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Kaposi's Sarcoma-Associated Herpesvirus RTA Promotes Degradation of the Hey1 Repressor Protein through the Ubiquitin Proteasome Pathway▽

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The Kaposi's sarcoma-associated herpesvirus (KSHV) replication and transcription activator (RTA) protein regulates the latent-lytic switch by transactivating a variety of KSHV lytic and cellular promoters. RTA is a novel E3 ubiquitin ligase that targets a number of transcriptional repressor proteins for degradation by the ubiquitin proteasome pathway. Herein, we show that RTA interacts with the cellular transcriptional repressor protein Hey1. We demonstrate that Hey1 is a target for RTA-mediated ubiquitination and is subsequently degraded by the proteasome. Moreover, a Cys-plus-His-rich region within RTA is important for RTA-mediated degradation of Hey1. We confirm that Hey1 represses the RTA promoter and, furthermore, show that Hey1 binds to the RTA promoter. An interaction was observed between Hey1 and the corepressor mSin3A, and this interaction was abolished in the presence of RTA. Additionally, mSin3A associated with the RTA promoter in nonreactivated, but not reactivated, BCBL1 cells. Small interfering RNA knockdown of Hey1 in HEK 293T cells latently infected with the recombinant virus rKSHV.219 led to increased levels of RTA expression upon reactivation but was insufficient to induce complete lytic reactivation. These results suggest that other additional transcriptional repressors are also important in maintenance of KSHV latency. Taken together, our results suggest that Hey1 has a contributory role in the maintenance of KSHV latency and that disruption of the Hey1 repressosome by RTA-targeted degradation may be one step in the mechanism to regulate lytic reactivation.

Kaposi's sarcoma-associated herpesvirus (KSHV)/human herpesvirus 8 is a gamma-2 herpesvirus associated with the AIDS-related malignancies Kaposi's sarcoma (KS), primary effusion lymphoma, and multicentric Castleman's disease (4, 5, 9, 56). In common with all herpesviruses, KSHV has two distinct phases within its life cycle, latency and lytic replication. During latency a limited subset of genes is expressed to ensure that the virus remains undetected by the host immune system (3, 8, 30, 40, 44, 46). This is important for formation of a persistent infection within the host. Upon reactivation the virus enters lytic replication, where lytic genes are transcribed, and new virus particles are produced, facilitating virus propagation.

The latent and lytic phases of the KSHV life cycle are both implicated in KS development. Latently expressed viral proteins have been shown to promote tumorigenesis by subverting the cellular mechanisms which would normally protect cells from aberrant proliferation. KSHV latency-associated nuclear antigen (LANA) exerts antiapoptotic and antiproliferative effects on cells by interacting with and inhibiting p53 (14, 55), retinoblastoma protein (43), and glycogen synthase kinase 3β (15). KSHV vFLIP has been shown to induce expression of the antiapoptotic transcription factor NF-κB, leading to antiapoptotic effects and a change in cellular morphology (6, 20, 31). It is this change in morphology that gives the characteristic spindle-shaped cells observed in KS lesions. While latent proteins have oncogenic properties, latency alone does not appear to be strongly immortalizing, suggesting that lytic replication has a role in KS pathogenesis. Moreover, clinical studies showed that ganciclovir, a drug that inhibits lytic replication of KSHV, successfully reduced the occurrence of new KS tumors in patients with advanced AIDS, signifying that lytic replication has a critical role in KS tumorigenesis (39). There are several ways in which the lytic cycle is likely to aid tumorigenesis, for example, through propagation of the viral infection by production of new virus particles and/or expression of paracrine signaling molecules (16). Specifically, the presence of active KSHV replication and increased viral load in the peripheral blood predicts the pathogenic outcome of the infection, leading to increased risk of progression and severity of KS (42). Moreover, lytic gene expression potentially contributes to the development of KS through expression of viral lytic genes, such as growth modulators and immune evasion genes, which play vital roles in KS disease pathogenesis (10). Finally, lytic replication sustains the population of latently infected cells that would otherwise be reduced due to the poor persistence of the KSHV episome during spindle cell division (21).

The switch between latency and lytic replication is regulated by the virally encoded replication and transcription activator RTA (ORF50). RTA is conserved and has an essential role in the latent-lytic switch within all known gammaherpesviruses including murine herpesvirus 68, herpesvirus saimiri, and Epstein-Barr virus (17, 48, 57). Expression of RTA in cells latently infected with KSHV is necessary and sufficient to induce lytic

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replication (18, 37). RTA controls lytic replication through transcriptional activation of a number of lytic genes including polyadenylated nuclear (PAN) RNA, K8, and viral interleukin-6 (18, 37, 45, 46, 49, 50, 66) and is thought to function as a tetramer (2). Transactivation of lytic genes by RTA occurs by a number of mechanisms either through direct binding to promoter DNA sequences or through interactions with other transcriptional control proteins including RBP-Jκ (35), C/EBPα (52), AP1 (54), and HMGB1 (25, 47). Additionally, viral and cellular factors have been identified that suppress the transcriptional activation of lytic genes. Such factors include RBP-Jκ, CREB-binding protein, KSHV K-bZIP, KSHV LANA, KSHV RTA binding protein (K-RBP), and interferon regulatory factor 7 (IRF7) (1, 22, 23, 29, 33, 53, 61). Interestingly, recent findings have shown that two such factors, IRF7 and K-RBP, are both targeted for proteasomal degradation via a novel E3 ubiquitin ligase activity of RTA (62, 64). KSHV LANA and K-bZIP also undergo proteasomal degradation in the presence of RTA although it has not been determined whether the E3 ubiquitin ligase activity of RTA is responsible (62).

E3 ubiquitin ligases can act as "adapter proteins," allowing E2 ubiquitin-conjugating enzymes to come into close proximity with substrate proteins. E2 ubiquitin-conjugating enzymes then modify the target protein by the addition of a ubiquitin molecule (26). Four distinct classes of E3 ubiquitin ligase have been observed: HECT domain, RING domain, PHD domain, and U-box-containing E3. RTA does not possess any of these well-characterized domains; instead, it has a noncanonical Cys-plus-His-rich region which is responsible for its ubiquitin ligase activity (64). Site-directed mutagenesis studies identified two cysteine residues (Cys131 and Cys141) and one histidine residue (His145) that were essential for ubiquitination of IRF7 (64).

Herein, we identify a third target of RTA ubiquitin ligase activity, a cellular transcriptional repressor known as Hey1. Hey1 belongs to a family of basic helix-loop-helix (bHLH) transcriptional repressor proteins that are highly conserved throughout evolution (59). Three *Hey* genes have been identified (*Hey1*, *Hey2*, and *HeyL*), all of which are Notch inducible and have important roles in development (27, 32, 34, 41); however, the role of these genes in adulthood is unknown. The molecular mechanism of Hey1 repressor activity is not fully understood, but both DNA binding and non-DNA binding models exist (11). Hey1 dimerizes to form either homodimers or heterodimers with a closely related protein, Hes. Hey proteins can also associate with a wide range of other bHLH proteins and have been shown to recruit corepressors such as histone deacetylases (HDACs), nuclear receptor corepressor (NCoR), and mSin3A (28) to form a repressosome on target promoters. Hey proteins have also been shown to inhibit transcriptional activators including GATA4 and GATA6, leading to downregulation of atrial natriuretic factor, a protein involved in cardiac development (12). Intriguingly, Hey1 was recently shown to repress the KSHV RTA promoter, but the mechanism of repression was not elucidated (60).

In this report we demonstrate that RTA downregulates Hey1 protein levels via the ubiquitin-proteasome pathway. Moreover, we show that Hey1 and the corepressor mSin3A associate with the RTA promoter in latently infected, but not lytically infected, BCBL1 cells. We confirm that Hey1 represses the RTA promoter, and we also show that mSin3A interacts with Hey1 and that this interaction is abolished in the presence of RTA. These findings suggest that Hey1 may have a role in the maintenance of latency by repressing the RTA promoter and that RTA may help to relieve repression on its own promoter; nevertheless, small interfering RNA (siRNA) knockdown of Hey1 can enhance RTA expression upon reactivation but is not sufficient to reactivate latent virus. Therefore, our results provide another example of RTA-mediated downregulation of a repressor protein, which may contribute to the switch between KSHV latency and lytic replication.

MATERIALS AND METHODS

Plasmids. The yeast two-hybrid RTA bait plasmid was constructed by PCR amplification of the RTA second exon minus its conserved carboxy-terminal transactivation domain (24, 38) using primers 5′-CGC AGA TCT GGT AAG AAG CTT CGG CGG and 5′-CGC CTC GAG TCA AAG CCT TAC GCT TCT. The primers incorporated BglII and XhoI restriction sites, allowing the PCR fragment to be cloned into pGBT9 (Clontech) to derive the GAL4 DNA-binding domain (DBD) fusion, pDBD-RTA. The green fluorescent protein (GFP)-RTA fusion construct was produced by PCR amplification of the complete RTA cDNA with primers 5′-GGA AGA TCT AAG CTT CGG CGG TCC TGT GT-3′ and 5′-AAA CTG CAG TCA GTC TCG GAA GTA ATT ACG-3′. These oligonucleotides incorporated BglII and PstI restriction sites, respectively, allowing the PCR fragment to be inserted downstream of the enhanced GFP (EGFP) coding region of pEGFP-C1 to derive pEGFP-RTA. Plasmids pSEW-R01 and pYXY6 encoding RTA and RTA with the mutation H145L (RTA^{H145L}), respectively, were kindly provided by G. Hayward, Johns Hopkins School of Medicine (64). The RTA promoter-luciferase reporter construct pLucORF50Δ9 contains a promoter fragment encompassing 169 bp upstream of the transcription start site, cloned into the luciferase reporter construct (58). The Hey1 expression constructs were kind gifts from M. Gessler, University of Wurzburg, and E. Olson, The University of Texas Southwestern Medical Center (13, 41).

Yeast two-hybrid screen for RTA-interacting proteins. The GAL4-based yeast two-hybrid system screening technique (7) was used to identify proteins that interacted with RTA. A human kidney cDNA-GAL4 activation domain (AD) fusion library in the vector pACT2 ($GAL4_{768–881}$AD *LEU2*) (Clontech) was used to identify RTA-interacting proteins. The bait plasmid was transformed into *Saccharomyces cerevisiae* strain HF7c (*MATα ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 gal4-542 gal80-538 LYS2*::$GAL1_{UAS}$-$GAL1_{TATA}$-*HIS3 URA3*::$GAL4_{17\text{-}MERS(x3)}$-$CYC1_{TATA}$-*lacZ*) (Clontech). Clones were selected on minimal synthetic dropout medium in the absence of tryptophan. Yeast clones harboring the bait plasmids were then sequentially transformed with the prey library. Positive clones potentially harboring RTA-interacting species were identified both by their ability to grow on medium without tryptophan, leucine, and histidine and by the detection of β-galactosidase activity, as specified by the manufacturer (Clontech). Plasmids were isolated from positive yeast clones, with selection for pACT2 cDNA library plasmids by transformation of the leucine auxotroph into *Escherichia coli* strain HB101. Clones were then grouped by size and restriction analysis.

To confirm the specificity of the interactions, pACT2 library plasmids were transformed in yeast strains harboring no plasmid, yeast containing pGBT9 vector only, yeast containing pLAM5 (a GAL4 human lamin C fusion), or pDBD-RTA. Only library plasmids demonstrating a requirement for the pDBD-RTA plasmid for induced expression of *HIS3* or *lacZ* reporter genes were considered further and selected for DNA sequencing.

Cell culture and transfection. HEK 293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Paisley, United Kingdom) supplemented with 10% fetal calf serum (FCS; Invitrogen) and penicillin-streptomycin. HEK 293T cells infected with the recombinant KSHV construct rKSHV.219 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FCS, penicillin-streptomycin, and 1 μg/ml puromycin. BCBL1 cells were cultured in RPMI medium (Invitrogen) supplemented with 10% FCS and penicillin-streptomycin. HEK 293T plasmid transfections were performed using Lipofectamine 2000 (Invitrogen) following the manufacturer's directions. Transfection of scrambled siRNA and Hey1 siRNAs (5′-AAU GCU GCA UAC GGC AGG ATT-3′ and 5′-CAG UUU GUC UGA GCU GAG ATT-3′

[Ambion]) into rKSHV.219-infected HEK 293T cells was carried out with Lipofectamine 2000, following the manufacturer's protocol.

Luciferase assays. Transfections were carried out in triplicate on HEK 293T cells in a 24-well format with Lipofectamine 2000, according to the Invitrogen protocol. The pLucORF50Δ9 reporter construct was transiently transfected into HEK 293T cells alongside 0 to 0.4 μg of Hey1-Myc construct or 0 to 0.4 μg of RTA construct. The total DNA was kept constant using the pCS2-Myc or pCDNA3.1 vector, respectively. Cells were harvested at 24 h posttransfection and lysed with passive lysis buffer (Promega), according to the manufacturer's instructions. Luciferase activities of the lysates were determined using LARII reagent (Promega) on a FLUOstar Optima microplate reader (BMG Labtech, Ltd.) following the manufacturers' protocols. The luciferase activity of each lysate was measured in triplicate, the values were averaged, and standard deviations were calculated.

Coimmunoprecipitations. Protein coimmunoprecipitation assays were carried out with exogenously expressed proteins from transfected HEK 293T cell lysates and endogenous proteins from BCBL1 cell lysates. HEK 293T cells (1×10^6) transfected with appropriate constructs were harvested at 24 h posttransfection. Harvested BCBL1 cells (1×10^7) and HEK 293T cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed with modified radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 1% [vol/vol] NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1× complete protease inhibitors [Roche]). Cell lysates were precleared with protein A-agarose for 30 min at 4°C. After the preclearing the cell lysates were incubated with 10 μg of the appropriate polyclonal immunoglobulin G (IgG) for 2 h at 4°C. Protein A-agarose was added to the cell lysate and incubated for a further 2 h at 4°C. The agarose was washed three times with modified radioimmunoprecipitation assay buffer and once with PBS to remove unbound proteins. Washed agarose was boiled in Laemmli sample buffer with 10 mM dithiothreitol (DTT) for 4 min, and the solubilized proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent Western blotting using an appropriate primary IgG and species-specific horseradish peroxidase-conjugated secondary IgG.

ChIP assays. Chromatin immunoprecipitation (ChIP) assays were carried out using a ChIP kit (Millipore), as previously described (19). Briefly, either 1×10^6 HEK 293T cells, cotransfected with the pLucORF50Δ1 and Hey1-Myc or Myc alone, or 1×10^7 BCBL1 cells were used per assay. At 24 h posttransfection or 12 h postinduction, respectively, the cells were UV cross-linked (CL-1000 UV cross-linker [UVP, Inc.]) and washed twice with ice-cold PBS with protease inhibitors. The cells were lysed in 400 μl of the provided SDS lysis buffer, with protease inhibitors added, and incubated for 20 min on ice. The lysates were centrifuged at 13,000 rpm for 10 min at 4°C, and 3.6 ml of the provided ChIP dilution buffer was added to the cleared lysates. The diluted cell lysates were precleared with 25 μl of the provided protein A-agarose–salmon sperm DNA (50% slurry) for 30 min at 4°C. An appropriate polyclonal IgG or an equivalent volume of ChIP dilution buffer was added to the cell lysate and incubated for 2 h at 4°C. Following this incubation, 60 μl of protein A-agarose–salmon sperm DNA (50% slurry) was added and incubated for 1 h at 4°C. The agarose was pelleted by low-speed centrifugation (at 800 rpm for 30 s) and washed sequentially with 1 ml of each of the buffers provided, i.e., low-salt immune complex wash buffer, high-salt immune complex wash buffer, and LiCl immune complex wash buffer, and this step was followed by two final washes with Tris-EDTA buffer. The histone complex was eluted from the agarose by incubation with 250 μl of freshly prepared elution buffer (1% SDS–0.1 M $NaHCO_3$) for 15 min at room temperature. The elution step was repeated, and the eluates were combined before the addition of 20 μl of 5 M NaCl, 10 μl of 0.5 M EDTA, 20 μl of 1 M Tris-HCl, pH 6.5, and 2 μl of 20 mg/ml proteinase K. The eluates were incubated for 1 h at 45°C. The DNA was purified by phenol-chloroform extraction and ethanol precipitation and resuspended in 30 μl of sterile distilled H_2O. PCRs were performed on the immunoprecipitated DNA using *Taq* polymerase (Invitrogen) and primers toward the RTA promoter (5′-CCC AAG CTT TTT TGT GGC TGC C-3′ and 5′-GGA AGA TCT CAC ACC TCC ATG TTC AGT CAC-3′), ORF73 promoter (5′-TCA GCA CGG GGC GTG ATG GCG-3′ and 5′-GCA TTT CAA AGA TAA GGG TG-3′), and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (5′-CCA CCC ATG GCA AAT TCC ATG GCA-3′ and 5′-TCT AGA CGG CAG GTC AGG TCC ACC-3′).

Fluorescence microscopy. A total of 1×10^6 rKSHV.219-infected HEK 293T cells were transfected with 75 pmol of scrambled siRNA or Hey1 siRNAs or were lytically induced with 3 mM sodium butyrate (NaB). The cells were visualized at 24 and 48 h posttransfection using the GFP and red fluorescent protein (RFP) filters on a Zeiss Axiovert 135TV microscope.

RESULTS

RTA yeast two-hybrid assays show an interaction with cellular Hey1. In order to identify cellular proteins that interact with RTA, 1.5×10^6 independent cDNA clones from a human kidney cDNA library fused to the GAL4 AD were screened. A total of 18 clones activated expression of histidine and beta-galactosidase reporter genes upon expression of the RTA-DBD fusion protein (pDBD-RTA). Specificity of these interactions was confirmed by transforming the putative RTA binding cellular clones into yeast harboring no plasmid, pGBT9 (vector only), pLAM5 (Gal4 human laminin C fusion), or pDBD-RTA. Only clones that induced expression of histidine and beta-galactosidase reporter genes in the presence of pDBD-RTA were sequenced and subjected to sequence comparison against the EMBL/GenBank database. Sequence analysis revealed that 10 of the RTA binding clones correspond to the cellular transcriptional repressor Hairy/E(spl)-related with YRPW (Hey1), which is also known as HERP2, Hesr1, HRT1, and CHF2.

RTA interacts with Hey1 in coimmunoprecipitation assays. Hey1 is a cellular transcriptional repressor involved in the Notch signaling pathway (41). The interaction between Hey1 and RTA is particularly intriguing due to their opposing functions: RTA is a transcriptional activator, and Hey1 is a transcriptional repressor. Therefore, to confirm that the interaction between RTA and Hey1 could be observed in mammalian cells, coimmunoprecipitation assays were performed. HEK 293T cells were cotransfected with Hey1-Myc and GFP-RTA or GFP, and the transfected cells were cultured in the absence or presence of the proteasome inhibitor, MG132. The cell lysates were incubated with a Myc-specific IgG and protein A-agarose, and immunoprecipitated proteins were analyzed by immunoblotting using a GFP-specific IgG. Figure 1A shows that Hey1 was coimmunoprecipitated with GFP-RTA; however, this interaction was observed only in cells cultured in the presence of MG132. The interaction was confirmed by the reciprocal immunoprecipitation, incubating the cell lysates with GFP-specific polyclonal IgG and protein A-agarose, and subsequent immunoblotting with a Myc-specific monoclonal IgG (Fig. 1B). To exclude the possibility that this interaction was an artifact of overexpression, we repeated the coimmunoprecipitation experiment with endogenously expressed proteins in reactivated versus nonreactivated BCBL1 cells. BCBL1 cells harbor latent KSHV, and the virus can be stimulated to enter lytic reactivation by addition of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and NaB. Upon reactivation RTA protein levels are markedly upregulated as the virus enters lytic replication. Figure 1C shows that RTA was coimmunoprecipitated with Hey1 in reactivated BCBL1 cells in the presence of MG132. The interaction is observed only in coimmunoprecipitations performed in the presence of MG132 and is not evident in latently infected cells. The coimmunoprecipitation data show that RTA interacts with Hey1 both in vitro and in vivo. Significantly, this interaction was observed only in the presence of the proteasome inhibitor MG132.

RTA downregulates Hey1 protein levels. RTA was recently shown to downregulate the levels of IRF7 (64) and K-RBP (62) by means of a novel E3 ubiquitin ligase activity. The observed E3 ubiquitin ligase activity of RTA was attributed to

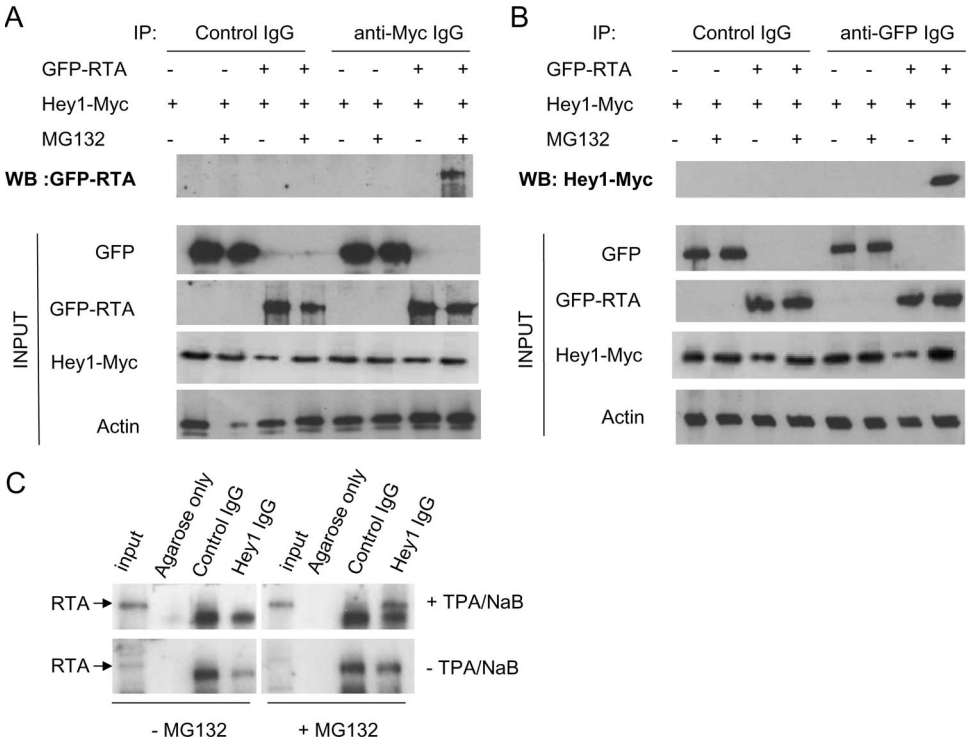

FIG. 1. Hey1 and RTA interact in HEK 293T and BCBL1 cells. (A) Immunoblot analysis of the coimmunoprecipitation of Hey1 with RTA. HEK 293T cells were cotransfected with Hey1-Myc and pEGFP-RTA or pEGFP-C1, in the absence or presence of 5 μM MG132 added at 12 h posttransfection. The cell lysates were incubated with Myc tag-specific polyclonal IgG or a control IgG and precipitated using protein A-agarose. Immunoprecipitated proteins were analyzed by immunoblotting using a GFP-specific monoclonal IgG. Total cell lysates from transfected cells served as input controls for GFP, GFP-RTA, and Hey1-Myc. (B) The experiment was performed as in panel A except that proteins were immunoprecipitated using GFP-specific IgG, and Western blot analysis was performed with a Myc tag-specific IgG. (C) Co-immunoprecipitation assays on endogenous Hey1 and RTA from reactivated versus nonreactivated BCBL1 cells. BCBL1 cells induced with TPA (20 ng/ml) and NaB (3 mM) were compared against uninduced BCBL1 cells in the absence and presence of 5 μM MG132. Proteins were immunoprecipitated from the BCBL1 cell lysates using a Hey1-specific polyclonal IgG and protein A-agarose. Immunoblot analysis was carried out using an RTA-specific polyclonal IgG. The band running at a slightly lower M_r than RTA is due to cross-reactivity from the heavy chain of the immunoprecipitating IgG. IP, immunoprecipitation.

a Cys-plus-His-rich domain, which was required for downregulation of both IRF7 and K-RBP. Mutations within the Cys-plus-His-rich domain generated three E3 ubiquitin ligase-defective mutants, RTA^{C131S}, RTA^{C141S}, and RTA^{H145L} (62, 64). RTA is thought to promote degradation of IRF7 and K-RBP to stem their repressive effects on the transactivation activity of RTA (62). In light of these findings and given the opposing functions of RTA and Hey1 (Hey1 is a transcriptional repressor, and RTA is a transcriptional activator), we set out to determine whether Hey1 is a novel substrate for RTA-mediated ubiquitination. Cell lysates were prepared from HEK 293T cells transfected with Hey1-Myc in the presence of increasing amounts of wild-type (wt) RTA or RTA^{H145L} plasmid DNA. The lysates were analyzed for Hey1-Myc protein levels by immunoblotting using a Myc tag-specific IgG (Fig. 2). Expression levels of RTA and RTA^{H145L} were determined using an RTA-specific IgG, and an actin-specific IgG was used to demonstrate equal loading. As RTA levels increased, Hey1 levels decreased in a dose-responsive manner. However, in the presence of increasing amounts of RTA^{H145L}, Hey1-Myc protein levels remained unaffected. The observed effect of RTA expression on Hey1 protein levels was not due to nonspecific

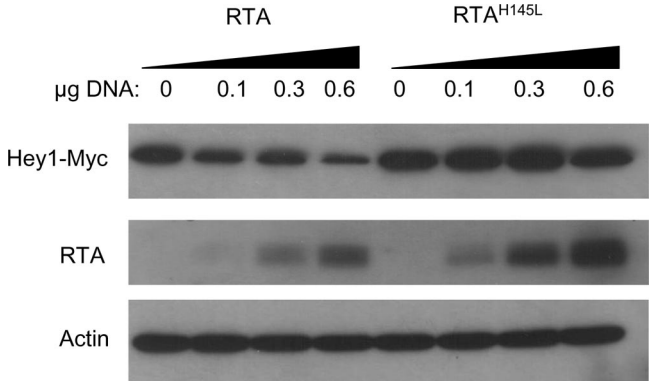

FIG. 2. Hey1 is downregulated in the presence of RTA in a dose-responsive manner. HEK 293T cells were transfected with Hey1-Myc in the presence of increasing amounts (0 to 0.6 μg) of RTA or RTA^{H145L} plasmid DNA. Cell lysates were analyzed by immunoblotting using a Myc tag-specific monoclonal IgG to determine the relative levels of Hey1-Myc. RTA-specific polyclonal IgG was used to confirm the expression levels of RTA and RTA^{H145L}, and an actin-specific monoclonal IgG was used as a loading control.

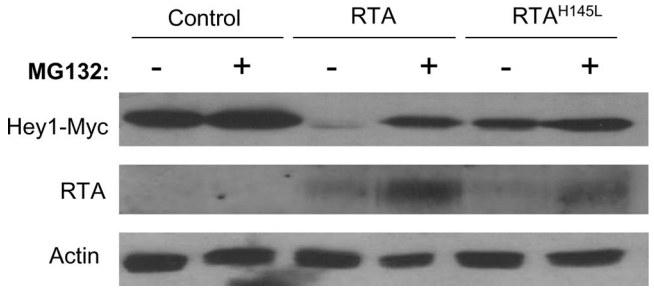

FIG. 3. Hey1 is degraded by a proteasome-dependent mechanism in the presence of RTA. HEK 293T cells were cotransfected with Hey1-Myc and RTA or RTA^{H145L} in the absence and presence of the proteasome inhibitor MG132 (5 μM) added at 12 h posttransfection. Cell lysates were analyzed at 36 h posttransfection by immunoblotting using a Myc tag-specific monoclonal IgG to determine the levels of Hey1-Myc. RTA-specific polyclonal IgG was used to confirm expression of RTA and RTA^{H145L}. An actin-specific monoclonal IgG was used as a loading control.

reductions in protein levels as cellular actin levels were unaffected by the presence of RTA. The ubiquitin ligase-defective mutant RTA^{H145L} was expressed at higher levels than wt RTA. We suspect that this is due to the reported auto-ubiquitination activity of wt RTA, which promotes its own degradation. These data show that Hey1 protein levels were downregulated in the presence of RTA but not RTA^{H145L}. Therefore, efficient downregulation of Hey1 appears to be dependent on the Cys-plus-His-rich domain of RTA that is critical for the E3 ubiquitin ligase activity of RTA.

RTA targets Hey1 for proteasomal degradation. Ubiquitination of target proteins can have a number of outcomes, depending on the nature of the ubiquitin modification. Ubiquitination of IRF7 and K-RBP by RTA leads to proteasomal degradation of both of these target proteins (62, 64). To determine if Hey1 is also targeted for proteasomal degradation by RTA, we investigated whether RTA was still able to downregulate Hey1 protein levels in the presence of a proteasome inhibitor, MG132. HEK 293T cells were cotransfected with Hey1-Myc and RTA or RTA^{H145L} in the absence and presence of MG132, which was added to the cell medium at 12 h posttransfection. The cells were harvested 24 h later, and the cell lysates were subjected to SDS-PAGE and subsequent immunoblotting with a Myc tag-specific IgG to observe the levels of Hey1 protein (Fig. 3). In the presence of RTA Hey1 levels were markedly reduced; however, in the presence of MG132 and RTA, Hey1 levels were comparable to levels in the RTA^{H145L}-expressing control cells. These results suggest that the observed reduction in expression of Hey1 in the presence of wt RTA is proteasome dependent.

RTA ubiquitinates Hey1. Degradation of IRF7 and K-RBP in the presence of RTA has been attributed to a novel E3 ubiquitin ligase activity of RTA (62, 64). To confirm that the observed proteasomal degradation of Hey1 in the presence of RTA is directly related to the E3 ubiquitin ligase activity of RTA, immunoblotting was performed to detect ubiquitinated Hey1 in lytically reactivated BCBL1 cells. BCBL1 cells were treated with TPA and NaB to induce KSHV lytic reactivation and subsequent RTA expression. The experiment was carried out in the absence and presence of MG132. At 18 h postinduction the cells were harvested and boiled in a modified Laemmli sample buffer containing 2% SDS, 10 mM *N*-ethylmaleimide, and 10 mM DTT. The samples were separated by SDS-PAGE and subjected to immunoblotting with a Hey1-specific IgG. Figure 4 clearly shows the presence of a ladder of ubiquitinated Hey1 species upon reactivation of the virus from latency. This was observed only in the presence of MG132 and only in reactivated cells. Ubiquitination of Hey1 correlated with expression of RTA as ubiquitinated Hey1 species were not observed in the nonreactivated cells, where RTA expression was markedly lower. The data strongly suggest that, in the presence of RTA, Hey1 is ubiquitinated and that this ubiquitination targets Hey1 for proteasomal degradation.

Hey1 represses the RTA promoter. Hey1 is a transcriptional repressor involved in the Notch signaling pathway. Based on our findings that RTA promotes degradation of Hey1, we hypothesized that Hey1 might repress the RTA promoter since this would provide an advantage for RTA to downregulate Hey1. Interestingly, a recent study by Yada et al. (60) suggested that Hey1 does in fact repress the RTA promoter in reporter assays. To test our hypothesis and confirm the findings by Yada et al. (60), luciferase reporter assays were performed on HEK 293T cells cotransfected with an RTA promoter-luciferase construct (pLucORF50Δ9) in the presence of 0 to 0.4 μg of Hey1-Myc plasmid DNA. The total DNA transfected into the cells was kept constant at 0.9 μg by addition of pCS2-Myc. Figure 5A shows that RTA promoter activity is repressed by Hey1 in a dose-responsive manner. In the presence of 0.4 μg of Hey1-Myc DNA, the RTA promoter is repressed by almost 75% compared to the control; this level of repression is considerably higher than that observed by Yada et al. for a similar region of the RTA promoter. Furthermore, expression of RTA alongside Hey1 rescues the transcriptional activity of the RTA promoter in a dose-responsive manner (Fig. 5B). Our data confirm that Hey1 represses RTA promoter activity and that expression of RTA ablates this repressive effect although the exact mechanism through which this takes place has not been deduced. These observations provide a rationale behind the targeting of Hey1 for proteasomal degradation by RTA and suggest a mechanism whereby Hey1 helps in the repression of RTA gene expression; however, degradation of Hey1 enhances reactivation, specifically increasing RTA expression during the early stages of lytic replication.

Hey1 and associated corepressors bind the RTA promoter. The molecular mechanism by which Hey1 controls transcription has not yet been fully elucidated. A number of models have been described suggesting how Hey1 may repress its target promoters, including both DNA binding and non-DNA binding mechanisms (11). To determine whether Hey1 represses RTA gene expression through interactions with the RTA promoter, we performed ChIP assays. HEK 293T cells were cotransfected with an RTA promoter construct in the presence of either Hey1-Myc or Myc. A Myc tag-specific polyclonal IgG was used to immunoprecipitate Hey1-Myc and its associated DNA. Proteinaceous material was removed from the immunoprecipitate by proteinase K digestion, and the purified DNA was used as a template in a PCR reaction with primers against the RTA or the ORF73 promoter region. Figure 6A and B show that Hey1 is associated with the RTA promoter, but it does not bind to the ORF73 latent gene

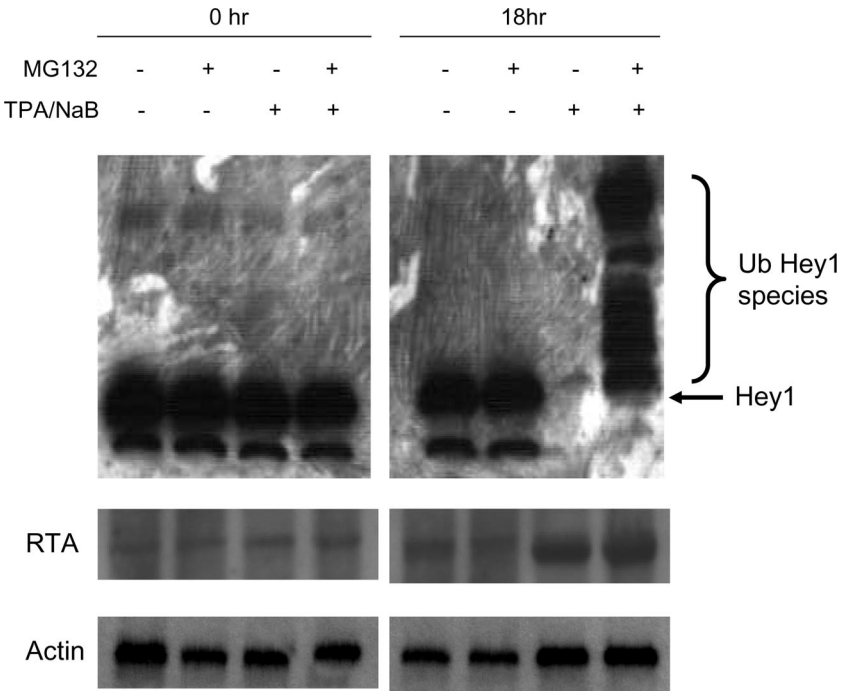

FIG. 4. Hey1 is ubiquitinated in reactivated BCBL1 cells. BCBL1 cells were induced using 20 ng/ml TPA and 3 mM NaB in the presence and absence of 5 μM MG132. The cells were harvested at 0 and 18 h postreactivation and lysed in Laemmli buffer containing 2% SDS, 10 mM *N*-ethylmaleimide, 10 mM DTT, and protease inhibitor cocktail. Immunoblot analysis using a Hey1-specific polyclonal IgG was carried out to detect ubiquitinated Hey1 species. RTA-specific polyclonal IgG was used to show the upregulation of RTA protein levels in the reactivated cells. An actin-specific monoclonal antibody was used as a control for equal loading.

promoter or GAPDH gene sequence. To confirm that the interaction was not an artifact of overexpression, ChIP assays were repeated using nonreactivated versus reactivated BCBL1 cells (Fig. 6C). In nonreactivated BCBL1 cells, endogenous Hey1 interacted with the RTA promoter but not with the ORF73 promoter or GAPDH gene sequence. This interaction was abolished when the cells were reactivated with TPA and NaB.

Effective repression often requires formation of a multiprotein complex on the target promoter, referred to as a repressor complex or repressosome. Thus, Hey1 is likely to be one of a number of proteins that form a repressosome on the RTA

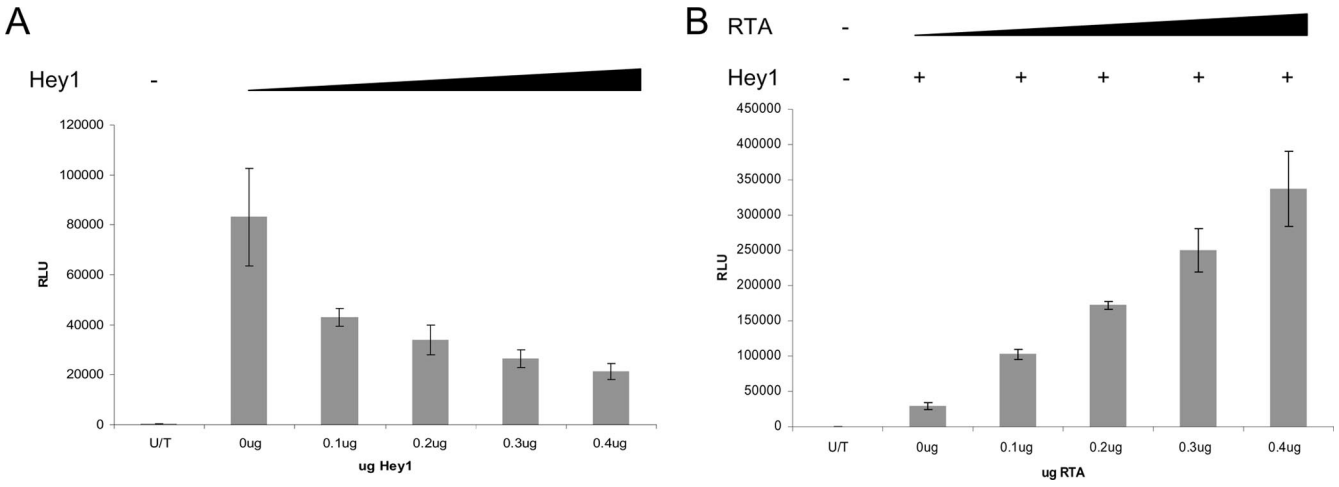

FIG. 5. Hey1 represses the RTA promoter, and repression is released upon RTA expression. (A) HEK 293T cells were transfected with 0.4 μg of pLucORF50Δ9 and increasing amounts (0 to 0.4 μg) of Hey1-Myc plasmid DNA. Total DNA was kept constant with pCS2-Myc parent vector. (B) HEK 293T cells were transfected with 0.3 μg of pLucORF50Δ9 and 0.3 μg of Hey1, alongside increasing amounts (0 to 0.4 μg) of RTA plasmid DNA. The total DNA was kept constant with pCDNA3.1. All transfections were carried out in triplicate. Cells were harvested at 24 h posttransfection, and the luciferase activity of each cell lysate was measured in triplicate. The readings were averaged for each condition, and the standard error was calculated. RLU, relative light units; U/T, untransfected control.

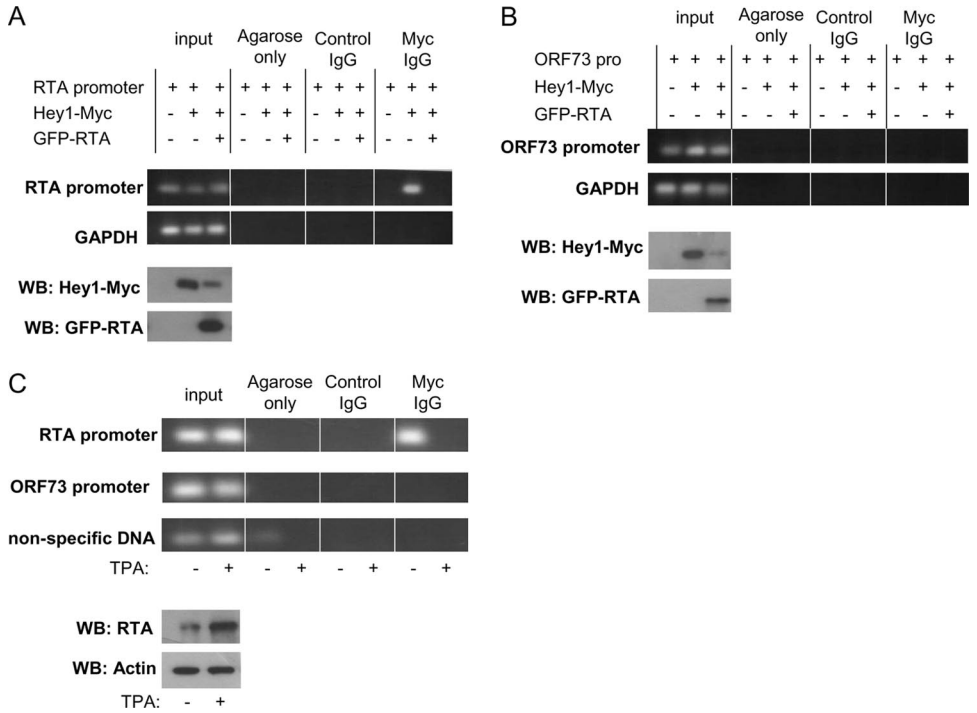

FIG. 6. Hey1 associates with the RTA promoter in HEK 293T and BCBL1 cells. ChIP assays were carried out on HEK 293T cells transfected with Hey1-Myc or Myc and an RTA promoter construct (A) or an ORF73 promoter construct (B) in the absence and presence of GFP-RTA. The chromatin complexes were immunoprecipitated using a Myc tag-specific polyclonal IgG and protein A-agarose alongside agarose-only and IgG controls. The presence of RTA promoter DNA in the input and the immunoprecipitates was determined by PCR amplification using primers specific to the RTA promoter or the ORF73 promoter and also using primers toward GAPDH. Western blotting (WB) was carried out to confirm expression of GFP-RTA and Hey1-Myc using GFP- and Myc-specific monoclonal IgGs, respectively. (C) ChIP assays were performed on nonreactivated versus reactivated BCBL1 cells. Reactivated cells were treated with 20 ng/ml TPA and 3 mM NaB. Endogenous Hey1 and associated DNA fragments were coimmunoprecipitated using a Hey1-specific polyclonal IgG and protein A-agarose alongside agarose-only and IgG controls. PCR amplification using primers against the RTA promoter or ORF73 promoter was used to determine whether endogenous RTA or ORF73 promoter DNA, respectively, was present in the immunoprecipitates. A GAPDH control PCR was also performed. Immunoblotting was carried out with an RTA-specific IgG to confirm RTA expression following reactivation and with an actin-specific IgG to demonstrate equal loading.

promoter. The Hey family of proteins has been shown to interact with corepressor proteins including HDACs and mSin3A (28). However, Hey1 itself has not previously been shown to interact with HDAC1 or mSin3A. Therefore, we performed coimmunoprecipitation assays to determine whether Hey1 interacts with these corepressors. 293T cells were transfected with Hey1-Myc in the absence or presence of RTA, and the cell lysates were subjected to coimmunoprecipitation using antibodies against endogenous HDAC1, NCoR, and mSin3A. Immunoblot analysis with a Myc-specific antibody revealed an interaction between Hey1 and mSin3A, which was observed only in the absence of RTA; in contrast, this interaction was abolished upon expression of RTA (Fig. 7). Levels of mSin3A were unaffected in the absence or presence of RTA, suggesting that RTA-mediated degradation of Hey1 was responsible for the inability to observe the interaction between Hey1 and mSin3A in RTA-expressing cells.

It is possible that Hey1 may recruit mSin3A to the RTA promoter to initialize formation of a repressosome. To help determine whether the Hey1-mSin3A interaction has a role in RTA promoter repression, we performed ChIP assays using an mSin3A-specific polyclonal IgG in nonreactivated versus reactivated BCBL1 cells. DNA was extracted and purified from the

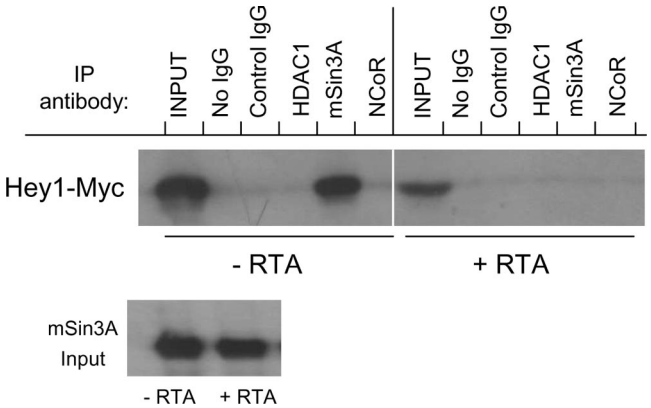

FIG. 7. Hey1 associates with mSin3A. Coimmunoprecipitations were carried out on HEK 293T cells transiently transfected with Hey1-Myc in the absence or presence of RTA. Cell lysates were incubated with protein A-agarose and antibodies specific to either HDAC1, mSin3A, or NCoR. The precipitated proteins were analyzed by Western blotting using a monoclonal Myc tag-specific IgG. Input levels of mSin3A are shown in cells transfected in the absence and presence of RTA (lower panel). IP, immunoprecipitation.

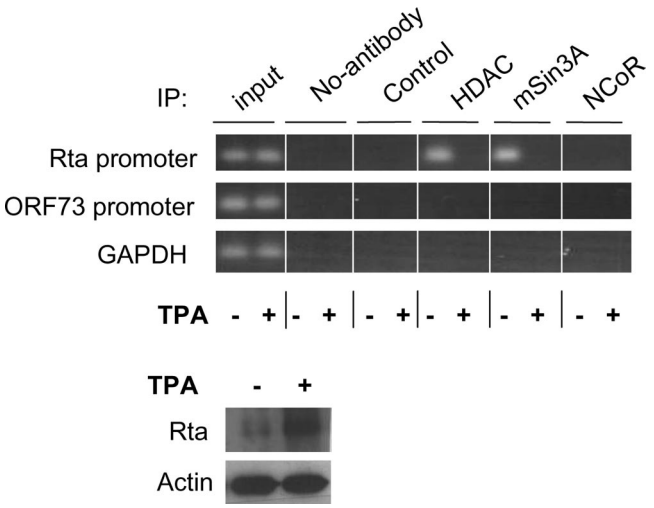

FIG. 8. HDAC1 and mSin3A corepressors associate with the RTA promoter in BCBL1 cells. ChIP assays were carried out on nonreactivated versus reactivated BCBL1 cells using antibodies specific to endogenous HDAC1, mSin3A, and NCoR. Reactivated BCBL1 cells were treated with 20 ng/ml TPA and 3 mM NaB. PCR amplification was carried out on the immunoprecipitates using primers against the RTA promoter region to determine whether endogenous RTA promoter DNA was associated with the candidate repressors. Control PCR amplifications were carried out using GAPDH and ORF73 promoter-specific primers. RTA expression was detected following TPA/NaB reactivation by immunoblotting using RTA-specific IgG, and equal loading was shown using actin-specific IgG (lower panel). IP, immunoprecipitation.

immunoprecipitated protein-DNA complexes and used as a template in PCRs with primers to the RTA promoter or the latent ORF73 promoter. The RTA promoter was coimmunoprecipitated with mSin3A in nonreactivated BCBL1 cells but not reactivated BCBL1 cells (Fig. 8). Although no interaction was observed between Hey1 and HDAC1 or NCoR, it is still possible that they are components of the repressosome via indirect interactions with Hey1. Therefore, we also carried out ChIP assays with HDAC1 and NCoR-specific antibodies (Fig. 8). HDAC1 was associated with the RTA promoter in latent but not reactivated BCBL1 cells. In contrast, no interaction was observed with NCoR in either nonreactivated or reactivated cells.

These data suggest that Hey1 binds the RTA promoter and recruits corepressors such as mSin3A to the RTA promoter. Furthermore, the observed interactions take place only in the absence of RTA or in nonreactivated BCBL1 cells. These results support our hypothesis that Hey1 represses RTA expression by binding to the RTA promoter and recruiting a repressor complex. Furthermore, the repressosome dissociates from the RTA promoter upon lytic reactivation, releasing the repression on the RTA promoter.

Hey1 knockdown is insufficient to induce lytic reactivation but enhances RTA expression upon reactivation. The formation of a Hey1 repressosome on the RTA promoter suggests that Hey1 has a role in maintenance of latency. Moreover, the targeted degradation of Hey1 by RTA indicates that removal of the Hey1-associated repressosome may be an important step in initiation of the lytic replication cycle. Effective Hey1 depletion has previously been observed using siRNAs (65). Therefore, to test this hypothesis, we performed experiments using siRNAs to knock down Hey1 expression in cells latently infected with KSHV. Due to the poor transfection efficiency of BCBL1 cells, these experiments were carried out in rKSHV.219-infected HEK 293T cells. These cells are latently infected with recombinant KSHV that expresses GFP constitutively under the control of the cellular EF-1α promoter and RFP under the control of the lytic PAN promoter (51). The aim of the experiment was to determine whether KSHV could be reactivated following loss of Hey1. To confirm the effectiveness of the Hey1 siRNAs upon transfection into the rKSHV.219-infected HEK 293T cells, immunoblotting was carried out to detect endogenous Hey1 levels at 0, 24, and 48 h posttransfection (Fig. 9A). Upon effective depletion of Hey1 using siRNAs, we assessed what effect depletion of Hey1 had on reactivation and lytic gene expression. Initially, we assessed whether depletion of Hey1 could reactivate lytic gene expression. To this end, rKSHV.219-infected HEK 293T cells were transfected with Hey1-specific or scrambled siRNAs, and cells were harvested at 0, 24, and 48 h posttransfection. Western blotting for the lytic ORF57 gene product was then performed using an ORF57-specific IgG. Results shown in Fig. 9B demonstrate that Hey1 depletion is not sufficient to reactivate lytic gene expression. Further studies were also performed using the Hey1-specific siRNAs in the presence of TPA. rKSHV.219-infected HEK 293T cells were transfected with Hey1-specific or scrambled siRNAs and induced with TPA at 24 h posttransfection. Immunoblotting was then performed on cell lysates harvested at 24 h postreactivation using an RTA-specific IgG. Results demonstrated a modest increase in RTA levels in the presence of Hey1-specific siRNAs upon reactivation (Fig. 9C). These results suggest that although depletion of Hey1 is not sufficient to reactivate KSHV, it may enhance RTA levels during early stages of lytic replication.

To confirm the results observed by immunoblotting, we also performed fluorescence microscopy on siRNA-treated rKSHV.219-infected HEK 293T cells (58). This stable cell line is an excellent tool for studying KSHV lytic reactivation as the cells display RFP fluorescence only when the virus is undergoing lytic replication and actively transcribing from the PAN promoter. The rKSHV.219-infected HEK 293T cells were transfected with scrambled or Hey siRNAs or chemically reactivated using NaB as a positive control for reactivation. The cells were then observed for GFP and RFP expression at 24 and 48 h posttransfection (Fig. 9C). These results show that knockdown of Hey1 leads to modest increases in RTA expression upon reactivation but is insufficient to effectively reactivate KSHV in HEK 293T cells latently infected with rKSHV.219. This suggests that Hey1 degradation is not solely responsible for maintenance of latency but could be one of multiple mechanisms in place to keep lytic reactivation under tight control, perhaps by regulating RTA expression.

DISCUSSION

The latent-lytic switch is essential for KSHV infectious virion propagation and has critical implications in KS disease pathogenesis (16). KSHV RTA is the key regulator of the latent-lytic switch since RTA expression alone is sufficient to

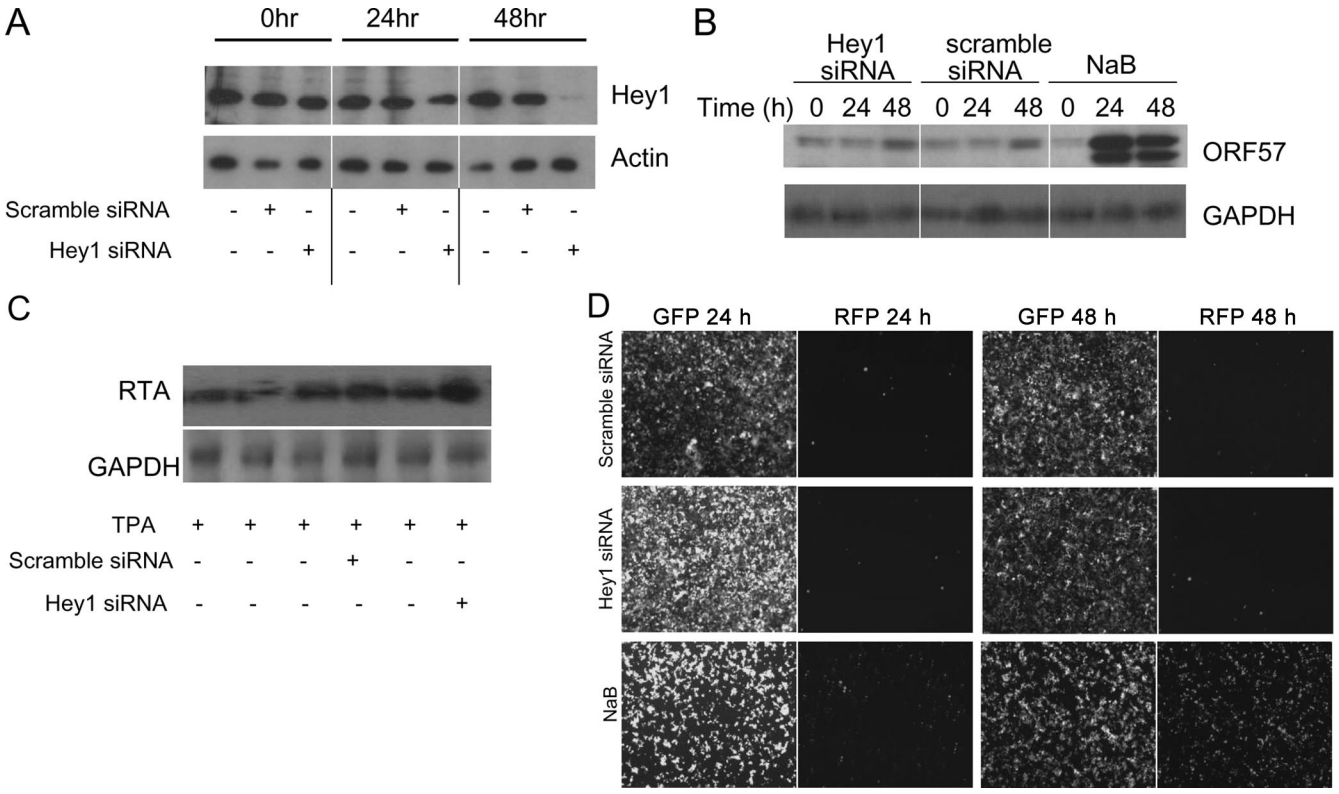

FIG. 9. Depletion of Hey1 enhances RTA expression upon reactivation but is insufficient to reactivate a latent KSHV infection. (A) rKSHV.219-infected HEK 293T cells were transfected with Hey1-specific or scrambled siRNAs, and the cells were harvested at 0, 24, and 48 h posttransfection. Cell lysates were analyzed by Western blotting using a Hey1-specific polyclonal IgG and actin-specific IgG. (B) rKSHV.219-infected HEK 293T cells were transfected with Hey1-specific or scrambled siRNAs or chemically induced using with 20 ng/ml TPA and 3 mM NaB. Cell lysates were harvested at 0, 24, and 48 h posttransfection and analyzed by immunoblotting using an ORF57-specific polyclonal IgG and actin-specific IgG loading control. (C) rKSHV.219-infected HEK 293T cells were transfected with mock, scrambled, or Hey1-specific siRNAs and at 24 h posttransfection reactivated with 20 ng/ml TPA. Cell lysates were harvested at 24 h postreactivation and analyzed by Western blotting with an RTA-specific IgG and GAPDH-specific IgG for a loading control. (D) HEK 293T cells latently infected with rKSHV.219 were transfected with Hey1-specific or scrambled siRNAs or chemically induced with NaB and TPA. At 24 and 48 h posttransfection, cells were analyzed for GFP and RFP fluorescence by fluorescence microscopy. Typical fields, singly excited to detect cells expressing GFP (virus-infected cells) or RFP (lytically reactivated virus) are shown.

drive KSHV lytic reactivation in latently infected cells (37). During latency the RTA promoter is thought to be repressed by many proteins including NF-κB (1), KSHV LANA (36), and Hey1 (60). Conversely, X-box binding protein 1 (XBP-1), Oct-1 and C/EBPα are associated with the RTA promoter during lytic replication and promote RTA transcription (45, 52, 63). Herein, we provide evidence that RTA interacts with the transcriptional repressor protein, Hey1, and promotes its degradation via the ubiquitin-proteasome pathway. Hey1 has been shown to repress the RTA promoter; therefore, this targeted degradation may have implications for the regulation of the KSHV latent-lytic switch and initiation of lytic replication (Fig. 10).

RTA has recently been shown to function as a novel E3 ubiquitin ligase and ubiquitinates IRF7 and K-RBP, targeting them for proteasomal degradation (62, 64). Our results suggest that Hey1 is an additional target for RTA's ubiquitin ligase activity. Hey1 is a cellular transcriptional repressor and primary downstream target of the Notch signaling pathway (11). We have shown that RTA can promote ubiquitination and degradation of Hey1 via the ubiquitin-proteasome degradation pathway. The E3 ubiquitin ligase activity of RTA has been attributed to a noncanonical Cys-plus-His-rich domain as mutants within this domain could not facilitate ubiquitination and degradation of IRF7 (64). Similarly, we also found that these mutants were unable to target Hey1 for degradation. We cannot conclusively determine from our data if RTA has intrinsic E3 ligase activity or whether it interacts with a cellular E3 ubiquitin ligase. However, RTA can auto-ubiquitinate, leading to proteasomal degradation (59). This is a common feature of E3 ubiquitin ligases, suggesting that RTA has intrinsic E3 ligase activity.

Hey1 can now be added to the growing list of proteins including K-RBP, NF-κB, K-bZIP, and LANA, which repress KSHV lytic gene expression and are also downregulated in the presence of RTA. The proteasome inhibitor MG132 inhibits RTA-dependent downregulation of Hey1 (shown herein), LANA, and K-bZIP, suggesting that these proteins are downregulated by the ubiquitin-proteasome pathway; however, MG132 does not inhibit NF-κB downregulation (62). This suggests that RTA utilizes more than one mechanism to downregulate target proteins.

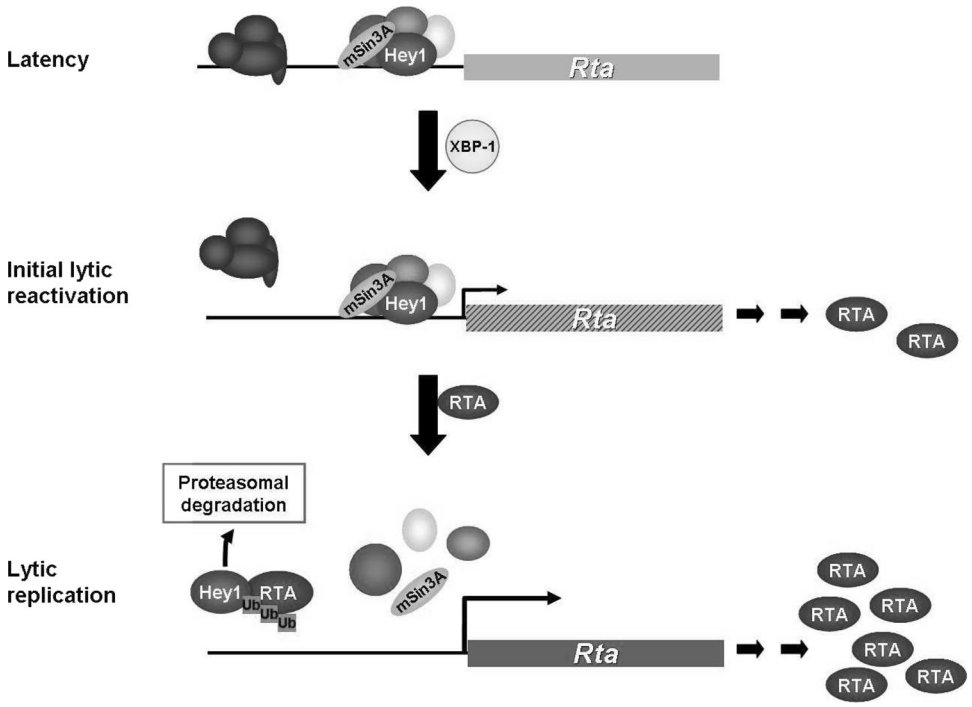

FIG. 10. Proposed role of Hey1 in KSHV reactivation and lytic replication. During latency the RTA promoter is tightly repressed by the Hey1-induced repressosome and other repressors (indicated as a complex to the left of the Hey1 repressosome), preventing RTA expression. Upon plasma cell differentiation, XBP-1s is expressed and activates the RTA promoter, causing low-level RTA expression and thus triggering initial lytic reactivation. Once a small amount of RTA is expressed, it ubiquitinates Hey1, leading to proteasomal degradation of Hey1 and dissociation of the repressosome from the RTA promoter. Loss of the Hey1-induced repressosome from the RTA promoter enhances RTA expression, allowing complete lytic replication to proceed. Ub, ubiquitin.

The Hey family of bHLH proteins has been shown to recruit HDACs, mSin3A, and NCoR corepressors to their target promoters, thereby initiating the formation of a repressosome on target promoters (11). Results herein and previous observations (55) have shown that Hey1 can repress the RTA promoter. Moreover, we demonstrate that Hey1, mSin3A, and HDAC1 all interact with the RTA promoter in BCBL1 cells latently infected with KSHV. Interestingly, coimmunoprecipitation assays showed an interaction between Hey1 and mSin3A, which was abolished in the presence of RTA. Therefore, one may speculate that Hey1 recruits mSin3A and other corepressors to the RTA promoter to form a repressosome on the RTA promoter during latency. Our data showed that HDAC1 is associated with the RTA promoter in BCBL1 cells latently infected with KSHV-; however, we did not detect an interaction between Hey1 and HDAC1. This may suggest that HDAC1 is not part of the Hey1-induced repressosome on the RTA promoter. This theory is perhaps supported by the observations of Yang et al., who observed no reduction in HDAC1 levels in the presence of RTA (62).

The observation that Hey1 represses the RTA promoter and may initiate the formation of a repressosome in cells latently infected with KSHV implicates Hey1 in the maintenance of latency. Therefore, we reasoned that RTA-dependent targeted degradation of Hey1 might result in the initiation of lytic replication due to dissociation of the Hey1 repressosome. However, siRNA-mediated depletion of Hey1 in HEK 293T cells latently infected with rKSHV.219 did not trigger lytic reactivation. Nevertheless, Hey1 depletion does enhance RTA expression upon reactivation. A possible explanation for not triggering reactivation is that depletion of Hey1 is insufficient to induce lytic reactivation because other repressor proteins, such as LANA and NF-κB, interact with the RTA promoter. However, it must also be noted that although the majority of Hey1 was depleted in siRNA-treated cells, this is not complete knockdown.

We have previously published data to suggest that XBP-1 has a role in reactivation of KSHV (58). During plasma cell differentiation, a highly active spliced form of XBP-1(XBP-1s) is expressed. XBP-1s binds to and activates the RTA promoter (58), and it is possible that this may be the mechanism through which lytic reactivation is initiated. We believe that XBP-1s and Hey1 both play a role in lytic reactivation, whereby the RTA promoter is initially activated by XBP-1s, leading to low-level RTA expression, and RTA then targets Hey1 for proteasomal degradation, releasing the Hey1-induced repressosome from the RTA promoter and enhancing RTA expression. The high-level RTA expression would then be sufficient to induce complete lytic reactivation of the virus (Fig. 10).

Herein, we demonstrate that Hey1 is targeted by RTA for ubiquitin-mediated proteasomal degradation; however, a previous study identified Hey1 as a possible RTA-responsive gene (55). Moreover, RTA has been shown to activate the Hey1 promoter in luciferase reporter assays (59). This suggests that RTA and Hey1 expression may be intrinsically linked, provid-

ing a complex feedback loop mechanism to fine-tune the switch between latency and lytic replication.

In summary, we show that the transcriptional repressor protein Hey1 binds and represses the RTA promoter during latent KSHV infection in BCBL1 cells. The corepressor mSin3A may be one component of the Hey1-induced multiprotein repressosome, which is formed on the RTA promoter. Furthermore, RTA ubiquitinates Hey1 and targets it for proteasomal degradation via the ubiquitin-proteasome pathway, releasing its repressive effect on the RTA promoter. We found that depletion of Hey1 by siRNAs enhanced RTA levels upon reactivation but was not sufficient to induce complete lytic reactivation. These results suggest that RTA-induced degradation of Hey1 has a contributory role in the highly regulated process of lytic reactivation.

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