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Kaposi's Sarcoma-Associated Herpesvirus Transactivator RTA Promotes Degradation of the Repressors To Regulate Viral Lytic Replication[▽]

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Kaposi's sarcoma-associated herpesvirus/human herpesvirus 8 (KSHV/HHV-8) RTA is an important protein involved in the induction of KSHV lytic replication from latency through activation of the lytic cascade. A number of cellular and viral proteins, including K-RBP, have been found to repress RTA-mediated transactivation and KSHV lytic replication. However, it is unclear as to how RTA overcomes the suppression during lytic reactivation. In this study, we found that RTA can induce K-RBP degradation through the ubiquitin-proteasome pathway and that two regions in RTA are responsible. Moreover, we found that RTA can promote the degradation of several other RTA repressors. RTA mutants that are defective in inducing K-RBP degradation cannot activate RTA responsive promoter as efficiently as wild-type RTA. Interference of the ubiquitin-proteasome pathway affected RTA-mediated transactivation and KSHV reactivation from latency. Our results suggest that KSHV RTA can stimulate the turnover of repressors to modulate viral reactivation. Since herpes simplex virus type 1 transactivator ICP0 and human cytomegalovirus transactivator pp71 also stimulate the degradation of cellular silencers, it is possible that the promotion of silencer degradation by viral transactivators may be a common mechanism for regulating the lytic replication of herpesviruses.

Kaposi's sarcoma-associated herpesvirus (KSHV)/human herpesvirus 8 (HHV-8) is a recently discovered human gammaherpesvirus that is frequently found in AIDS patients (8). KSHV plays an important role in the development of Kaposi's sarcoma (KS), which is the most common malignancy in AIDS patients (14). KSHV is not only the etiological agent of KS but is also associated with two other lymphoproliferative disorders, primary effusion lymphoma and multicentric Castleman's disease in human immunodeficiency virus type 1-infected individuals (6, 36). Similar to other herpesviruses, there are two key stages in the KSHV life cycle, the establishment of latency and reactivation from latency, which lead to lytic replication (22, 33). Even though latency enables the virus to establish persistent infection and plays a critical role in tumorigenesis (31), lytic reactivation also contributes significantly to the development of disease, either through the spread of infection to new target cells or by activating the expression of cytokines, such as interlukin-6 (10, 15). The transition from latency to lytic replication is controlled by the KSHV replication and transcription activator (RTA), encoded by KSHV gene open reading frame 50 (ORF50) (29, 37). RTA expression is necessary and sufficient to disrupt viral latency and induce lytic replication. It has been shown that RTA is able to activate the expression of a number of viral genes during its lytic replication, including the early gene ORF57 (11, 28, 42).

The transactivation function of RTA was shown to be suppressed by a number of viral and cellular factors, which limits the extent of lytic replication and leads to latent and persistent infection. These cellular and viral factors, including histone deacetylase 1 (HDAC1), poly(ADP-ribose) polymerase 1 (PARP-1), Ste20-like kinase hKFC, interferon regulatory factor 7 (IRF-7), K-RBP (for KSHV-RTA binding protein), nuclear factor-κB (NF-κB), KbZIP (or K8), and latent nuclear antigen (LANA) have been shown to downregulate RTA-mediated transactivation through different mechanisms. The downmodulation of RTA-mediated transactivation by these factors also plays an important role in suppressing RTA-mediated KSHV lytic replication (2, 16, 17, 21, 25, 39, 43). However, RTA can still efficiently activate the expression of its target genes to induce KSHV lytic replication when needed, even in the presence of the many identified and perhaps other unidentified repressors. How KSHV RTA can overcome these barriers, and the mechanism involved in maintaining a balance between lytic replication and latency is unclear.

In the past decade, a number of viral proteins were found to direct host cell protein degradation through the proteasome, and the degradation is required for various aspects of viral life cycle (13). The proteasome is a multicomponent macromolecule that is ubiquitous in eukaryotic cells and works as a cellular machinery for degradation of proteins (38). The proteasome degradation is usually ubiquitin dependent. Polyubiquitin is conjugated to the target proteins through a process involving ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2, and ubiquitin ligase E3 (19). For herpesviruses, the transactivators of the alphaherpesvirus herpes simplex virus type 1 (HSV-1) and the betaherpesvirus human cytomegalovirus (HCMV) have been shown to promote cellular protein degradation through proteasomes (1, 12, 24). The HSV-1 transactivator ICP0 is required for efficient initiation of viral lytic infection and reactivation from latency (5). In the absence of functional ICP0 expression, the cellular repression mechanism silences viral transcription (23). ICP0 is thought to

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counteract this process by stimulating the degradation of a number of cellular repressor proteins via the ubiquitin-proteasome pathway (12). ICP0 harbors two distinct E3 ubiquitin ligase domains (HUL-1 and RING finger), and both are involved in mediating ubiquitination and degradation (1, 18). For HCMV, the transactivator pp71 was found to direct cellular pRb, p107, p130, and Daxx degradation through a ubiquitin-independent proteasome pathway. The degradation of these transcriptional repressors was suggested to enhance viral transcription and/or reactivation (20, 24).

It is likely that KSHV RTA also uses similar mechanism for its transactivation function. Both IRF-7 and K-RBP have been shown to suppress RTA-mediated transactivation and gene expression in our studies (39, 43). Recently, RTA was shown to induce IRF-7 degradation to evade the innate immune response mediated by IRF-7 (44). In the present study, we demonstrate that RTA can promote K-RBP degradation through the ubiquitin-proteasome pathway. Moreover, RTA can downregulate several other repressors via the proteasome pathway. We further demonstrate that efficient transactivation and reactivation of viral lytic replication by RTA require the ability of RTA to induce degradation. Our results suggest for the first time that the regulation of KSHV transition between latency and lytic reactivation involves a balance between repression of lytic viral gene expression by repressors and the degradation of repressors by viral transactivators. In concert with the studies on HSV-1 and HCMV, our studies suggest that KSHV RTA shares a common mechanism in the degradation of repressors to lead to efficient viral transcription, lytic infection, and reactivation. This may be a mechanism used by a number of other herpesviruses, and a further understanding of such mechanism in herpesvirus pathogenesis may lead to new strategies to treat herpesvirus-related diseases.

MATERIALS AND METHODS

Plasmids. The RTA expression plasmids pcDNAORF50 and pCMVTag50, which encode full-length RTA and Flag-tagged RTA, respectively, were described previously (40, 41). Plasmids pCMVTag50678, pCMVTag50665, pCMVTag50641, and pCMVTag50621, which encode Flag-tagged RTA amino acids 1 to 678, 1 to 665, 1 to 641, and 1 to 621, respectively, were generated by inserting the PCR amplified corresponding DNA fragments into suitable sites of pCMV-Tag2A. Plasmid pcDNA50K_{152}E was described elsewhere (45) and was kindly provided by Luwen Zhang (University of Nebraska, Lincoln). Several RTA mutant plasmids of pcDNA-ORF50 and pCMV-Tag50 were generated by using a QuikChange II site-directed mutagenesis kit (Stratagene, La Jolla, CA). The oligonucleotide primers used for mutant plasmids pcDNA50H_{145}L and pcDNA50C_{141}S were as follows: C_{141}S sense, 5′-GCCTGCCTCCAGCCATAT**C**TAAGCTACTACACGAAATATAC-3′; C_{141}S antisense, 5′-GTATATTTCGTGTAGTAGCTTA**G**ATATGGCTGGAGGCAGGC-3′; H_{145}L sense, 5′-GCCATATGTAAGCTACTAC**T**CGAAATATACACTGAAATG-3′; and H_{145}L antisense, 5′-CATTTCAGTGATTATTTCG**A**GTAGTAGCTTACATATGGC-3′. The oligonucleotide primers used for generating pCMVTag50m627-30, pCMVTag50m631-34, and pCMVTag50m635-38 were as follows: mRTA627-30 sense, 5′-CTGTACCAG**C**TGG**C**C**G**CG**G**CA**G**CGCCTCTGCGGTCAC-3′; mRTA627-30 antisense, 5′-GTGACCGCAGAGGCG**C**TG**C**CG**C**G**G**CCAGCTGGTACAG-3′; mRTA631-34 sense, 5′-CTGGACACGCCACCG**G**CT**G**C**G**G**C**G**G**CACCCTCCCCCG**C**TTC-3′; mRTA631-34 antisense, 5′-GAAGCGGGGGAGGGTG**C**C**G**CC**G**C**A**G**C**CGGTGGCGTGTCCAG-3′; mRTA635-38 sense, 5′-CCGCCTCTGCGGTCA**G**CC**G**CC**G**CC**A**CTTCCTTCGGCCCG-3′; and mRTA635-38 antisense, 5′-CGGGCCGAAGGAAG**T**GG**C**GG**C**GG**C**TGACCGCAGAGGCGG-3′. Nucleotides in boldface indicate the mutated sites.

Plasmids pcDNAK-RBP and pcDNAHisK-RBP, which encode full-length and His_6-tagged K-RBP, respectively, were described elsewhere (41). Plasmids pGFPLANA, pFlagKbZIP, and pcDNANF-κB p65 encoding green fluorescent protein (GFP)-tagged LANA, Flag-tagged KbZIP, and NF-κB p65 subunit were kind gifts from Kenneth M. Kaye (Harvard Medical School, Boston, MA), Hsing-Jien Kung (University of California, Davis), and Thomas M. Petro (University of Nebraska Medical Center), respectively. Plasmid pMT123, which encodes hemagglutinin (HA)-tagged ubiquitin was obtained from Dirk Bohmann (University of Rochester, Rochester, NY). The β-galactosidase expression plasmid pCMV-β used for the normalization of transfection efficiency was purchased from BD Clontech (Mountain View, CA). ORF57 promoter reporter plasmid p57Pluc1 was described previously (11).

All clones with inserts that were amplified by PCR were confirmed by DNA sequence analysis.

Antibodies and inhibitors. The anti-K-RBP antibody has been described previously (43) or was purchased (PTGLAB, Chicago, IL). The RTA antibody was prepared by immunizing rabbit with insect cell expressed recombinant RTA (Lampire Biological Laboratories, Pipersville, PA). The mouse monoclonal anti-NF-κB p65 antibody and the rabbit anti-GALBD polyclonal antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The mouse anti-γ-tubulin monoclonal antibody was purchased from Sigma (St. Louis, MO). The mouse anti-Flag M2 monoclonal antibody was purchased from Stratagene. The rabbit anti-HA polyclonal antibody and mouse anti-His monoclonal antibody were purchased from BD Clontech. The rat anti-KSHV LANA monoclonal antibody was purchased from Advanced Biotechnologies, Inc. (Silverdale, WA). The mouse anti-KSHV K8 and anti-KSHV ORF45 monoclonal antibodies were purchased from Novus Biologicals (Littleton, CO). The mouse anti-KSHV K8.1 monoclonal antibody was obtained from Bala Chandran (Rosalind Franklin University, Chicago, IL). MG132, lactacystin, chloroquine, and leupeptin were purchased from Sigma.

Cell culture, transfection, and luciferase assays. Human 293T cells were maintained in Dulbecco modified Eagle medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and 100 μg penicillin-streptomycin (Mediatech, Manassas, VA)/ml at 37°C with 5% CO_2. The Ts20 cell line, which has a temperature-sensitive E1 ubiquitin-activating enzyme that is inactive at 39°C, was obtained from Harvey Ozer (New Jersey Medical School) (9). This cell line was maintained in Dulbecco modified Eagle medium supplemented with 10% FBS with 5% CO_2 at 35 or 39°C. Transfection of 293T cells and Ts20 cells was carried out by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's recommendations. Luciferase activities were determined by a luciferase assay system (Promega, Madison, WI) according to the manufacturer's procedure. The data were averaged from the results of at least three independent experiments. The transfection efficiency for each experiment was normalized by cotransfecting β-Gal expression plasmid pCMV-β as the internal control. BJAB is a KSHV-negative cell line. BCBL-1 is a KSHV-positive primary effusion lymphoma cell line. TRExBJABRTA, TRExBJAB, TRExBCBL-1RTA, and TRExBCBL-1 are BJAB and BCBL-1 cell lines with or without a tetracycline- (or doxycycline)-inducible RTA gene (7, 32). These cell lines were provided by Jae Jung (Harvard Medical School). These cell lines were maintained in RPMI 1640 (Invitrogen) containing 10% FBS, 100 μg of penicillin-streptomycin/ml, and 200 μg of hygromycin B/ml at 37°C with 5% CO_2.

IP and Western blot analysis. Cells transfected with various expression plasmids were harvested and lysed in ice-cold immunoprecipitation (IP) buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, protease inhibitor cocktail [Pierce], and 1 mM phenylmethylsulfonyl fluoride in phosphate-buffered saline) at 48 h posttransfection. MG132 at 5 μM was used to treat cells for 12 h when needed. After centrifugation at 16,000 × *g* for 10 min, the cell lysates were precipitated with 2 to 3 μg of specific antibodies, followed by incubation overnight at 4°C. Protein G beads (Amersham, Piscataway, NJ) were used to catch antibody-protein complex at 4°C for 2 h. The beads were then washed four times with IP buffer. The immunoprecipitated proteins were eluted by heating in sample buffer, analyzed by sodium dodecyl sulfate–7 to 10% polyacrylamide gel electrophoresis, and transferred to a polyvinylidene difluoride membrane (Amersham) for Western blot analysis as described previously (43). The amounts of proteins relative to control tubulin were quantified by using the NIH ImageJ software.

In vitro ubiquitin conjugation assay. The reaction (20 μl) was performed in buffer (25 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, dithiothreitol, NaCl, 50 nM MG132), 40 ng of purified E1 protein (Boston Biochem, Cambridge, MA), 200 ng of UbcH5a protein (Boston Biochem), 4 μg of human flag-ubiquitin (Boston Biochem), 5 μM Ub aldehyde (Boston Biochem), 1× ATP energy solution (Boston Biochem), and appropriate amounts of *E. coli*-expressed purified His-tagged K-RBP protein. To test the E3 ubiquitin ligase activity of RTA, RTA expressed and purified from insect cells or expressed from a TNT-coupled transcription and translation kit (Promega) was used. Reaction mixtures were incubated for 2.5 to 3 h at 37°C with agitation. The reaction solution was resolved

by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7% gel and detected by Western blot analysis.

RNA extraction and RT-PCR. Total cellular RNA was extracted by using RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's procedure. Reverse transcription-PCR (RT-PCR) was performed by using a SuperScript III One-Step RT-PCR System (Invitrogen) according to the manufacturer's instructions. Quantitative RT-PCR was performed by using a iScript One-Step RT-PCR kit with Sybr green (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. The relative amounts of DNA were calculated by using Bio-Rad iCycler software (version 3.1; Bio-Rad Laboratories).

Protein stability analysis. For the analysis of the protein stability, 293T cells transfected with plasmids expressing different proteins of interest were treated with 75 μg of cycloheximide (Sigma)/ml at 24 h after transfection. The cells were then harvested at various time points after cycloheximide treatment, the proteins of interest were analyzed by Western blotting to determine the amount of proteins at various time points using specific antibodies, and the amounts of protein detected were quantified by using the NIH ImageJ software.

RESULTS

RTA expression promotes K-RBP degradation. We have previously demonstrated that K-RBP interacts with KSHV RTA (41) and functions as a repressor for RTA-mediated transactivation to negatively regulate RTA-mediated KSHV lytic replication in KSHV-infected cells (43). During the course of our studies, we frequently observed that the K-RBP protein levels were much lower in the presence of RTA. Further study demonstrated that K-RBP protein levels were reduced when increasing amounts of RTA were coexpressed, and very low level of RTA was sufficient to reduce K-RBP steady-state levels (Fig. 1A). Interestingly, there was a portion of K-RBP protein that is resistant to RTA-induced reduction since even a very high level of RTA expression could not completely remove all of the K-RBP protein (Fig. 1A). This effect was specific since the levels of the control tubulin protein remained unchanged. In addition, a cotransfected GAL4BD protein levels were not reduced by RTA expression. The GAL4BD levels were even increased when a high level of RTA was expressed (Fig. 1A). The downregulation of K-RBP by RTA is not at the transcriptional level because similar levels of K-RBP mRNA were detected in cells that were cotransfected with both K-RBP and RTA expression plasmids compared to cells transfected with K-RBP and control plasmids (Fig. 1B). These results suggest that RTA expression reduces the steady-state levels of K-RBP protein at the posttranslational level. We then examined the turnover rate of K-RBP in the presence or absence of RTA. The levels of K-RBP in 293T cells cotransfected with RTA or control plasmids, in the presence of cycloheximide treatment to prevent new protein synthesis, were determined at different time points after cycloheximide treatment using tubulin as a control. The turnover rate of K-RBP was found to be higher in the presence of RTA expression (Fig. 1C). The half-life of K-RBP in the presence of RTA was about 40 min compared to about 80 min in its absence. Taken together, these results demonstrate that RTA can enhance the K-RBP turnover rate.

We next examined whether the endogenous K-RBP protein level was also reduced when RTA was expressed in TRExB JABRTA cells, in which the His-tagged RTA gene is integrated into the genome, and RTA expression can be induced by doxycycline or tetracycline (7). After induction, the levels of K-RBP and a control protein tubulin were examined at various time points by Western blot analysis. The results showed that the K-RBP protein levels were reduced upon RTA expression, but the levels of tubulin were unaffected (Fig. 1D). With the control TRExBJAB cells without the integrated RTA gene, K-RBP levels were found to remain constant through the studied period (Fig. 1D). Our results suggest that the reduction of K-RBP protein is due to RTA expression and not a nonspecific effect induced by doxycycline.

K-RBP degradation is mediated by the ubiquitin-proteasome pathway. To determine the mechanism responsible for the degradation of K-RBP in the presence of RTA, different inhibitors specific for either lysosome- or proteasome-dependent degradation were used to treat 293T cells transfected with K-RBP and RTA expression plasmids. Chloroquine and leupeptin, which are lysosome-dependent degradation inhibitors, did not block RTA-mediated K-RBP turnover, whereas the proteasome inhibitor MG132 blocked the degradation of K-RBP when RTA was coexpressed (Fig. 2A). Our results suggest that RTA promotes K-RBP degradation via the proteasome-dependent pathway. To further study the mechanism involved in RTA-mediated K-RBP degradation, we investigated whether K-RBP undergoes normal proteasomal degradation in the absence of RTA expression. 293T cells transfected with K-RBP expression plasmid were treated with MG132 or vehicle control dimethyl sulfoxide (DMSO) and then treated with cycloheximide. K-RBP levels were determined by Western blot analysis at various time points. The results showed that the K-RBP turnover rate was much slower in MG132-treated cells (Fig. 2B), suggesting that K-RBP undergoes normal proteasomal degradation but the presence of RTA accelerates the process.

Ubiquitination of target proteins and subsequent degradation by the proteasome is a well-characterized pathway for proteasome-dependent degradation. Since K-RBP appears to be degraded via the proteasome pathway, we investigated whether K-RBP can be modified by ubiquitin. 293T cells were cotransfected with His-tagged K-RBP together with HA-tagged ubiquitin in the presence or absence of MG132 treatment. The His-tagged K-RBP protein was immunoprecipitated with anti-His antibody and analyzed by immunoblotting with anti-K-RBP serum to detect all K-RBP species (Fig. 3A) or with an anti-HA antibody to specifically detect ubiquitinated K-RBP (Fig. 3B). In both cases, higher-molecular-weight ubiquitinated K-RBP molecules were detected, suggesting that K-RBP is modified by ubiquitin. Similarly, the higher-molecular-weight species of K-RBP was also found in cells cotransfected with both K-RBP and HA-tagged ubiquitin expression plasmids when lysates were immunoprecipitated with anti-HA antibody and then Western blotted by anti-K-RBP antibody to specifically detect ubiquitinated K-RBP (Fig. 3C). The most robust ubiquitination of K-RBP was found in cells transfected with both HA-ubiquitin and K-RBP expression plasmids in the presence of MG132 treatment (Fig. 3, the third lane in each panel), which inhibited degradation of the highly ubiquitinated proteins. Taken together, these results suggest that K-RBP protein can be ubiquitinated and undergoes ubiquitin-dependent proteasomal degradation in vivo.

Ubiquitination of K-RBP in the presence of RTA. RTA has been reported to promote IRF-7 degradation by stimulating IRF-7 ubiquitination (44). Since our results demonstrated that K-RBP can be modified by ubiquitin and degraded by RTA through the proteasome pathway, they suggest that RTA may

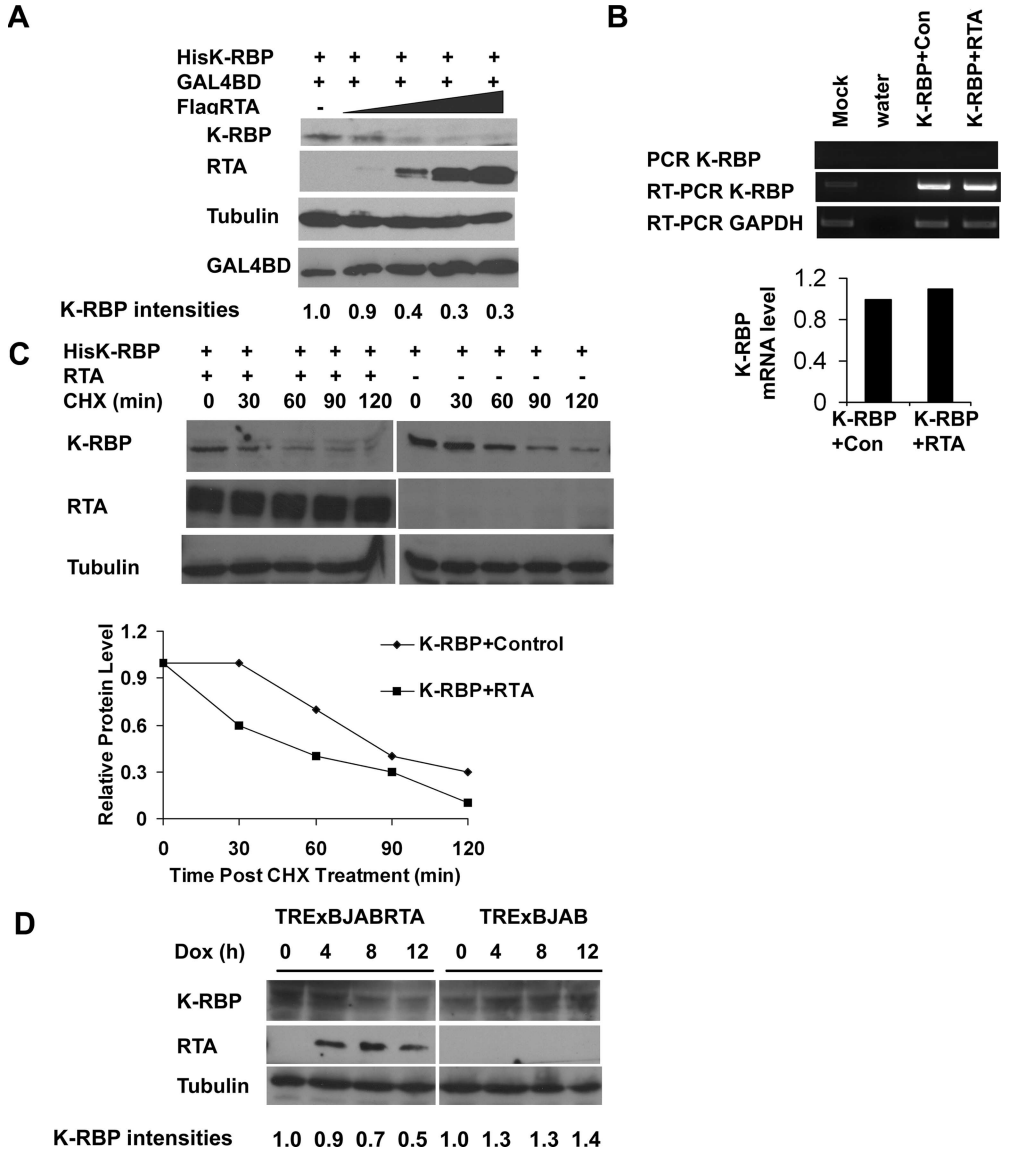

FIG. 1. Expression of RTA reduces K-RBP protein level. (A) Degradation of K-RBP by RTA. 293T cells were cotransfected with fixed amount of His-tagged K-RBP (1 μg), GAL4BD (0.3 μg), and increasing amounts of Flag-tagged RTA plasmids (0, 0.1, 0.25, 0.5, and 1.0 μg). The cell lysates were prepared and immunoblotted with anti-K-RBP (top), anti-Flag (upper middle), anti-γ-tubulin (lower middle), or anti-GAL4BD (bottom) antibodies. The numbers below the figure indicate the relative intensities of the K-RBP protein. (B) In the top panel are shown the results of an RT-PCR analysis of the K-RBP mRNA isolated from cells either mock-transfected, transfected with K-RBP plus RTA, or control plasmids. Equivalent amounts of RNA were used in the RT-PCR for each experiment, as confirmed by RT-PCR of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA, which was used as an internal control. The bottom panel shows the results of a quantitative RT-PCR analysis of K-RBP mRNA in the presence or absence of RTA expression. Con, control. (C) The top panel shows the turnover of K-RBP in the presence or absence of RTA. 293T cells transfected with His-tagged K-RBP expression plasmid in the presence of control or RTA plasmids were harvested at different time points after pretreatment with 75 μg of cycloheximide (CHX)/ml. The cell lysates were immunoblotted using anti-K-RBP (top), anti-RTA (middle), or anti-γ-tubulin (bottom) antibodies. In the bottom panel, a graph depicts the densitometric analysis of the band intensities. The K-RBP band intensities normalized to tubulin at time zero were fixed as 1. (D) RTA expression reduces endogenous K-RBP levels. TREXBJABRTA or TREXBJAB cells were treated with 1 μM doxycycline, and cells were harvested at various time points. The cell lysates were analyzed by Western blot analysis using anti-K-RBP (top), anti-His (middle), or anti-γ-tubulin (bottom) antibodies. The numbers below the figure indicate the relative intensities of the K-RBP protein.

enhance the degradation of K-RBP by promoting ubiquitination of K-RBP. To explore this possibility, we examined the ubiquitination state of K-RBP in the presence or absence of RTA expression in 293T cells. In the absence of MG132 treatment, the expression of RTA led to a lower level of polyubiquitinated K-RBP detected, possibly due to enhanced degradation of ubiquitinated K-RBP. In the presence of proteasome inhibitor MG132, higher levels of ubiquitinated K-RBP were detected when RTA was coexpressed (Fig. 4A). To confirm the induction of K-RBP ubiquitination by RTA, we determined the ubiquitination state of K-RBP in the presence of RTA in vitro. K-RBP protein expressed in and purified from *E. coli* can be

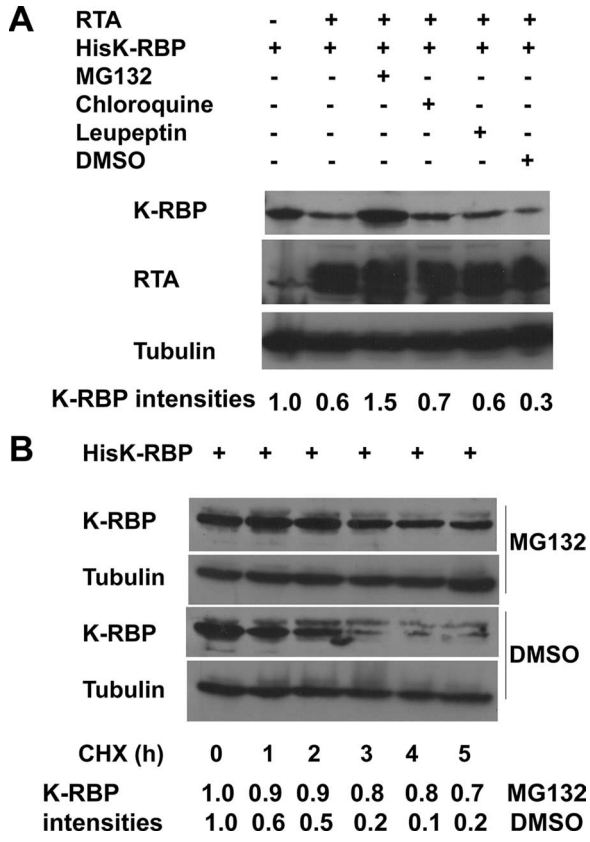

FIG. 2. K-RBP undergoes proteasomal degradation. (A) RTA induces K-RBP degradation via proteasome pathway. 293T cells were cotransfected with 1.5 μg of His-tagged K-RBP plus 0.5 μg of Flag-tagged RTA or control plasmids in the presence or absence of indicated inhibitors after treating for 12 h. The cell lysates were prepared and immunoblotted with anti-K-RBP (top), anti-RTA (middle), or anti-γ-tubulin (bottom) antibodies. The numbers below the figure indicate the relative intensities of the K-RBP protein. (B) K-RBP was targeted for proteasomal degradation in the absence of RTA. 293T cells were transfected with His-tagged K-RBP plasmid, treated with 75 μg of cycloheximide/ml in the presence of MG132 or vehicle control DMSO, and harvested at various time points after cycloheximide (CHX) treatment. The cell lysates were analyzed by Western blotting with anti-K-RBP and anti-γ-tubulin antibodies. The numbers below the figure indicate the relative intensities of the K-RBP protein.

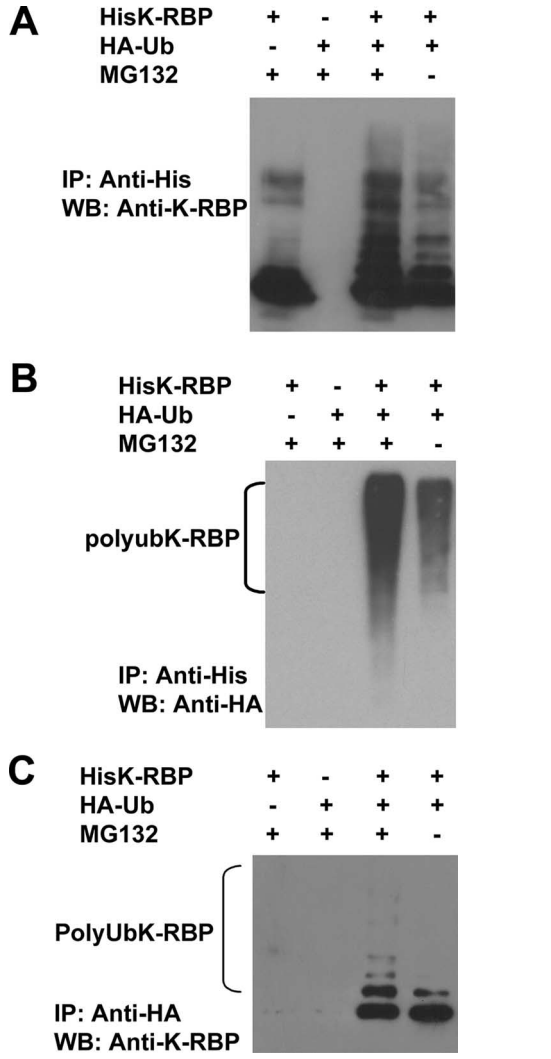

FIG. 3. K-RBP undergoes ubiquitin modification. (A) K-RBP is ubiquitinated in vivo. 293T cells were transfected with His-tagged K-RBP and/or HA-tagged ubiquitin plasmids as indicated. Cells were treated with 5 μM MG132 or vehicle control DMSO for 12 h before harvesting. The cell lysates were immunoprecipitated using anti-His antibody, and the immunoprecipitates were analyzed by Western blotting with anti-K-RBP antibody. (B) K-RBP is ubiquitinated in vivo. The immunoprecipitate described in panel A was analyzed by Western blotting with anti-HA antibody. (C) K-RBP was ubiquitinated in vivo. Transfection and treatment were carried out as described for panel A. The cell lysates were immunoprecipitated with anti-HA antibody and analyzed by using anti-K-RBP antibody.

ubiquitinated in the presence of purified RTA protein, E1, UbcH5a, and ubiquitin (Fig. 4B), further suggesting that RTA can simulate K-RBP ubiquitination. Furthermore, ubiquitination of K-RBP was observed using in vitro transcription and translation lysate in the presence of ubiquitin, E1, and UbcH5a (Fig. 4C). The presence of in vitro-expressed RTA protein also led to enhancement of ubiquitination of K-RBP (Fig. 4C, lanes 3 and 4). Higher levels of ubiquitinated K-RBP were detected after IP of the His-tagged K-RBP using anti-His antibody, followed by Western blot analysis with anti-Flag antibody to detect ubiquitinated K-RBP (Fig. 4D, lanes 2 and 3). However, there are also nonspecific proteins precipitated by anti-His antibody, and they are present even in the absence of K-RBP (Fig. 4D, lane 4).

The Cys/His-rich domain and C terminus of RTA are required to induce K-RBP degradation. It has been suggested that the Cys/His-rich domain between amino acids 118 and 207 of RTA is important for IRF-7 degradation, and several mutants of RTA in this domain, such as $RTAC_{141}S$ and $RTAH_{145}L$, are inactive in promoting IRF-7 degradation (44). We therefore tested whether $RTAC_{141}S$ and $RTAH_{145}L$ also lose their abilities to induce K-RBP degradation. Interestingly, both mutants were found to induce K-RBP degradation but were not as efficient as wild-type RTA in the transfected 293T cells (Fig. 5A). Another RTA mutant with a single amino acid change in this region, $RTAK_{152}E$, completely lost its ability to induce K-RBP degradation (Fig. 5A). These results suggest that efficient degradation of K-RBP requires the Cys/His-rich domain of RTA.

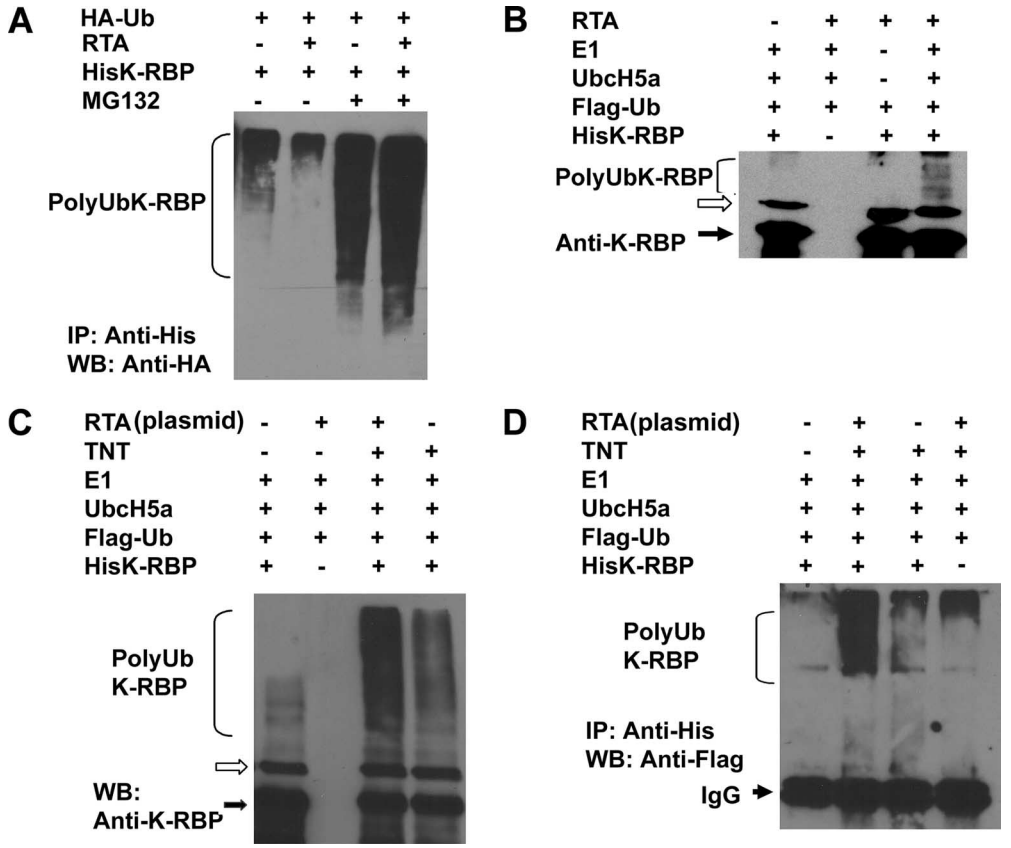

FIG. 4. RTA promotes K-RBP ubiquitination in vivo and in vitro. (A) 293T cells were transfected with His-tagged K-RBP plasmid, HA-tagged ubiquitin plasmid plus RTA, or control plasmids. Cells were treated with 5 μM MG132 or vehicle control DMSO for 12 h before harvesting. The cell lysates were immunoprecipitated by anti-His antibody and then analyzed by Western blotting with anti-HA antibody. (B) In vitro K-RBP ubiquitination was carried out as described in Materials and Methods using E1, UbcH5a, insect cells expressed purified RTA, *E. coli* expressed His-tagged K-RBP, and Flag-tagged ubiquitin as indicated. Western blot analysis of the lysates was performed with anti-K-RBP antibody. The solid arrow indicates the unmodified K-RBP, and the empty arrow indicates a nonspecific band. (C) In vitro K-RBP ubiquitination was performed as described for panel B except that RTA was expressed from an in vitro transcription and translation system (TNT) and the RTA/TNT lysate was used. The reaction mixtures were analyzed by Western blotting with anti-K-RBP antibody. The solid arrow indicates the unmodified K-RBP, and the empty arrow indicates a nonspecific band. (D) In vitro K-RBP ubiquitination was performed as described for panel C. The reaction mixtures were immunoprecipitated with anti-His antibody and analyzed using anti-Flag antibody.

In addition to the Cys/His-rich domain, the C-terminal domain of RTA was also found to be critical for K-RBP degradation. The RTA truncation mutant RTA621 was inactive in inducing K-RBP degradation, and mutant RTA641 can only induce K-RBP degradation at a high concentration (0.5 μg) but not at a lower concentration (0.25 μg) of the expression plasmid (Fig. 5B and C). This result suggests that the ability of RTA641 to induce K-RBP degradation is partially impaired. Deletion mutant RTA665 still induced degradation of K-RBP even at a lower concentration similar to wild-type RTA. These results suggest that the domain between amino acids 621 and 641 of RTA plays an important role in mediating K-RBP degradation. To further identify the amino acids in this region that are involved, several other mutants with multiple amino acid substitutions were tested (Fig. 5B and D). The importance of this domain was further confirmed with the clones mRTA631-634 and mRTA635-638 containing four amino acid substitutions. Both were partially impaired in their abilities to degrade K-RBP (Fig. 5D). Another mutant with changes from amino acids 627 to 630 of RTA had no effect and degraded K-RBP as efficiently as wild-type RTA (Fig. 5D). Notably, most of these RTA mutants have higher expression levels than that of wild-type RTA, a finding consistent with the previous finding that RTA also regulates its own stability (44). To rule out the possibility that the different abilities to induce degradation of K-RBP is due to the different expression levels among RTA and its mutants, we coexpressed K-RBP expression plasmid with 1 μg of RTA or 0.25 or 0.5 μg of RTA621 expression plasmid to compensate for their differences in expression levels and found that only wild-type RTA can degrade K-RBP when the RTA and RTA621 protein levels were comparable (Fig. 5E). In addition, using in vitro-translated RTA mutants, we found that these mutants could not enhance K-RBP ubiquitination as efficiently as wild-type RTA (data not shown). We also examined the in vivo K-RBP ubiquitination levels in the presence of RTA621 and RTAK_{152}E and found that they did not affect K-RBP ubiquitination levels (Fig. 5F). Together, these results confirm the importance of these regions of RTA in inducing K-RBP degradation.

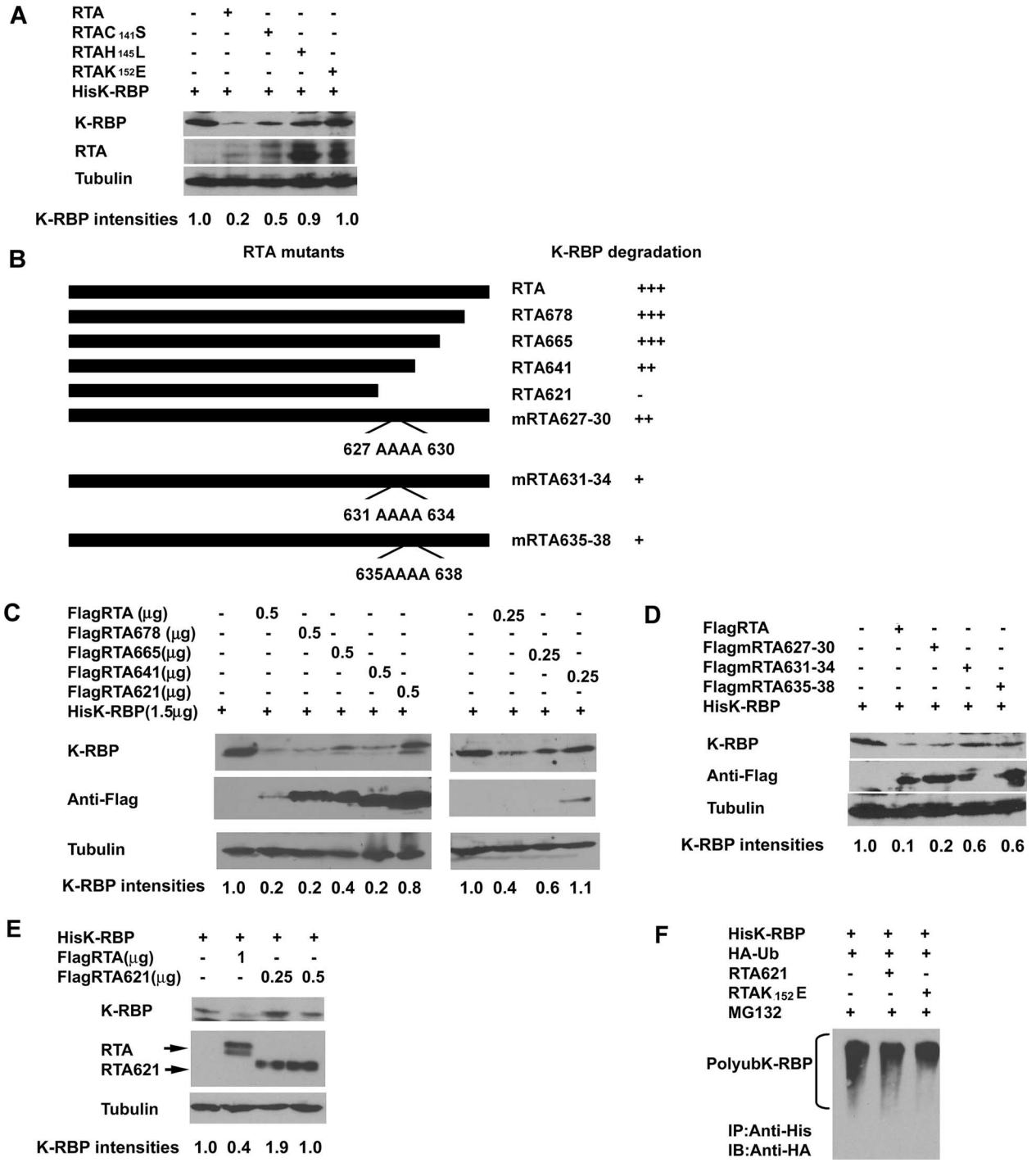

FIG. 5. RTA domains responsible for K-RBP degradation. (A) 293T cells were transfected with His-tagged K-RBP plus RTA or its mutants the $RTAC_{141}$ S, $RTAH_{145}L$, and $RTAK_{152}E$ plasmids. The cell lysates were analyzed by Western blotting with anti-K-RBP antibody (top), anti-RTA antibody (middle), or anti-γ-tubulin antibody (bottom). The numbers below the figure indicate the relative intensities of the K-RBP protein. (B) Schematic diagrams of the RTA C-terminus mutants. The number of "+" symbols after each mutant indicates its ability to induce K-RBP degradation. The "−" symbol represents no degradation. (C) 293T cells were transfected with the indicated amounts of His-tagged K-RBP and Flag-tagged RTA or its mutant plasmids. The cell lysates were then analyzed by Western blotting with anti-K-RBP antibody (top), anti-Flag antibody (middle), or anti-γ-tubulin antibody (bottom). The numbers below the figure indicate the relative intensities of the K-RBP protein. (D) 293T cells were transfected with His-tagged K-RBP plus Flag-tagged RTA or mutant plasmids. The cell lysates were analyzed by Western blotting with anti-K-RBP antibody (top), anti-Flag antibody (middle) and anti-γ-tubulin antibody (bottom). The numbers below the figure indicate the relative intensities of the K-RBP protein. (E) 293T cells were transfected with His-tagged K-RBP plus Flag-tagged RTA or RTA641 plasmids at the indicated amounts. The cell lysates were analyzed by Western blotting with anti-K-RBP antibody (top), anti-Flag antibody (middle), and anti-γ-tubulin antibody (bottom). The numbers below the figure indicate the relative intensities of the K-RBP protein. (F) RTA621 and $RTAK_{152}E$ did not enhance K-RBP ubiquitination. 293T cells were transfected with His-tagged K-RBP plasmid, HA-tagged ubiquitin plasmid plus RTA621, $RTAK_{152}E$, or control plasmids. Cells were treated with 5 μM MG132 for 12 h before harvesting. The cell lysates were immunoprecipitated by anti-His antibody and then analyzed by Western blotting with anti-HA antibody.

Ability of RTA to mediate degradation correlates with its transactivation ability. We next tested whether the degradation ability of RTA correlates to its transactivation function, since both IRF-7 and K-RBP can repress RTA-mediated transactivation (39, 43). If there is a correlation, we expect that increasing the amounts of RTA will overcome K-RBP suppression. We used the KSHV ORF57 promoter, which can be activated by RTA, to test this possibility. A fixed amount of K-RBP expression plasmid, an increasing amount RTA expression plasmid or control plasmid, and the ORF57 luciferase reporter were cotransfected into 293T cells. The repression function of K-RBP was examined. The result showed that an increasing amount of RTA diminished the repression by K-RBP from 74 to 17% (Fig. 6A). We also tested the transactivation ability of the ORF57 promoter by RTA mutants that are impaired in inducing K-RBP degradation. As expected, there is a good correlation between the ability of RTA to activate ORF57 promoter and to degrade K-RBP. The three mutants, $RTAC_{141}S$, $RTAH_{145}L$, and $RTAK_{152}E$, that were impaired in K-RBP degradation were also impaired in their transactivation function compared to wild-type RTA (Fig. 6B and 5A). Similarly, the C-terminal RTA mutants, including RTA621, RTA641, mRTA631-635, and mRTA635-638, were also not able to transactivate the ORF57 promoter efficiently compared to wild-type RTA (Fig. 6C). The impairment in transactivation of these mutants was not due to defects in the protein expression since all mutants were expressed at a similar or higher level than the wild-type RTA (Fig. 5C). Since RTA641 could induce K-RBP degradation at high levels, we tested the transactivation ability of RTA641 at lower (250 ng) and higher (500 ng) concentrations of the expression plasmid and compared that to wild-type RTA. The results indicated that the presence of more RTA641 showed an increase in transactivation but not to the same level as the wild-type RTA (Fig. 6D). It is possible that there are additional factors, in addition to its ability to degrade K-RBP, that may have affected the transactivation function of this RTA mutant.

To further confirm a correlation between ubiquitin-proteasome degradation and the RTA transactivation function, two other studies were carried out. The first was the overexpression of ubiquitin, and the second was the use of Ts20 cells with a temperature-sensitive E1 ubiquitin-activating enzyme. We expected that overexpression of ubiquitin would enhance RTA function, whereas its function would be impaired in Ts20 cells at the restrictive temperature. Indeed, when the 293T cells were cotransfected with ubiquitin, RTA, and ORF57 promoter constructs, RTA-mediated transactivation of the ORF57 promoter was enhanced up to fivefold upon overexpression of ubiquitin (Fig. 6E). Cotransfection of Ts20 with RTA expression plasmid and ORF57 promoter constructs at 39°C when E1 is inactive resulted in a lower level of transactivation of the ORF57 promoter by RTA compared to that at 35°C when E1 is active (Fig. 6F) (9), even though the basal level of ORF57 promoter activity was similar at both temperatures (data not shown). These results together confirmed that the ubiquitin-proteasome pathway plays an important role in RTA-mediated transactivation.

RTA promotes degradation of other repressors. In addition to K-RBP and IRF-7, a number of other cellular and viral proteins have been shown to repress RTA-mediated transactivation and/or KSHV lytic replication. They include KSHV latent protein LANA, early protein KbZIP, and cellular proteins NF-κB, HDAC1, PARP-1, and hKFC (2, 16, 17, 21, 25). It was of interest to determine whether RTA also mediates degradation of other repressors. Plasmids encoding KbZIP, LANA, and NF-κB were cotransfected with RTA expression plasmid or control plasmid in the absence or presence of MG132. Western blot analysis showed that the presence of RTA readily downregulated the steady-state levels of these proteins (Fig. 7). However, we found that RTA was not able to reduce HDAC1 expression level (data not shown), which suggests that RTA downregulates some but not all repressors of RTA. For KbZIP, LANA and NF-κB, it is unlikely that RTA acts by repressing the expression of these repressors at the transcriptional level because the expression of these proteins is under the control of the CMV promoter similar to K-RBP. As shown in Fig. 1B, there is no repressive effect of RTA on K-RBP expression mediated by the CMV promoter. Interestingly, we also found that MG132 can prevent RTA-mediated degradation of KbZIP and LANA (Fig. 7A and B), suggesting that proteasomal degradation is involved in the degradation of these two proteins by RTA. Similarly the expression of RTA was found to reduce NF-κB expression level in cotransfection study (Fig. 7C). However, in contrast to KbZIP and LANA, MG132 was unable to block NF-κB degradation induced by RTA (Fig. 7C). This suggests that the reduction of NF-κB levels may involve other mechanism.

Involvement of ubiquitin-proteasome pathway in RTA-mediated KSHV reactivation. Based on our findings that KSHV RTA induces the degradation of K-RBP and several other repressors through the proteasome pathway, we hypothesized that interference with the proteasomal degradation pathway would affect RTA-mediated KSHV reactivation from latency. We then determined whether K-RBP is downregulated when RTA is expressed in the context of KSHV lytic replication. We again used KSHV-positive TRExBCBL-1RTA and TRExBCBL-1 cells for this purpose. In TRExBCBL-1RTA cells, DNA encoding His-tagged RTA is integrated into the cellular genome, and the expression of RTA is tightly controlled by doxycycline (32). The induction of RTA expression initiates KSHV lytic replication. The cell line TRExBCBL-1 without integrated RTA gene was used as a control. Upon RTA induction in these cells by doxycycline, K-RBP levels were detected at various time points. Western blot analysis showed a reduction of K-RBP level upon RTA expression in TRExBCBL-1 RTA cells but not in control TRExBCBL-1 cells (Fig. 8A). We then investigated whether KSHV lytic replication can be affected if the degradation of repressors through the proteasome is inhibited. TRExBCBL-1RTA cells were treated by MG132 to inhibit the ubiquitin-proteasomal degradation pathway or by control DMSO before the cells were treated with doxycycline. Cells were collected at various time points after doxycycline treatment and Western blot analysis was performed to detect cellular and viral protein expression. The expression of viral lytic proteins, KbZIP, ORF45, and K8.1 as indicators for lytic replication, was significantly reduced in MG132-treated cells (Fig. 8B). The His-tagged RTA expression levels from integrated RTA gene were equivalent in both MG132- and DMSO-treated cells during the time course we examined (Fig. 8B) and suggests that MG132 did not affect doxycycline-in-

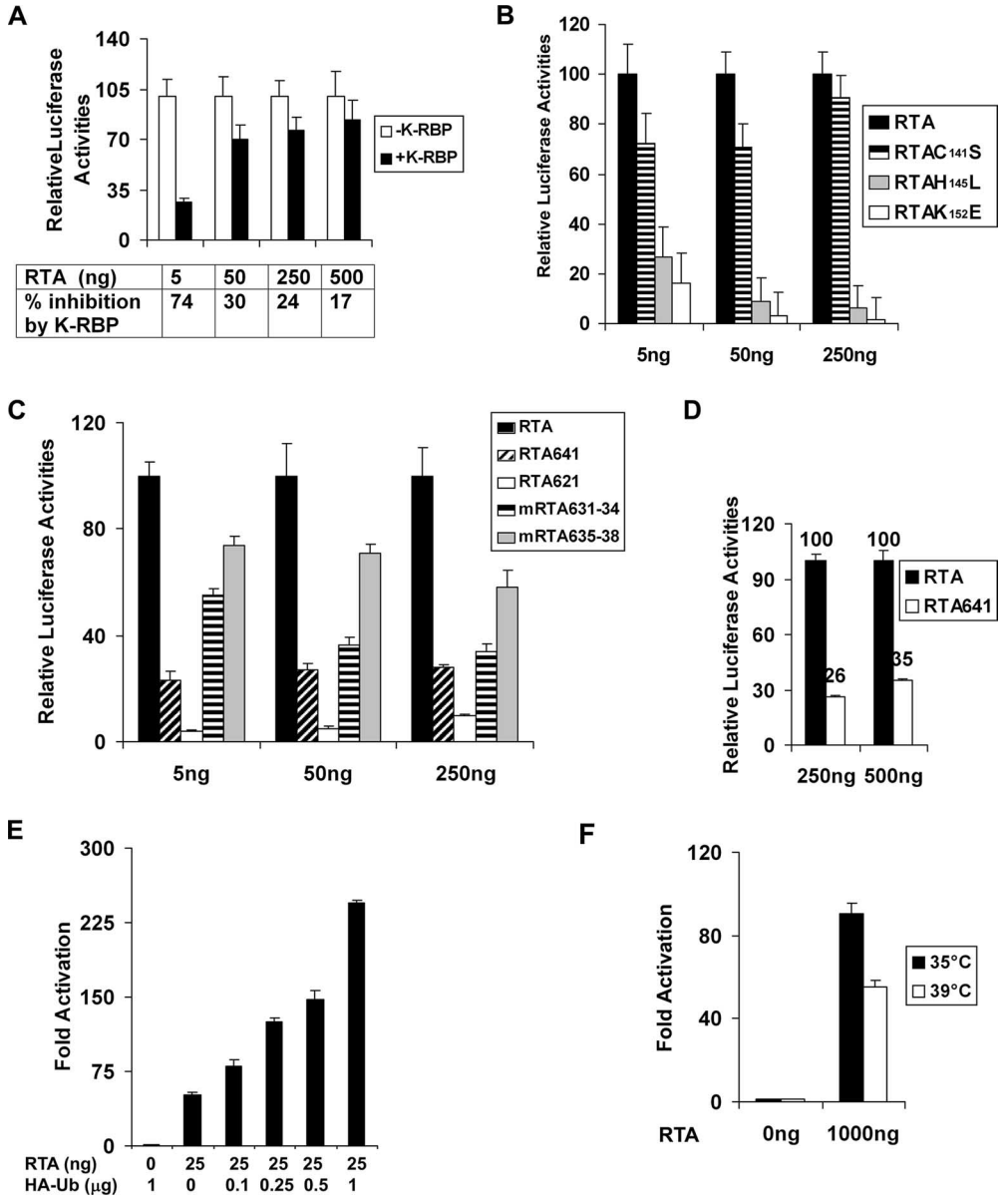

FIG. 6. Correlation of RTA-mediated transactivation and degradation. (A) 293T cells were transfected with the reporter construct p57Pluc1 (10 ng), an increasing amounts of pCMVTag50, and with a fixed amount (1,000 ng) of pcDNAK-RBP or control plasmid. The luciferase activities were measured at 24 h after transfection. The luciferase reporter activities are expressed as a percentage of activation, with activation by RTA alone equal to 100% at each concentration of RTA expression plasmid. The error bars indicate the standard deviations. The percentages of inhibition of RTA-mediated ORF57 promoter activation by K-RBP at each concentration of RTA expression plasmid are indicated. (B) 293T cells were transfected with the reporter construct p57Pluc1 (10 ng) and various amounts of pcDNAORF50 or the indicated mutants. The luciferase activities were measured at 24 h after transfection. Luciferase reporter activities are expressed as percentages of activation, with activation by wild-type RTA equal to 100% for each concentration of RTA expression plasmid used. The error bars indicate the standard deviations. (C) 293T cells were transfected with the reporter construct p57Pluc1 (10 ng) and various amounts of pCMVTag50 or the indicated mutants. The luciferase activities were measured at 24 h after transfection. Luciferase reporter activities are expressed as percentages of activation, with activation by wild-type RTA equal to 100% for each concentration of RTA expression plasmid used. The error bars indicate the standard deviations. (D) 293T cells were transfected with the reporter construct p57Pluc1 (10 ng) and the indicated amounts of pCMVTag50 or pCMVTag50641. The luciferase activities were measured at 24 h after transfection. Luciferase reporter activities are expressed as percentages of activation, with activation by wild-type RTA equal to 100% for each concentration of RTA expression plasmid used. The error bars indicate the standard deviations. (E) Ubiquitin enhances RTA-mediated transactivation. 293T cells were transfected with the reporter construct p57Pluc1 (10 ng), pcDNAORF50, and increasing amounts of ubiquitin expression plasmid pMT123. Luciferase activities were measured at 24 h after transfection. The error bars indicate the standard deviations. (F) RTA-mediated transactivation was reduced in cells with an inactivated ubiquitin pathway. Ts20 cells were transfected with the reporter construct p57Pluc1 (100 ng) and pCMVORF50. The cells were incubated at either 39 or 35°C. Luciferase activities were measured at 24 h after transfection. The error bars indicate the standard deviations.

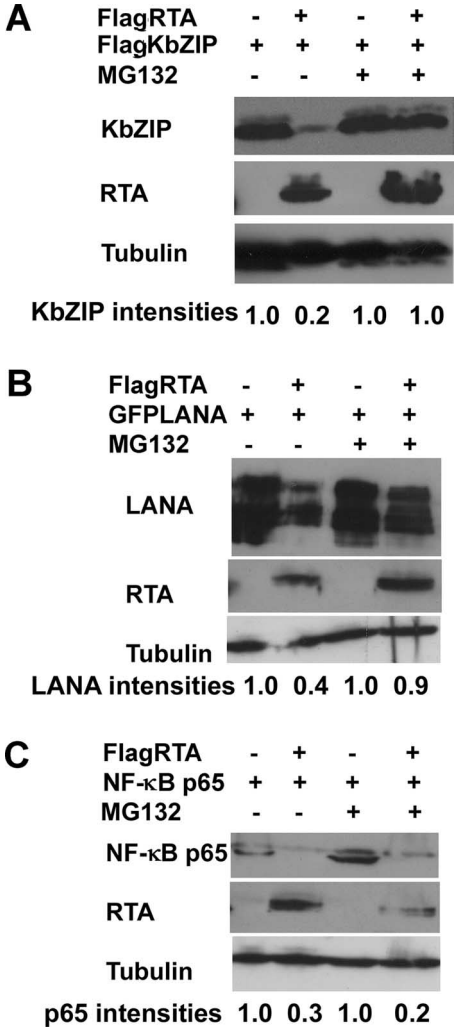

FIG. 7. Expression of RTA reduces several other RTA repressor levels. (A) RTA expression reduces KbZIP protein level in a proteasome-dependent manner. 293T cells were cotransfected with 1.0 μg of Flag-tagged KbZIP plus 1.0 μg of Flag-tagged RTA or control plasmids, with or without 5 μM MG132 for 12 h. The cell lysates were prepared and immunoblotted with anti-Flag (top and middle) or anti-γ-tubulin (bottom) antibodies. The numbers below the figure indicate the relative intensities of the KbZIP protein. (B) Experiments were performed as described for panel A except that green fluorescent protein (GFP)-tagged LANA plasmid was used, and anti-LANA antibody was used for Western blotting to detect LANA expression. (C) Experiments were performed as described for panel A except that NF-κB p65 plasmid was used, and anti-p65 antibody was used for Western blotting to detect NF-κB p65 expression.

duced RTA expression. In addition, a more specific proteasome inhibitor lactacystin treatment also inhibited KSHV reactivation in TRExBCBL-1 RTA cells (data not shown). As a control, the cellular protein tubulin was not affected (Fig. 8B). Interestingly, the levels of latent protein LANA were also similar in MG132-treated and untreated cells. This could be due to a combination of the effects of RTA-mediated degradation and the activation of LANA expression by RTA (26, 30). To further demonstrate that the reduction in KSHV reactivation is due to a block of the lytic gene expression at the transcriptional level, we examined the mRNA levels of viral lytic genes. The results showed that the mRNA levels of KbZIP and K8.1 were lower in the presence of proteasome inhibitor (Fig. 8C), suggesting that the reduction in lytic viral protein levels is due to less-efficient transcription of these genes in the presence of proteasome inhibitor. Our overall results indicate that proteasomal degradation is involved in regulating KSHV switch between latency and lytic replication. Since RTA appears to be the sole transactivator of KSHV lytic replication and we show that RTA also induces repressor degradation, it is likely that RTA utilizes the proteasomal degradation pathway to acquire maximal lytic replication during reactivation.

DISCUSSION

The mechanism and domains of RTA involved in promoting K-RBP degradation. It has been reported that RTA harbors E3 ubiquitin ligase activity and induces IRF-7 ubiquitination and degradation (44). In the present study, we found that RTA can promote ubiquitination of K-RBP by in vitro and in vivo ubiquitin conjugation assays, suggesting a similar mechanism is used by RTA to degrade K-RBP. However, we do not know at this point whether this degradation is mediated by RTA functioning as an E3 ubiquitin ligase itself or it is via a cellular E3 ubiquitin ligase that associates with RTA. Since the RTA was expressed from TNT system or insect cells in our in vitro K-RBP ubiquitination assay, we could not rule out the possibility that certain E3 ubiquitin ligases may be copurified with RTA from insect cells or presence in the TNT lysates. In fact, RTA is known to interact with a number of cellular factors (42). We found that two domains in RTA are required for the degradation of K-RBP, the C-terminal region located at the end of activation domain and the Cys/His-rich region at the N terminus that was suggested to be required for IRF-7 degradation (44). It is likely that the mutations in these regions that render RTA inactive in inducing degradation are due to the disruption of the E3 ubiquitin ligase activity of RTA or disruption of the association with cellular factors that are important for the ubiquitination of target proteins by RTA, since these mutants cannot enhance K-RBP ubiquitination.

We have consistently observed that RTA itself can be stabilized by proteasome inhibitor and several RTA mutants that cannot induce K-RBP degradation have much higher expression levels than wild-type RTA. These results are consistent with previous findings from another study demonstrating that there is an enhanced level of RTA in the presence of proteasome inhibitors (44). We have thus carried out an experiment to test whether RTA is modified by ubiquitin in transfected cells. However, we did not find ubiquitinated RTA species in this experiment, suggesting that RTA itself may not be conjugated by ubiquitin but can be degraded by proteasome (data not shown).

RTA-mediated degradation of other repressors. In addition to K-RBP, the steady-state levels of several other RTA repressors, NF-κB, KbZIP, and LANA (2, 21, 25), were also found to be downmodulated in the presence of RTA, either through proteasome-dependent or a proteasome-independent pathway. Thus, K-RBP is only one of a number of repressors downmodulated by RTA. It is interesting that RTA can induce LANA and KbZIP degradation since RTA can activate KbZIP

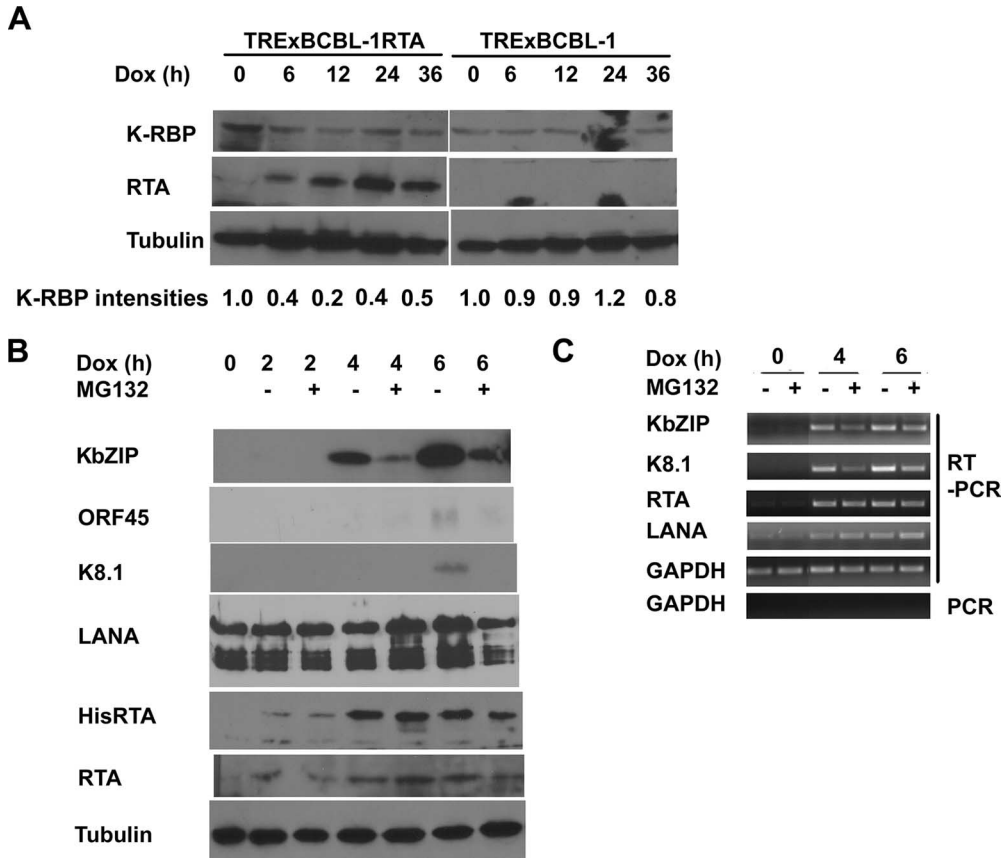

FIG. 8. The ubiquitin-proteasome pathway is involved in RTA-mediated lytic replication. (A) RTA expression reduces endogenous K-RBP levels during KSHV lytic replication. TRExBCBL-1RTA or TRExBCBL1 cells were treated with 1 μM doxycycline, and cells were harvested at various time points. The cell lysates were analyzed by Western blot analysis with anti-K-RBP (top), anti-RTA (middle), or anti-γ-tubulin (bottom) antibodies. The numbers below the figure indicate the relative intensities of the K-RBP protein. (B) MG132 treatment inhibits KSHV lytic replication. TRExBCBL-1RTA cells were treated with or without 5 μM MG132 and 1 μM doxycycline. Cells were harvested at various time points. The cell lysates were analyzed by Western blot analysis with different antibodies as indicated. (C) MG132 treatment inhibits KSHV lytic replication. TRExBCBL-1RTA cells were treated with or without 5 μM MG132 and 1 μM doxycycline. Cells were harvested at various time points. RT-PCR analysis of the indicated mRNA isolated from different time points was performed with equivalent amounts of total RNA. GAPDH mRNA was used as an internal control.

and LANA expression at the transcriptional level during KSHV lytic replication (4, 26, 30). It is possible that RTA positively regulates LANA and KbZIP at the transcriptional level but negatively regulates them at the posttranslational level to maintain different levels of these two proteins at different stages of viral life cycle. In fact, even though LANA transcription increases during KSHV lytic replication (30), the protein level is not significantly enhanced (32). In addition, the KbZIP protein levels were found to be stable during the later part of KSHV lytic replication (32). It is likely that RTA evokes multiple regulation strategies during KSHV lytic replication.

Another interesting finding is that RTA can reduce NF-κB p65 level, whereas high NF-κB activity is required for efficient KSHV virion production during lytic replication (35). It is possible that the level of p65 protein may vary during various stages of lytic replication. Its levels may be low during the onset of lytic replication to favor KSHV lytic replication but could increase again during the late stage of lytic replication, which is required for virion production (35). We indeed found that NF-κB p65 was reduced early upon KSHV reactivation in TRExBCBL1 RTA cells, but its level increased at later time points (unpublished data), suggesting that other viral and cellular factors may counteract the effect of RTA-mediated downregulation of p65 during KSHV lytic replication. Moreover, even though p65 levels were reduced in the presence of RTA, the reduction is independent of the proteasome degradation pathway since MG132 had no effect. This suggests that the proteasome-dependent pathway is likely to be only one of several mechanisms that RTA utilizes for the degradation of its target proteins. It will be interesting to elucidate the other pathways that are involved.

A potential problem in the use of proteasome inhibitors in our study is they may affect cellular transcription and KSHV reactivation independent of RTA. However, since RTA is the key activator of KSHV lytic gene expression and lytic viral replication, and it has been shown to be involved in the ubiquitination and proteasome pathway, it is likely that the RTA-dependent proteasomal degradation pathway is playing a role. However, other mechanisms that affect lytic viral replication may also be involved. These could include proteasome pathways independent of RTA. It will be of interest to determine

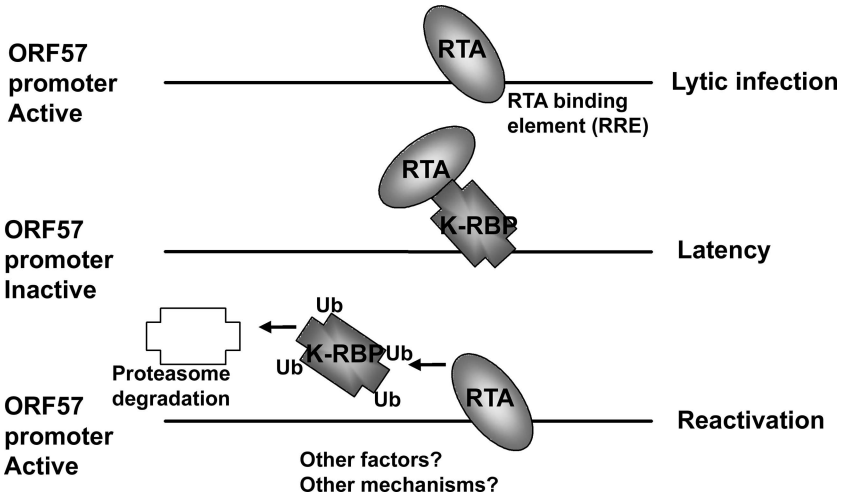

FIG. 9. Proposed model on how RTA promotes degradation of repressors for transactivation and KSHV lytic replication.

what other pathways are affected by proteasome inhibitors in KSHV replication.

Correlation between RTA-mediated degradation and transactivation. It is likely that the ability of RTA to transactivate the expression of its target promoter is coupled to its ability to degrade the negative regulators that may be associated with RTA or with the transcriptional complex. For example, the mutants that cannot induce degradation or partially lose their ability to induce K-RBP degradation could not activate the KSHV ORF57 promoter efficiently compared to wild-type RTA. In fact, RTA is associated with a number of cellular factors other than K-RBP, including IRF-7, PARP-1, LANA, KbZIP, and HDAC1. Many of these factors inhibit RTA-mediated transactivation (16, 17, 21, 25, 39, 43, 44). The degradation of these proteins by RTA could counteract the inhibitory effect caused by these factors for RTA-mediated transactivation. At this point, it is not clear whether RTA induces repressor degradation to achieve transactivation specifically or indirectly via activating cellular degradation pathways, which in turn induces the degradation of these repressors nonspecifically. The results from our group and others favor the former possibility, since RTA induces degradation selectively and does not induce HDAC1 degradation (data not shown) and IRF-3 degradation (44). However, it is likely that the degradation of these repressors by RTA is one of a number of mechanisms that RTA utilizes to achieve efficient transactivation and activate lytic viral replication from latency. The other mechanisms that RTA utilizes to acquire efficient transactivation include, but may not be limited to, binding to DNA, binding to cellular cofactor, and oligomerization (3, 11, 27), and one or more of these mechanisms could be involved. A better understanding of these mechanisms may lead to new strategies to block KSHV lytic replication.

Other potential consequences of RTA-mediated degradation. It is likely that RTA-mediated degradation may affect other aspects of the KSHV replication in addition to transactivation. The degradation of IRF-7 by RTA was found to suppress IRF-7-mediated IFN-β promoter activation (44), and it was suggested that it antagonizes antiviral type I interferon production. In addition, we found that RTA can mediate NF-κB downregulation. NF-κB is known to be involved in the regulation of type I interferon production in concert with IRFs (34). We actually found that RTA suppresses NF-κB-dependent luciferase reporter promoter in BJAB cells (unpublished data). The results together suggest that RTA-mediated IRF-7 and NF-κB degradation may play a role in modulating the innate immune response. It is also likely that RTA may direct the degradation of cellular proteins that have other regulatory functions in KSHV infection, replication, and pathogenesis.

Role of repressor degradation in KSHV reactivation from latency in KSHV-infected cells. Based on the findings in the present study, we proposed that the degradation of cellular and viral repressors by RTA is required for its maximal transactivation and lytic replication induction ability (Fig. 9). With this model, using ORF57 promoter as examples of an RTA target promoter, cellular and viral repressors such as K-RBP suppress RTA-mediated transactivation and reactivation. However, RTA can promote the degradation of these repressors to activate KSHV gene expression and lytic replication. The establishment of either latency or lytic viral replication thus involves a regulatory loop to maintain a balance between RTA and repressors. During the onset of KSHV reactivation, when RTA expression levels are low, the repression by these repressors may lead to abortion of the lytic replication. However, RTA expression may be robust enough in some cells to degrade those repressors, overcome the suppression, and induce lytic replication. It is possible that RTA induces degradation of proteins in addition to those identified here and uses other mechanisms to induce degradation. Further studies will be needed to substantiate this model, as well as to identify other RTA degradation targets and other potential mechanisms RTA uses to induce degradation.

Alphaherpesvirus HSV-1 transactivator ICP0 and betaherpesvirus HCMV transactivator pp71 have been shown to utilize the proteasome-dependent pathway to induce cellular protein degradation to modulate viral replication. The degradation of proteins that silence viral gene expression was proposed as a mechanism for the lytic infection of these viruses (12, 20, 24). Our study suggests that gammaherpesvirus KSHV also uses its transactivator RTA to eliminate the cellular and viral silencers

for efficient transactivation and lytic replication. Thus, it is likely that the degradation of repressors through various mechanisms by transactivators of herpesviruses to acquire full lytic infection may be a common mechanism for herpesviruses. Further studies will be needed to examine whether other herpesviruses also use similar mechanism.

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