Kaposi’s Sarcoma-Associated Herpesvirus K7 Protein Targets a Ubiquitin-Like/Ubiquitin-Associated Domain-Containing Protein To Promote Protein Degradation

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Pathogens exploit host machinery to establish an environment that favors their propagation. Because of their pivotal roles in cellular physiology, protein degradation pathways are common targets for viral proteins. Protein-linking integrin-associated protein and cytoskeleton 1 (PLIC1), also called ubiquilin, contains an amino-terminal ubiquitin-like (UBL) domain and a carboxy-terminal ubiquitin-associated (UBA) domain. PLIC1 is proposed to function as a regulator of the ubiquitination complex and proteasome machinery. Kaposi’s sarcoma-associated herpesvirus (KSHV) contains a small membrane protein, K7, that protects cells from apoptosis induced by various stimuli. We report here that cellular PLIC1 is a K7-interacting protein and that the central hydrophobic region of K7 and the carboxy-terminal UBA domain of PLIC1 are responsible for their interaction. Cellular PLIC1 formed a dimer and bound efficiently to polyubiquitinated proteins through its carboxy-terminal UBA domain, and this activity correlated with its ability to stabilize cellular IκB protein. In contrast, K7 interaction prevented PLIC1 from forming a dimer and binding to polyubiquitinated proteins, leading to the rapid degradation of IκB. Furthermore, K7 expression promoted efficient degradation of the p53 tumor suppressor, resulting in inhibition of p53-mediated apoptosis. These results indicate that KSHV K7 targets a regulator of the ubiquitin- and proteasome-mediated degradation machinery to deregulate cellular protein turnover, which potentially provides a favorable environment for viral reproduction.

Protein degradation is important for a variety of cellular events. While eukaryotes have evolved numerous ways to control protein degradation at different levels temporally and spatially, altered protein degradation potentially results in a range of clinical presentations from mild inflammation to life-threatening Alzheimer’s disease (1, 19). Cytosolic protein degradation is carried out mainly by two different but closely related machineries, the ubiquitination machinery and the proteasome. These two machineries are tightly coupled to control an array of cellular processes, including signal transduction, development, apoptosis, cell cycle progression, endocytosis, and immune response (2, 22).

Marking a target protein with ubiquitin involves three catalytic enzymes: the E1 ubiquitin-activating enzyme, the E2 ubiquitin-conjugating enzyme, and the E3 ubiquitin ligase. The Nedd4-like proteins, RING finger-containing proteins, and HECT-containing proteins represent three families of E3 ligases (37). However, the E3 ligase family has been further extended to the suppressor of cytokine signaling molecules and the U-box proteins (25, 36). The ubiquitin moiety is attached to a specific lysine of E1 through a thioester bond, whose energy is derived from cellular ATP. The ubiquitin is subsequently conjugated to E2 and transferred to either the E3 ligase, which delivers ubiquitin to the correct target protein, or directly to the substrate, with E3 ligase functioning as an adaptor (37, 48). This selectivity is achieved through specific interactions between E3 ligases and their substrates.

Once polyubiquitinated, a target protein is delivered to the 26S proteasome and degraded within the organelle. Various proteins are suspected to functionally link these two machineries for coordinated protein degradation (7, 26). Protein-linking integrin-associated protein and cytoskeleton (PLIC), also called ubiquilin (29), has recently been reported to be involved in protein degradation (26, 50). There are two different PLIC isoforms, and both harbor a ubiquitin-like (UBL) domain at the amino terminus and a ubiquitin-associated (UBA) domain at the carboxyl terminus. Human PLIC1 and PLIC2 are type 2 UBL-containing proteins whose UBL domain is an integral part of the protein and cannot be covalently linked to target proteins. Other members of this family include Saccharomyces cerevisiae Dsk2p and Rad23, Xenopus laevis DXRP, and mouse PLIC1 and PLIC2 (13, 14, 26, 43). The fact that these proteins are highly conserved from S. cerevisiae to humans suggests that they have crucial and similar roles in diverse organisms. The UBL domain of PLIC1 interacts with various components of the ubiquitin-proteasome system, including the S5a protein of the 19S subunit of proteasome and E3 ligases, such as E6AP, βTRCP, and Nedd4 (26, 32). Therefore, PLIC proteins may

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provide a link between the ubiquitination complex and the proteasome machinery. However, a recent biochemical characterization of the yeast Rad23 UBA domain put a constraint on the proposed delivery function of UBL and UBA domain-containing proteins (39). Thus, the mechanism is not yet known in detail.

Upon viral infection, cells can be targeted by host immune responses or can go through a programmed cell death process, called apoptosis, as defense mechanisms to limit the ability of the virus to replicate. To prevent this, viruses have evolved elaborate mechanisms to subvert the apoptotic process to facilitate a persistent infection or prolong the survival of lytically infected cells. As seen with most DNA viruses (10), gamma 2 herpesviruses, which include Kaposi’s sarcoma-associated herpesvirus (KHSV), rhesus monkey rhadinovirus, herpesvirus saimiri, and murine gamma herpesvirus 68, are genetically equipped to prevent cellular apoptosis (21). A notable example is KHSV K7, which is a small membrane protein that functions as an antiapoptotic factor during KHSV lytic replication. Wang et al. and we have shown that K7 apparently targets multiple cellular factors to allow escape from host antiviral destruction (12, 47). These cellular targets include calcium-modulating cyclophilin ligand (CAML), a protein that regulates the intracellular Ca\(^{2+}\) concentration (12), and Bcl-2 and caspase 3, proteins that are involved in the regulation of cell death (47). Similar to CAML, K7 expression significantly enhances the kinetics and amplitude of the intracellular Ca\(^{2+}\) rise upon apoptotic stimulus, suggesting that K7 targets cellular CAML to increase the cytosolic Ca\(^{2+}\) response, which consequently protects cells from mitochondrial damage and apoptosis (12). In addition, K7 also binds to Bcl-2 via its putative Bcl-2 homology domain and to caspase 3 via its baculovirus inhibitor-of-apoptosis repeat domain (47). In particular, the BH2-like domain of K7 is crucial for the inhibition of caspase 3 activity and is therefore essential for its antiapoptotic function. Furthermore, K7 bridges Bcl-2 and activates caspase 3 into a protein complex. Thus, this indicates a novel viral antiapoptotic strategy in which the K7 protein targets a cellular Ca\(^{2+}\)-modulating protein to confer resistance to apoptosis and also targets Bcl-2 and caspase 3 proteins to inhibit caspase activity. The sum of these activities may allow the completion of viral lytic replication and, eventually, the maintenance of persistent infection in the host.

We report here that the KSHV K7 interacts with an additional cellular protein, PLIC1, through its central hydrophobic region. PLIC1 binds efficiently to polyubiquitinated proteins through its carboxy-terminal UBA domain, and this activity is correlated with its ability to stabilize IxB. In contrast, K7 interaction inhibited PLIC1 activity, resulting in rapid degradation of IxB and p53 and inhibition of p53-dependent apoptosis. These results indicate that KSHV K7 antagonizes cellular PLIC1 to facilitate the degradation of cellular antiviral proteins, which eventually leads to the establishment of an environment that favors viral replication and persistent infection.

**MATERIALS AND METHODS**

**Cell culture and transfection.** 293T cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 µg of streptomycin per ml (complete medium). BJAB and BCBL-1 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, L-glutamine, and antibiotics as described above (RPMI 10). Fugene 6 (Roche) or calcium phosphate (Clontech) was used for transient expression of K7 in COS-1 and 293T cells. Electroporation at 220 V and 975 µF was used for transient expression of K7 in BJAB cells. Stable transfection-regulated-inducible cell lines were established as previously described (34) and maintained with hygromycin at a concentration of 400 µg/ml for BJAB, 200 µg/ml for BCBL-1, and 100 µg/ml for 293 cells.

**Plasmid construction.** A DNA fragment corresponding to the KSHV K7 coding sequence was amplified from BCBL-1 genomic DNA by PCR as previously described (12). PCR-amplified DNA was cloned into vector pEGFP-C1/C2 oligonucleotide (Invitrogen) or pcDNA-FRT/TO (Invitrogen). To express an enhanced green fluorescent protein (EGFP)-PLIC1 fusion protein, PLIC1 was PCR amplified and ligated to pEGFP1 digested with BamHI and SalI. K7 and PLIC1 were completely sequenced to verify 100% agreement with the original sequence (Prism 377 automated DNA sequence; ABI). Mutations in the K7 gene were generated by PCR with oligonucleotide-directed mutagenesis. Each K7 mutant was completely sequenced to verify the presence of the mutation and the absence of any other changes and then subcloned into pEGFP-C2.

To express glutathione S-transferase (GST) fusion proteins from Escherichia coli, PLIC1 sequences were amplified by PCR and cloned into pGEX-4T-1 at the BamHI and SalI sites as described previously (29). The mammalian GST expression vector pGHOST was derived from pcDNA-FRT/TO that had been digested with BamHI and NotI, followed by ligation with a GST DNA fragment cut by BglII and NotI. Subsequently, the UBA of PLIC1 was PCR amplified, digested with BamHI and NotI, and ligated to pGHOST cut with the same set of enzymes. All constructs were sequenced to verify the lack of unwanted mutations.

**Yeast two-hybrid screen.** S. cerevisiae transformation with library cDNA was performed by a method described previously (11). S. cerevisiae strain AH109 bearing a plasmid carrying a Gal4-K7 fusion gene was grown overnight in synthetic dropout (SD) medium without tryptophan to a density of approximately 10⁷ cells/ml, then diluted in 1 liter of warm YPD to an optical density at 600 nm (OD₆₀₀) of 0.2 to 0.3, and grown to the exponential stage. The cells were harvested and washed with 100 ml of water twice and with Tris-EDTA (Clontech) once. The pellet was resuspended in 8 ml of 10 mM Tris-HCl (pH 7.5)-1 mM EDTA-0.1 M lithium acetate. The suspension was mixed with 1 ml of transforming DNA and 20 µg of single-stranded salmon sperm DNA, after which 60 ml of a solution of 40% polyethylene glycol 4000 in Tris-EDTA-lithium acetate was added and mixed thoroughly, followed by incubation at 30°C for 30 min. After a heat pulse at 42°C for 15 min, the cells were pelleted, washed with 50 ml of Tris-EDTA, and plated on selective medium. Library screening and recovery of plasmids were performed according to the manufacturer’s instructions (Clontech).

**Immunoblotting and immunoprecipitation.** Cells were harvested and then lysed with NP-40 buffer (0.15 M NaCl, 1% Nonidet P-40, 50 mM Tris, pH 7.5) containing 0.1 mM Na₂VO₄, 1 mM NaF, and protease inhibitor cocktail (Roche, Mannheim, Germany). In order to demonstrate PLIC dimerization, cells were lysed with phosphate-buffered saline (pH 7.4) (Sigma) supplemented with NP-40 to a final concentration of 0.2% and protease inhibitor cocktail. After being cleared with protein A-protein G agarose beads for 1 h at 4°C, whole-cell lysates were used for immunoprecipitation. For immunoblotting, polypeptides were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Bio-Rad). Immunoblot detection was performed with anti-Xpress, anti-V5 antibody (1:5,000) (Invitrogen), anti-Flag antibody (1:5,000) (Sigma, anti-p53 (DO-1), anti-GFP, anti-GST, antinithamagglutinin (HA) (1:1,000), anti-IXBα (1:500) (Santa Cruz), anti-ubiquitinated protein antibody FK2 (1:2,000) (Abnitt Research Products, Mannheim, United Kingdom), or anti-PLIC1 antibody (1:500) (29). The protein was visualized with a chemiluminescent detection reagent (Pierce) and detected with a Fuji PhosphorImager.

**GST purification and in vitro GST pulldown.** GST or GST fusion proteins were expressed in E. coli BL21 grown to log phase. After induction with 2 mM isopropylthiogalactopyranoside (IPTG) for 2 h, E. coli was harvested, lysed with phosphate-buffered saline containing 0.1% Sarkosyl, 1% Triton X-100, and protease inhibitor cocktail, and mixed with glutathione-Sepharose beads at 4°C for 1 h. The beads were washed three times with lysis buffer.

The GST pulldown experiment was carried out as previously described (11). PLIC, K7, and EGFP-K7 fusions were expressed from 293T cells and lysed in NP-40 buffer as described above. However, Triton X-100 was added to a final concentration of 0.5% when the cell lysate was harvested. After lysis, the supernatant was mixed with purified GST fusion proteins for an additional hour at 4°C. Sepharose beads containing GST fusion proteins were washed with lysis buffer

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three times and subjected to SDS-PAGE, followed by immunoblotting with antibodies.

**Protein stability.** For K7 and p53 half-life measurement, cells were treated with cycloheximide at 20 μg/ml for up to 180 min. For 15g degradation, 293T cells were treated with tumor necrosis factor alpha (TNF-α) at a concentration of 10 ng/ml for up to 60 min. Equal numbers of cells were lysed, and 10 μg of total protein was used for SDS-PAGE and immunoblotting. Alternatively, BJAB cells stably transfected with vector pFR-T or pFR1 were used for the same experiment. Cells were induced to express K7 for 2 days with doxycycline at a concentration of 2 μg/ml. At various time points, 10^6 cells were collected and subjected to the p53 and IκB stability experiment as described above.

To examine the effect of K7 and PLIC1 on the expression of engineered GFP constructs, 293T cells were transfected with a construct expressing ubiquitin and GFP (Ub-X-GFP) in the presence of K7 and/or PLIC1 by the calcium phosphate method (Clontech). GFP expression levels were measured by either flow cytometry or Western blotting with anti-GFP polyclonal antibody (Santa Cruz Biotech).

**Apoptosis.** BJAB and BCBL-1 cells stably expressing K7 were grown with RPMI 10 and induced with 2 μg of doxycycline per ml for 2 days. The cells were then treated with etoposide (10 μg/ml) for 28 h (BJAB) or 48 h (BCBL-1). Cell viability was measured by trypan blue dye exclusion.

**RESULTS**

**Interaction of K7 with a cellular PLIC1.** To investigate the role of K7 in the virus-host interaction, we employed the yeast two-hybrid screen to identify cellular targets. A DNA fragment containing full-length K7 was fused in-frame to the GAL4 DNA-binding domain for use as bait. The target cDNA library was from Epstein-Barr virus-transformed B lymphocytes. Transformants were plated on selective plates lacking Leu, Trp, Ade, and His. Colonies were transferred to plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-α-Gal), and colonies that yielded a dark blue color were recovered and analyzed further.

Of the 10^5 transformants that were tested for growth on the selective plates and for color development in the presence of X-α-Gal, 20 colonies showed moderate growth and were positive for β-galactosidase and α-galactosidase expression. DNA sequence analysis showed that 7 of the 20 K7-interacting clones contained various fragments of PLIC1, all of which included its carboxy-terminal region (Fig. 1A). Transformation of any of these PLIC1 cDNA clones with a negative control bait (pGBK7 vector or pGBK7-lamin C) neither supported growth on selective plates nor yielded color development on X-α-Gal, suggesting that PLIC1 is an authentic K7-binding protein (data not shown).

To confirm the interaction between K7 and PLIC1, the
FIG. 2. PLIC1 expression enhances K7 protein expression. (A) Proteasome-dependent degradation of K7. At 36 h posttransfection with V5-tagged K7 expression vector, 293T cells were treated or not with 50 μM MG132 for 6 h, and whole-cell lysates were then used for immunoblotting with an anti-V5 antibody. Arrows indicate the K7 proteins. (B) Extended half-life of the K7 lysine mutant. 293T cells were transfected with a vector expressing wild-type K7 or K7 mutants. (Left panels) At 36 h posttransfection, cells were treated or not with 50 μM MG132 for 6 h and then used for immunoblotting with an anti-V5 antibody. Lane 1, wild-type K7; lane 2, K7 (K7R); lane 3, K7 (K82R); lane 4, K7 (K2,92,97,103R); lane 5, K7 (K2,92,97,103R, K5R). (Right panels) At 36 h posttransfection, cells were treated with cycloheximide (20 μg/ml) for various times, and K7 protein was examined by immunoblotting with an anti-V5 antibody. (C) PLIC1 enhances K7 expression. (Left panels) 293T cells were cotransfected with the K7 expression vector with increasing amounts of PLIC1 expression vector. At 36 h posttransfection, cells were treated with 50 μM MG132 for 6 h (bottom) or not treated (top) and then used for immunoblotting to examine K7 expression. Arrows indicate K7 proteins. (Right panel) Upon expression with GST-UBA, K7 half-life was examined as described for panel B.
all five lysine residues of K7 are likely subjected to ubiquitination.

Overexpression of PLIC1 and PLIC2 isoforms has been shown to deregulate the expression of various cellular proteins (3, 29, 51), particularly IκB and p53, which are the substrates of the 26S proteasome (26). This phenomenon is likely due to the interference of excess PLIC in the coupling of ubiquitination and proteasome degradation. To explore the role of PLIC1 in K7 degradation, 293T cells were transfected with the HA-tagged ubiquitin expression vector alone or together with the Flag-tagged PLIC1 expression vector. At 36 h posttransfection, cells were either untreated (left panel) or treated with 50 μM MG132 for 10 h (right panel). (Top panels) We probed 20 μg of whole-cell lysates with an anti-HA antibody to examine the level of polyubiquitinated protein. (Bottom panels) Flag-PLIC1 expression was detected by immunoblotting with an anti-Flag antibody. Cell lysates were used in a pulldown assay with GST or GST-PLIC1 fusion proteins. (Top panel) Recovered protein complexes were analyzed by immunoblotting with a horseradish peroxidase-conjugated antibody. (Bottom panel) GST and GST-PLIC1 fusion protein used for the binding assay. Lane 1, 5% of input; lane 2, GST; lane 3, GST-PLIC1 (382 to 589); lane 4, GST-PLIC1 (541 to 589); lane 5, GST-PLIC1 (441 to 589); lane 6, GST-PLIC1 (441 to 540). (C) PLIC1 and PLIC2 bind to polyubiquitinated protein in living cells. 293T cells were cotransfected with the Flag-tagged PLIC1 or PLIC2 expression vector and the HA-ubiquitin expression vector. At 36 h posttransfection, cells were treated with 50 μM MG132 for 12 h. Whole-cell lysates were used for immunoprecipitation with an anti-Flag antibody. An anti-Myc antibody (lanes 3 and 5) was included as a negative control. (Top panel) Immunoprecipitates were analyzed by SDS-PAGE and reacted with an anti-HA horseradish peroxidase-conjugated antibody. (Bottom panel) The membrane was stripped and reprobed with an anti-Flag antibody to examine PLIC proteins in immunoprecipitates. Lane 1, PLIC1; lane 2, PLIC2. (D) PLIC1 binds to polyubiquitinated protein through the carboxy-terminal UBA domain in living cells. (Left panel) 293T cells were cotransfected with the HA-tagged ubiquitin expression vector and the Flag-tagged wild-type (wt) PLIC1 and its mutant expression vector. At 36 h posttransfection, cells were treated with 50 μM MG132 for 12 h before harvest. Whole-cell lysates were used for immunoprecipitation with an anti-Flag antibody, followed by immunoblotting with an anti-HA antibody. The bottom panel shows the expression of wild-type PLIC1 and its mutants. F, full-length PLIC1; ΔL, PLIC1 ΔUBL; ΔA, PLIC1 ΔUBA. The arrow indicates the heavy chain of immunoglobulin. (Right panel) 293T cells were cotransfected with the HA-ubiquitin expression vector and GST (lane 1) or GST-UBA (lane 2) expression vector. At 36 h posttransfection, cells were treated with 50 μM MG132 for an additional 12 h. Whole-cell lysates were subjected to glutathione chromatography, followed by immunoblotting with an anti-HA antibody. The bottom panel shows GST and GST-UBA expression.

results indicate that K7 undergoes ubiquitin-dependent proteasomal degradation and that its protein level is also modulated by an interaction with PLIC1.

PLIC1 binds to polyubiquitinated protein through the UBA domain. The expression of yeast homologs of PLIC, Dsk2p and Rad23, has been shown to induce the accumulation of polyubiquitinated proteins (7, 14). Furthermore, the UBA domain of Dsk2p and Rad23 has been shown to bind efficiently to polyubiquitinated proteins, which ultimately deliver ubiquitinated substrates for 26S proteasome degradation (7, 14). To test whether PLIC1 has a function similar to that of its yeast homologs, 293T cells were transfected with the HA-tagged ubiquitin expression vector or together with the PLIC1 expression vector. At 24 h posttransfection, these cells were incubated in the presence or absence of MG132 for an additional

FIG. 3. PLIC1 binds to polyubiquitinated proteins. (A) PLIC1 induces the accumulation of polyubiquitinated proteins. 293T cells were transfected with the HA-tagged ubiquitin expression vector alone or together with the Flag-tagged PLIC1 expression vector. At 36 h posttransfection, cells were either untreated (left panel) or treated with 50 μM MG132 for 10 h (right panel). (Top panels) We probed 20 μg of whole-cell lysates with an anti-HA antibody to examine the level of polyubiquitinated protein. (Bottom panels) Flag-PLIC1 expression was detected by immunoblotting with an anti-Flag antibody. (B) The carboxy-terminal UBA domain of PLIC1 binds to polyubiquitinated proteins. At 36 h posttransfection with the HA-tagged ubiquitin expression vector, 293T cells were treated with 50 μM MG132 for 12 h before harvest. Cell lysates were used in a pulldown assay with GST or GST-PLIC1 fusion proteins. (Top panel) Recovered protein complexes were analyzed by immunoblotting with a horseradish peroxidase-conjugated antibody. (Bottom panel) GST and GST-PLIC1 fusion protein used for the binding assay. Lane 1, 5% of input; lane 2, GST; lane 3, GST-PLIC1 (382 to 589); lane 4, GST-PLIC1 (541 to 589); lane 5, GST-PLIC1 (441 to 589); lane 6, GST-PLIC1 (441 to 540). (C) PLIC1 and PLIC2 bind to polyubiquitinated protein in living cells. 293T cells were cotransfected with the Flag-tagged PLIC1 or PLIC2 expression vector and the HA-ubiquitin expression vector. At 36 h posttransfection, cells were treated with 50 μM MG132 for 12 h. Whole-cell lysates were used for immunoprecipitation with an anti-Flag antibody. An anti-Myc antibody (lanes 3 and 5) was included as a negative control. (Top panel) Immunoprecipitates were analyzed by SDS-PAGE and reacted with an anti-HA horseradish peroxidase-conjugated antibody. (Bottom panel) The membrane was stripped and reprobed with an anti-Flag antibody to examine PLIC proteins in immunoprecipitates. Lane 1, PLIC1; lane 2, PLIC2. (D) PLIC1 binds to polyubiquitinated protein through the carboxy-terminal UBA domain in living cells. (Left panel) 293T cells were cotransfected with the HA-tagged ubiquitin expression vector and the Flag-tagged wild-type (wt) PLIC1 and its mutant expression vector. At 36 h posttransfection, cells were treated with 50 μM MG132 for 12 h before harvest. Whole-cell lysates were used for immunoprecipitation with an anti-Flag antibody, followed by immunoblotting with an anti-HA antibody. The bottom panel shows the expression of wild-type PLIC1 and its mutants. F, full-length PLIC1; ΔL, PLIC1 ΔUBL; ΔA, PLIC1 ΔUBA. The arrow indicates the heavy chain of immunoglobulin. (Right panel) 293T cells were cotransfected with the HA-ubiquitin expression vector and GST (lane 1) or GST-UBA (lane 2) expression vector. At 36 h posttransfection, cells were treated with 50 μM MG132 for an additional 12 h. Whole-cell lysates were subjected to glutathione chromatography, followed by immunoblotting with an anti-HA antibody. The bottom panel shows GST and GST-UBA expression.
12 h. As seen with Dsk2p and Rad23 (7, 14), PLIC1 expression induced the dramatic accumulation of polyubiquitinated proteins, which was not significantly dependent on MG132 treatment (Fig. 3A).

To examine whether the UBA domain of PLIC1 was capable of binding to polyubiquitinated proteins, 293T cells were transfected with the HA-tagged ubiquitin expression vector. At 48 h posttransfection, cell lysates were mixed with GST-PLIC1 fusion proteins, followed by immunoblotting with an anti-HA antibody. The blot showed that GST-PLIC1 fusion proteins containing the UBA domain were capable of efficiently binding to polyubiquitinated proteins, whereas GST and GST-PLIC1 (441 to 540) were not under the same conditions (Fig. 3B). The interaction of full-length PLIC1/2 or the GST-UBA fusion with polyubiquitinated proteins was further demonstrated in living cells by coimmunoprecipitation and glutathione chromatography (Fig. 3C and D). Furthermore, PLIC1 mutants with a deletion of the amino-terminal UBL or the carboxy-terminal UBA were constructed and called PLIC1 ΔL and PLIC1 ΔA, respectively. These mutants were also tested for their interaction with polyubiquitinated proteins in cells. The results showed that wild-type PLIC1 and PLIC1 ΔL were capable of efficiently binding to polyubiquitinated proteins, whereas PLIC1 ΔA was not under the same conditions (Fig. 3D). Interestingly, the PLIC1 ΔL mutant had a higher affinity for polyubiquitinated protein than wild-type PLIC1. These results demonstrate that, as seen with yeast homologs (7, 14), mammalian PLIC1 is a polyubiquitin-binding protein and the carboxy UBA domain of PLIC1 is responsible for this activity.

Effect of K7 on PLIC1 dimerization and polyubiquitin binding. Structural analysis has demonstrated that the UBA domain has a three-helix bundle and forms a homodimer (4, 31). To examine the potential dimerization of PLIC1, cell lysates containing Flag-tagged PLIC1 were mixed with GST, GST-PLIC1 (382 to 589), GST-PLIC1 (441 to 589), and GST-PLIC1 (441 to 540), followed by immunoblotting with an anti-Flag antibody. The blot showed that GST-PLIC1 (382 to 589) and GST-PLIC1 (441 to 589) were capable of binding to PLIC1, whereas GST, GST-PLIC1 (541 to 589), and GST-PLIC1 (441 to 540) were not (Fig. 4A). This indicates that, besides the carboxy UBA domain, the additional region of PLIC1 that precedes the UBA domain is necessary for its homodimerization. Further mutational analysis showed that the carboxy-terminal UBA domain but not the amino-terminal UBL domain was required for homodimerization of PLIC1 in 293T cells (Fig. 4B). In addition, the heterodimerization between PLIC1 and PLIC2 was also readily detected in transfected cells (Fig. 4B).

Since K7 interacted efficiently with the carboxy-terminal UBA domain, its expression potentially affected the homodimerization of PLIC. To test this, 293T cells transfected with PLIC1 and/or the K7 expression vector were used for the GST pulldown assay with GST-PLIC1 (382 to 589). This assay showed that K7 expression considerably diminished the homodimerization of PLIC1 (Fig. 4C). To further confirm this result in mammalian cells, 293T cells were transfected with Xpress-tagged PLIC1 and Flag-tagged PLIC1 in the presence of increasing amounts of K7 expression vector. At 48 h posttransfection, cell lysates were used for immunoprecipitation with an anti-Flag antibody, followed by immunoblotting with an anti-Xpress antibody to detect the homodimerization of PLIC1. An anti-HA antibody was included in the immunoprecipitation as a negative control. As seen in an in vitro GST pulldown assay, K7 expression considerably reduced PLIC1 homodimerization in a dose-dependent manner (Fig. 4C).

Finally, we examined the effect of K7 expression on the polyubiquitin binding activity of PLIC1. 293T cells were individually transfected with the HA-tagged ubiquitin expression vector or with the K7 expression vector (Fig. 4D, left panel). Cell lysates containing HA-ubiquitin were mixed with increasing amounts of cell lysates containing K7, and mixed cell lysates were then used for the GST or GST-PLIC1 binding assay, followed by immunoblotting with an anti-HA antibody. The increasing amount of K7 led to a decreased level of polyubiquitinated proteins recovered by GST-PLIC1 (382 to 589) (Fig. 4D, left panel). Furthermore, HA-ubiquitin and Flag-tagged PLIC1 were also expressed in the presence and absence of K7 in 293T cells, and their lysates were used for immunoprecipitation with an anti-Flag antibody, followed by immunoblotting with an anti-HA antibody. The PLIC1 ΔA mutant was included as a negative control for ubiquitin binding (Fig. 4D, lane 1, right panel). This result also showed that K7 expression detectably suppressed the ability of PLIC1 to bind to polyubiquitinated proteins (Fig. 4D, right panel). These results collectively indicate that K7 expression suppresses the homodimerization and polyubiquitin binding activity of PLIC1.

K7 expression accelerates ubiquitin-dependent protein degradation. To examine the effect of K7 on proteasome-mediated protein degradation, we used GFP-based substrate fusion proteins, which allow rapid quantification of ubiquitin- and proteasome-dependent proteolysis in living cells (9). Ubiquitin-M-GFP contains no degradation signal and was therefore expected to be as stable as unmodified GFP. Ubiquitin-R-GFP contains an arginine residue at the amino terminus as the N-end rule–destabilizing amino acid signal, and ubiquitin-P-GFP contains a poorly cleavable ubiquitin as a ubiquitin fusion degradation signal (9). Both ubiquitin-R-GFP and ubiquitin-P-GFP were expected to be unstable because of their degradation signals. 293T cells were transfected with a set of ubiquitin-GFP fusion constructs in the presence of K7 and/or PLIC1. At 48 h posttransfection, the level of GFP expression was examined by immunoblotting and flow cytometry.

K7 expression resulted in a three- to fourfold reduction in the number of GFP-positive cells transfected with the ubiquitin-M-GFP, ubiquitin-P-GFP, or ubiquitin-R-GFP expression vector, whereas PLIC1 expression resulted in an approximately twofold increase in the number of GFP-positive cells under the same conditions (Fig. 5). In particular, the mean fluorescence intensity of the GFP-positive population of transfected cells was considerably increased by PLIC1 expression, whereas it was detectably reduced by K7 expression (Fig. 5). Finally, upon coexpression of K7 and PLIC1, K7 expression significantly antagonized PLIC1 activity in the degradation of ubiquitin-GFP protein, resulting in a mean fluorescence intensity that was similar to that of vector-transfected cells (Fig. 5). These results indicate that PLIC1 expression suppresses ubiquitin- and proteasome-dependent protein degradation, whereas K7 expression likely antagonizes PLIC1 activity.

Enhanced degradation of p53 and IκB by K7 expression. In order to cope with changes in the environment, the ubiquitin-
proteasome system is activated to degrade various cellular proteins, such as IκB and p53. Expression of PLIC isoforms has been shown to interfere with this process: specifically, PLIC1 expression inhibits the degradation of p53 and IκB (26). To investigate whether K7 affects the PLIC1-mediated inhibition of IκB degradation, 293T cells were transfected with the PLIC1 expression vector with or without the K7 expression vector. At 48 h posttransfection, cells were treated with TNF-α for various times to induce IκB degradation, followed by immunoblotting with an anti-IκB antibody. To further delineate PLIC1’s effect on IκB degradation, PLIC1 mutants were included: PLIC1 ΔUBL (with a deletion of the amino-terminal UBL domain), PLIC1 ΔUBA (with a deletion of the carboxy-terminal UBA domain), PLIC1 (UBL) (containing the aminoterminal UBL domain only), and GST-UBA fusion (containing the carboxy-terminal UBA domain only).

As shown previously (26), IκB underwent rapid degradation upon TNF-α treatment. The expression of wild-type PLIC1, PLIC1 ΔUBL, and GST-UBA significantly blocked IκB degradation upon TNF-α treatment, whereas the PLIC1 (UBL) and PLIC1 ΔUBA mutants were not able to do so under the same conditions (Fig. 6A). This indicates that the carboxy-terminal UBA domain is necessary and sufficient to block the IκB degradation induced by TNF-α treatment. In contrast, K7
expression induced the rapid degradation of IxB protein upon TNF-α treatment (Fig. 6A). Furthermore, when K7 and PLIC1 were coexpressed, K7 was capable of strongly antagonizing PLIC1 activity, resulting in enhanced degradation of IxB (Fig. 6A). Despite its efficient interaction, K7 had a minimal effect on the ability of PLIC1 UBL and GST-UBA to block IxB degradation, suggesting that full-length PLIC1 is necessary for the K7-mediated increase in IxB degradation (Fig. 6A). K7 and both the wild-type and mutant forms of PLIC1 were expressed at equivalent levels under these conditions (Fig. 6A).

To further examine the effect of K7 on the level of p53 tumor suppressor that is regulated by ubiquitin-dependent proteasomal degradation, K7 was expressed in KSHV-infected BCBL-1 cells or KSHV-negative BJAB B cells with the FLP recombinase target (FRT)-FLP recombination system (34). BJAB-FRT and BJAB-K7 cells were treated with cycloheximide, and the p53 protein level was examined by immunoblotting with anti-p53 antibody. The p53 protein was highly stable in BJAB-FRT cells; no significant reduction in p53 protein was detected after 45 min of cycloheximide treatment (Fig. 6B). In contrast, p53 protein was detectably reduced in BJAB cells expressing K7 after 45 min of cycloheximide treatment (Fig. 6B). Furthermore, K7 expression in both BJAB cells and KSHV-infected BCBL-1 cells significantly blocked the etoposide-induced apoptosis that was dependent on p53 activity (Fig. 6C). These results suggest that K7 antagonizes PLIC1 activity, resulting in enhanced degradation of p53 and thereby suppression of p53-dependent apoptosis.

**DISCUSSION**

We have demonstrated that the KSHV K7 protein interacts with the cellular PLIC1 protein through the hydrophobic region of K7 and the carboxy-terminal UBA domain of PLIC1. We showed that this interaction dramatically affects PLIC1’s function in ubiquitin-dependent proteasomal degradation. PLIC1 bound efficiently to polyubiquitinated proteins through its carboxy-terminal UBA domain, and this activity correlated with its ability to stabilize IxB. In contrast, K7 interaction antagonized PLIC1 activity, resulting in rapid degradation of IxB and p53. As a consequence, K7 expression efficiently blocked p53-dependent apoptosis. Vpr of human immunodeficiency virus type 1 has been shown to interact with the UBA domain of HHR23A, the human Rad23A homolog (49). A recent report demonstrated that hepatitis C virus RNA-dependent RNA polymerase interacts with PLIC1 and that this interaction significantly reduces the half-life of this polymerase (17). In contrast, we have found that PLIC interaction with K7 considerably increases K7 expression. Interestingly, PLIC expression stabilized the multiple forms of K7 protein, including unmodified, glycosylated, and ubiquitinated forms, whereas GST-PLIC1 UBA strongly stabilized only the unmodified K7 protein. This suggests that GST-PLIC1 UBA may bind preferentially to the unmodified K7, which prevents its glycosylation and ubiquitination. Nevertheless, this suggests that while PLIC1 is likely a common cellular target for viral proteins, it may have different effects on different viral proteins.

UBA domains are a recurring sequence motif of approximately 45 amino acid residues that are found in diverse proteins involved in the ubiquitin and proteasome pathway, DNA excision repair, and cell signaling via protein kinases (5, 28, 43). The UBA domains of Dsk2p and Rad23, which are yeast homologues of PLIC, have been shown to bind to polyubiquitinated proteins (8, 14). We demonstrated that PLIC1 also binds to polyubiquitin binding proteins and that this binding activity is attributed to its carboxy-terminal UBA domain. The UBA domain has been shown to form a compact three-helix bundle and adopt a module with an apparent hydrophobic surface, which presumably mediates hydrophobic interactions (31). Furthermore, the hydrophobic surface patch of UBA domains has been predicted to interact with the hydrophobic surface on the five-stranded beta-sheet of ubiquitin or the UBL domain (42). We found that, correlated with this, the central
leucine-rich hydrophobic region of K7 interacted with the UBA domain of PLIC1, suggesting that PLIC1 may interact with K7 and with ubiquitin in a similar way (31, 46).

While the specific function of UBL- and UBA-containing PLIC and its homologues remains largely controversial, the general consensus is that these proteins probably have regulatory roles during ubiquitin- and proteasome-mediated protein degradation. However, the functional activity of PLIC1 toward interacting proteins differs dramatically. PLIC1 and its homologues have been shown to enhance the protein expression of presenilin and the /H9253-aminobutyric acid receptor (3, 29), exhibit no effect on mTOR protein (51), and induce the degradation of cyclin A and hepatitis C virus RNA-dependent RNA polymerase proteins (13, 17). The best-characterized UBL and UBA domain-containing protein, Rad23, has been demonstrated to form homodimers and heterodimers via its UBA domains (4). Interestingly, the homodimer form of Rad23 is unable to bind to polyubiquitin chains, raising the possibility that dimerization through the UBA domain is crucial for regulating Rad23 activity (4).

We demonstrated that while PLIC1 also formed a dimer through the carboxy-terminal UBA domain, the additional 30 amino acids preceding the UBA domain were also required for this activity. We also found that K7 interacted with the carboxy-terminal UBA domain of PLIC1, which ultimately inhibited the homodimerization and polyubiquitin-binding activities of PLIC1. This indicates that the dimerization of each PLIC homologue contributes to the polyubiquitin binding activity in different ways: the dimerization of Rad23 blocks its polyubiquitin-binding activity (4), whereas the dimerization of PLIC1 likely enhances its polyubiquitin-binding activity. Similarly, the CUE domain of the Vps9p dimer has been proposed to bind to...
mono-ubiquitin, while the CUE domain of Vps9p monomer is not able to do so due to its lower affinity for single ubiquitins (23, 38). Nevertheless, this suggests that the equilibrium between monomer and dimer may be a key factor in regulating the polyubiquitin-binding activity of UBA domain-containing proteins. Thus, the inhibition of PLIC1 dimerization induced by K7 may be an important regulatory step to controlling its activity in ubiquitin-dependent protein degradation.

PLIC1 expression has been shown to interfere with the degradation of two model substrates of the 26S proteasome, p53 and IxB (26). We also showed that K7 induced IxB and p53 degradation. Consequently, K7 expression significantly inhibited p53-mediated apoptosis. By contrast, K7 expression showed little or no induction of NF-kB activity (data not shown). This indicates that despite its pleotropic effect on cellular protein stability, K7 biological activity may be more apparent in certain cellular signaling pathways, such as apoptosis. To further explore the potential contributions of K7 and PLIC1 in protein degradation, we used a set of GFP-based ubiquitin fusion proteins as substrates that allowed rapid quantification of ubiquitin- and proteasome-dependent proteolysis in living cells. This assay also showed that K7 expression facilitated the ubiquitin- and proteasome-dependent degradation of ubiquitin-GFP fusion proteins, whereas PLIC1 expression suppressed the ubiquitin- and proteasome-dependent protein degradation of ubiquitin-GFP fusions, consistent with the reported activity of PLIC1 (3, 26, 29).

Interestingly, the expression of PLIC1 and K7 appeared to modulate the protein degradation induced by both the N-end rule destabilization signal and ubiquitin fusion degradation signal (Fig. 5). This finding suggests that the K7-PLIC1 interaction has a broad spectrum of activity in protein degradation pathways. Consistent with this possibility, a yeast PLIC2 homolog (Chap1) has been shown to interact with the ATPase domain of Stch, an HSP70-like chaperone (24). Moreover, PLIC1 has also been shown to physically associate with oxidative-reductase-protein disulfide isomerase, suggesting its role in the endoplasmic reticulum’s unfolded-protein response (27).

Nevertheless, these results indicated that K7 antagonizes some, if not all, activities of PLIC1 associated with its UBA domain.

Consistent with a recent report, our results collectively suggested that PLIC1 functions as a negative regulator of ubiquitin- and proteasome-mediated protein degradation, while the KSHV K7 protein antagonizes its activity by binding to the UBA domain of PLIC1. Though PLIC proteins are structurally similar to Rad23, there are several differences between PLIC1 and Rad23 activity. First, Rad23 expression stabilizes model substrates, presumably through inhibition of polyubiquitin assembly and sequestration of polyubiquitinated proteins (8, 35), whereas PLIC1 expression induces the accumulation of polyubiquitinated proteins (Fig. 4C), suggesting that these proteins may regulate protein degradation in a similar but distinct way. Second, the expression of the amino-terminal UBL domain of PLIC1 has no effect on p53 and IxB degradation, while expression of the amino-terminal UBL domain of Rad23 exhibits remarkable interference with S. cerevisiae β-galactosidase degradation (7). Thus, while UBL- and UBA-containing proteins regulate the translocation of proteolytic substrates to the proteasome, the detailed molecular mechanisms of their actions may differ in various ways.

Pathogens exploit host machinery to establish an environment that ultimately favors their propagation (15, 16, 30). Because of the pivotal roles of the protein degradation machinery in cellular physiology, numerous viral proteins have been shown to selectively manipulate various components of this machinery (45, 52). In fact, p53 degradation has been a frequent target of a number of viral proteins, including human papilloma virus E6 (44), adenovirus E1B and E4 (6, 20, 41), and KSHV latent nuclear antigen and vIRF1 (18, 30, 33, 40).

We and others have previously shown that KSHV K7 functions as an antia apoptotic factor to efficiently protect cells from various damaging signals during KSHV lytic replication (12, 47). Here, we also demonstrate that K7 plays a role in protein degradation by interacting with the UBL- and UBA-containing PLIC1 protein. This indicates that KSHV K7 protein may not only protect cells from various types of damage but also regulate the cellular protein degradation machinery, which ultimately provides a favorable environment for viral replication and allows the completion of the viral life cycle. Future study of the molecular mechanisms of protein degradation modulated by KSHV K7 will lead to a better understanding of viral persistence and disease progression and also provide a novel means for investigating cellular regulatory systems.

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