPLF1 to the cullin CTD that also interacts with CAND1 and the failure to detect CAND1 in complex with cullins suggest that BPLF1 may interfere with CAND1 binding. To test this possibility, we took advantage of the published crystal structure of CAND1 in complex with Cul1 [PDB:1U6G], and of the CLUSTAL-W multiple sequence alignments of CAND1 from different species (Supplementary Figure S6), to search for possible similarities between helix-2 of BPLF1 and the CTD-binding domain of CAND1. As illustrated by the surface electrostatic potential representation shown in Figure 5C (upper panel), CAND1 helix-8 resembles BPLF1 helix-2 in that it contains an acidic core, formed by the conserved residues Glu153 and Asp160, which flanked at each side by basic residues. CAND1 helix-8 establishes an electrostatic interaction with helix-23 of the cullin CTD that exposes a complementary surface with alternating acidic, basic, and acidic domains (Figure 5C, lower panel, and Supplementary Figure S7). To test whether BPLF1 may also bind to this region of the CTD, bacterially expressed His-tagged CAND1 helix-8 and Cul4A helix-23 were tested for their capacity to compete for the binding of BPLF1 to Cul4A in in vitro pulldown assay. Pre-incubation of GST-Cul4A-D with increasing amounts of purified His-CAND1-h8, or His-BPLF1 with increasing amounts of His-Cul4A-h23, resulted in a dose-dependent inhibition of GST pulldown (Figure 5D). Collectively, these findings identify helix-23 as the binding site of BPLF1 on the cullin CTD and suggest that binding of BPLF1 to this site may interfere with the binding of CAND1.

**BPLF1 targets deneddylylated cullins for proteasomal degradation**

Although deneddylylation by the CSN and the sequestration of deneddylylated cullins by CAND1 inactivate CRLs in vitro, both events are required for sustained CRL activity in vivo, which led to a model where cycling between neddylylated, deneddylylated, and CAND1-bound states maintains the function and broadens the substrate repertoire of CRLs. Based on this model, deneddylylation by BPLF1 may not be sufficient to inactivate the CRLs. Thus, in the final set of experiments we sought to gain a better understanding of the effect of BPLF1 on the cullin cycle. Analysis of the expression levels of Cul1 and Cul4A, the substrate adaptors SKP1 and DDB1 and the E2-binding subunit RBX1 in western blots of cells expressing wild-type, catalytic mutant and binding mutant BPLF1 (Figure 6A, left panel) revealed a ~80% decrease in the cullin-specific bands in cells expressing the wild-type BPLF1, while the expression of SKP1, DDB1, and RBX1 was not affected. The cullin levels were fully restored by the addition of the proteasome inhibitor MG132 (Figure 6A, right panel, and Supplementary Figure S8A), indicating selective targeting of deneddylylated cullins for proteasomal degradation. In line with this finding, cullins immunoprecipitated from cells...
expressing the wild-type BPLF1 were efficiently polyubiquitinated (Supplementary Figure S8B).

In order to investigate whether the capacity of BPLF1 to compete the binding of CAND1 may explain the destabilization of cullins, BPLF1 transfected HeLa cells were co-transfected with increasing amounts of plasmids expressing full-length Myc-CAND1 or deletion mutants expressing the N-terminus that interacts with the cullin CTD (Myc-CAND1-N, amino acid 1-960) or the C-terminus that interacts with the substrate-adaptor-binding domain (Myc-CAND1-C, amino acid 563-1230). Both the full-length CAND1 and the N-terminus of CAND1 rescued the expression of endogenous Cul1 and Cul4A, while the C-terminus had only minor effects (Figure 6B and Supplementary Figure S9A). By lysing the cells in the presence of 1 mM o-phenantroline that preserve cullin neddylation, we observed that the cullins rescued by full-length CAND1 remained deneddylated while neddylated cullins were rescued in the presence CAND1-N, suggesting that, without the stabilizing contribution of the CTD, CAND1-N is displaced, exposing the neddylation site. Cullin degradation in the presence of BPLF1 was not rescued by overexpression of a dominant-negative RBX1 that efficiently blocks the CRL activity as assessed by stabilization of CDT1 and p21. Thus, the
degradation of cullins is not due to autoubiquitination (Supplementary Figure S9B). Collectively, these findings support the notion that the inhibition of CAND1 binding plays a critical role in determining the capacity of BPLF1 to interrupt the cullin neddylation cycle by targeting deneddylated cullins for degradation by the proteasome.

Discussion

The conserved cysteine proteases encoded in the N-terminus of the major tegument protein of herpes viridae promote the accumulation of nuclear CRL substrates and the establishment of an S-phase-like cellular environment that supports efficient replication of the viral genome (Gastaldello et al., 2010). Although the enzymes are highly active against both ubiquitin and NEDD8 conjugates in a variety of experimental conditions, the phenotype induced in transfected cells and during the activation of the productive virus cycle in EBV-infected cells is indistinguishable from that induced by MLN4924, a potent inhibitor of the NEDD8-activating enzyme that halts the neddylation cascade (Soucy et al., 2009), indicating that the deneddylation activity is functionally dominant in vivo. Here we have shown that binding to cullins and inhibition of the interaction of cullins with the regulatory protein CAND1 play a key role in determining the capacity of the viral deneddylation machinery to stably inactivate CRLs.

Using a strategy based on co-immunoprecipitation of tagged proteins in transfected cells, pulldown assays of purified polypeptides expressed in bacteria and mutation of candidate-binding residues, we have found that BPLF1 directly interacts with the conserved RBX1-binding domain of cullins and mapped the binding site to the exposed helix-2 of BPLF1 that is well conserved in this protein family. In particular, two Asp residues that point away from the surface of helix-2 establish electrostatic interactions with the cullin CTD since binding was abolished by mutations to Arg that change the surface charge without affecting the conformation and enzymatic activity of BPLF1. Most importantly, mutation of the cullin-binding residues hampered the capacity of the mutant to promote the stabilization of nuclear CRL substrates and the re-replication of cellular DNA (Figure 4). Together with the nuclear localization (Gastaldello et al., 2010), the targeting of BPLF1 to cullins provides an explanation for the dominant effect on the accumulation of CRL substrates involved in the regulation of the cell cycle. Interestingly, in line with the double specificity of BPLF1 for both ubiquitin and NEDD8 conjugates, the enzyme was shown to act as a potent ubiquitin deconjugase in the cytoplasm and, upon targeting to the endoplasmic reticulum, efficiently interfere with the translocation and degradation of ERAD substrates by promoting their deubiquitination (Ernst et al., 2011). Given the high degree of homology between ubiquitin and NEDD8 this dual specificity is not surprising and is indeed shared by many ubiquitin-specific proteases (Reyes-Turcu et al., 2009). Yet, the relevance of the deubiquitinating activity of BPLF1 during virus replication is unclear since, contrary to what may be predicted by the potency of the enzyme in in vitro assays and in transfected cells, preliminary results indicate that the progression of the replicative cycle is not accompanied by significant changes in the overall abundance of polyubiquitinated substrates in EBV-infected cells (unpublished data).

It remains unknown how the 3149 amino acid long tegument protein enters the nucleus and whether the intact protein may be capable of productive interactions with neddylated cullins. An interesting possibility is that the catalytic domain may be released by proteolytic processing during virus replication. If so, this would explain why only a ~55 kDa catalytic fragment of the BPLF1 homolog UL36 was detected by Kattenhorn et al. (2005) in HSV1-infected cells and a ~38 kDa catalytic fragment of the UL38 homolog was detected, together with the full-length protein, in HCMV-infected cells (Wang et al., 2006). In line with the possibility of proteolytic cleavage, we have observed that a 325 amino acid long N-terminal fragment of BPLF1 is processed in transfected HeLa cells to yield the catalytically active fragment that was used in this study (Gastaldello et al., 2010). We are currently investigating whether processing of the virus expressed BPLF1 occurs during the replicative cycle in EBV-infected cells.

Although binding to neddylated cullins provides a rationale for the preferential deneddylyase activity of BPLF1, it does not fully explain the inactivation of CRLs since deneddylation is required for sustained activity of the ligases (Pintard et al., 2003; Denti et al., 2006). Our findings suggest that, in addition to the deneddylyase activity, two properties of BPLF1 are instrumental for CRL inactivation. We have shown that BPLF1 halts the cullin...
neddylation cycle by inhibiting the recruitment of the CRL regulators CSN and CAND1; and we have further found that BPLF1 expression is accompanied by the selective degradation of cullins by the proteasome. While the failure to co-precipitate CSN subunits is likely to be explained by the preferential association of the CSN with neddylated CRLs, as confirmed by the rapid dissociation after acute cullin deneddylation in cells treated with MLN4924 (Bennett et al., 2010), the surprising absence of CAND1 prompted us to investigate whether BPLF1 may interfere with the activity of this cullin regulator. Two lines of evidence support this possibility. First, based on sequence alignment and analysis of the crystal structure of CAND1 bound to Cul1 we have found that the acidic helix-2 of BPLF1 resembles helix-8 of CAND1 that establishes electrostatic interactions with the basic helix-23 of the cullin CTD. The interaction of helix-23 with both CAND1 and BPLF1 was confirmed by the capacity of purified CAND1-h8 and Cul4A-h23 to prevent the binding of BPLF1. Second, the activity of BPLF1 was counteracted by the overexpression of CAND1 or its N-terminal domain. This excludes possible artifacts of misfolding that may be associated with the expression of short helical domains in in vitro competition assays. It is noteworthy that, although the binding of CAND1 to cullins is strengthened by multiple contacts involving different parts of the molecule, our findings suggest that inhibition of the electrostatic interaction between CAND1 helix-8 and cullin helix-23 may be sufficient to destabilize the complex. Interestingly, the competition for CAND1 binding has been recently shown to underlie the activation of multiple CRLs by the copper metabolism MURR1 domain-containing protein COMMD1 that plays an important role in the control of many ubiquitin regulated events, including NF-κB-mediated transcription, adaptation to hypoxia and electrolyte transport (Mao et al., 2011). While BPLF1 and COMMD1 have opposite effect on the activity of CRLs, their shared capacity to inhibit the binding of CAND1 further highlights the importance of this interaction in the regulation of CRLs.

Targeting of deneddylated cullins for proteasomal degradation appears to be critical for the capacity of BPLF1 to stably inactivate CRLs. Cullin proteolysis highlights an important difference between the effect of the viral enzyme and the chemical inhibition of the neddylation cascade induced by MLN4924 since the latter did not affect the abundance of cullins, even after prolonged treatment (Bennett et al., 2010). Interestingly, in both conditions the deneddylated cullins remained associated with the substrate adaptor and RBX1-binding subunits (Figure 5) (Bennett et al., 2010), whereas CAND1-containing complexes were detected only in MLN4924-treated cells although, contrary to expectations, deneddylation did not cause massive sequestration by CAND1. This underscores the importance of a dynamic equilibrium between binding of the substrate adaptor/receptor complexes and CAND1 in the assembly and function of CRLs. It is noteworthy that, akin the effect of BPLF1, concomitant knockdown of CAND1 and blockade of the deneddylation cascade were shown to promote the degradation of cullins in Drosophila (Kim et al., 2010) and human cells (Boh et al., 2011), and the effect was reversed in Drosophila by reintroduction of CAND1. Conceivably, by freezing the cullins in a deneddylated CAND1 unbound state BPLF1 could reveal the activity of a ligase that is physiologically involved in the turnover of a subpopulation of deneddylated and CAND1-free cullins. Alternatively, in keeping with the highjacking strategy adopted by many viral proteins, BPLF1 may recruit a new ligase. We have begun to explore this possibility but, so far, we have been unable to identify a candidate ligase by mass spectrometry analysis of proteins that co-precipitate with BPLF1 (unpublished data).

In conclusion, we have provided molecular evidence for the mechanism by which viral deneddylases modulate the activity of CRLs. Although many details of the process remain unresolved, the identification of the binding site of BPLF1 on cullins is a critical first step towards the design of strategies for specific interference with the activity of the viral enzyme that could result in selective inhibition of virus replication. In addition, the unique mode of action of the viral proteins provides an interesting tool for dissection of the cullin neddylation cycle whose details are still unknown due to the difficulty to capture distinct intermediate steps of the process.

Materials and methods
Cell lines and transfection
HeLa cells were cultured in Dulbecco’s minimal essential medium (Sigma-Aldrich), supplemented with 10% FCS (Gibco-Invitrogen), penicillin–streptomycin (Sigma-Aldrich) and l-glutamine (Sigma-Aldrich; complete medium) and maintained in a 37°C incubator in 5% CO2. Plasmid transfection was performed using the jetPEI kit (Polyplus transfection; Illkirch) as recommended by the manufacturer.

Immunoblotting and immunoprecipitation
HeLa cells transfected with the plasmids described in the Supplementary material were harvested after 48 h, washed in PBS, and resuspended in lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, pH 8.0, 0.5% NP-40, 20 mM NEM, and protease inhibitors). Protein concentration was measured with a protein assay kit (Bio-Rad Laboratories). For co-immunoprecipitation, aliquots of the cell lysates were incubated for 1 h with anti-Myc antibodies followed by protein-G coupled Sepharose beads (GE Healthcare) or for 2 h with anti-Flag agarose affinity gel (Sigma). Complexes were eluted with 100 ng/μl 3 × Flag or c-Myc peptides (Sigma) in washing buffer for 1 h at room temperature. Total cell lysates and IP eluates were denatured for 10 min at 100°C in loading buffer (NuPage 4 ×, Reducing Agent 10 ×) and fractionated in acrylamide Bis-Tris 4%–12% gradient gel (Invitrogen). After transfer to polyvinylidene fluoride membranes (Millipore), the filters were blocked in PBS saline buffer containing 0.1% Tween-20 and 5% non-fat milk or 3% BSA and incubated with primary antibodies for either 1 h at room temperature or overnight at 4°C followed by incubation for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibodies. The complexes were visualized by chemiluminescence.

Protein expression and purification
GST-tagged proteins were produced in BL21 bacteria after induction overnight at 23°C with 0.5 mM IPTG. After wash in cold PBS, the bacteria were lysed in buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM DTT, 1% Triton X-100, 1 mM PMSF, and the lysates were cleared by centrifugation for 10 min.
at 12000 g. GST-tagged proteins were purified with glutathione-Sepharose 4B beads (GE Healthcare) and eluted with 10 mM of glutathione (Sigma) in 0.2 M Tris–HCl, pH 7.4, followed by dialysis in a 8000 MW cut-off tube (Sigma) versus dialysis solution (100 mM NaCl, 20 mM Tris, pH 7.6, 1 mM EDTA). The GST tag was removed by digestion with 0.1 unit of protease X-factor/µg of protein (GE Healthcare) in reaction buffer (20 mM Tris–HCl, pH 6.5, 50 mM NaCl, 1 mM CaCl2, and 0.1% Triton X-100) for 16 h at 25 °C. His-tagged proteins were purified with HisTrap HP columns (GE Healthcare) according to manufacturer’s instructions and eluted with 200 mM imidazole. The imidazole was removed by centrifugation in a 3000 MW cut-off filter device (Microcon; Millipore) and proteins were resuspended in binding buffer (50 mM Tris–HCl, 100 mM NaCl, 1 mM DTT, 0.5% NP-40). The activity of purified BPLF1 was assayed by labeling with Ub-VS and Nedd8-VS functional probes (Boston Biochem) as described (Borodovsky et al., 2001).

**Protein binding and competition assays**

For *in vitro* binding and competition assays, equimolar amounts of purified GST and HIS tagged proteins were incubated for 15 min at 4 °C in binding buffer (50 mM Tris–HCl, 100 mM NaCl, 1 mM DTT, 0.5% NP-40) followed by capture of the complexes by incubation for 20 min at 4 °C with 50 µl of prewashed Glutathione Sepharose 4B beads or Ni-NTA agarose beads in gently rotation. The Glutathione Sepharose 4B beads were preincubated with 0.1% BSA to prevent unspecific binding. Competition assays were performed by preincubating the binding partner with 1:0.5, 1:1, and 1:3 molar ratio of the competitor for 20 min at 4 °C. For *in vivo* competition assays, HeLa cells were transfected with plasmids expressing Flag-BPLF1 and Myc-CAND1, Myc-CAND1-C or Myc-CAND1-N at 2:0, 2:0.5, 2:1, 2:2, and 2:4 ratios. After 36 h the cells were lysed in RIPA buffer (25 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 0.5% NP-40, 0.1% SDS, 0.5% DOC, protease inhibitors, 10 mM NEM, 20 mM iodoacetamide, 1 mM o-phenanthroline) and protein expression was analyzed in western blots.

**Supplementary material**

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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**References**


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