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By

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THE MOLECULAR MECHANISM OF VIRAL TRANSACTIVATOR RTA-MEDIATED KAPOSI'S SARCOMA-ASSOCIATED HERPESVIRUS (KSHV) LYTIC REPLICATION

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Kaposi's sarcoma-associated herpesvirus (KSHV) is a member of the gamma-herpesvirinae subfamily and displays two distinct life phases, latency and lytic replication. Infection with KSHV mostly results in a latent state. A small population of infected cells can spontaneously undergo lytic phase, which is marked by abundant viral gene expression and production of infectious viral progeny. Lytic replication is important for transmission of KSHV in the population and development of diseases. RTA (replication and transcription activator) is a master regulator of KSHV lytic replication. Expression of RTA alone is sufficient to disrupt KSHV latency and initiate the lytic replication cascade. The objective of this research is to understand how RTA stimulates lytic replication.

RTA utilizes its DNA binding ability and /or cooperates with co-activators to up-regulate vial lytic gene expression for lytic replication. In the present and previous studies, we identified three distinct RTA responsive elements (RREs) in an early gene (ORF57) promoter, and all of them are located in close proximity to each other in the ORF57 promoter. Current study demonstrated that two adjacent RREs and a co-activator (RBP-Jκ) binding site are required for optimal transactivation of the ORF57 promoter by RTA.

These results present the first evidence that RTA targets multiple DNA motifs in responsive viral promoter to achieve full transactivation.

We demonstrated that RTA is able to enhance the autophagy pathway through interference of the interaction between an autophagy-related protein Beclin 1 and an anti-apoptotic protein Bcl-2 by inducing Bcl-2 phosphorylation. This post-translational modification is a result of the induction of c-JUN N-terminal kinase (JNK) signaling pathway by RTA. In addition, a defective autophagy pathway specifically reduces RTA-mediated transactivation of lytic gene promoters. These results indicate that RTA activates autophagy to stimulate lytic gene expression and initiate lytic reactivation.

In conclusion, multiple non-conserved RREs and a co-activator binding site are required for RTA-mediated transactivation, indicating that a complex mechanism exists by which RTA transactivates lytic cycle gene expression. Additionally, RTA-induced autophagy may provide an optimal condition for RTA to mediate lytic gene expression and KSHV lytic activation, demonstrating that KSHV adopts cellular machinery for its viral DNA replication.

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LIST OF ABBREVIATIONS

AIDS: Acquired immune deficiency syndrome

AP-1: activator protein 1

Atg: autophagy-related genes

BAC: bacterial artificial chromosome

BCBL: body cavity-based lymphomas

Bcl-2: B-cell leukemia/lymphoma 2

BCLAF1: Bcl-2-associated transcription factor 1

BNIP3L: Bcl-2 adenovirus E1a nineteen kDa interacting protein 3-like protein

C/EBP-α: the CCAAT/enhancer binding protein α

ChIP: chromatin immunoprecipitation

DAPK: death-associated protein kinase

EBV: Epstein Barr virus

EMSA: electrophoretic mobility shift assay

FBS: fetal bovine serum

GPCR: G protein-coupled receptor

HAART: highly active antiretroviral therapy

HDAC: histone deacetylase

HHV8: human herpesvirus 8

HIF: hypoxia-inducible factor

HIV: human immunodeficiency virus

HMGB1: high-mobility group B1

HSV-1: Herpes simplex virus type 1

HUVEC: human umbilical vein endothelial cell

γHV68: murine gammaherpesvirus 68

JNK: c-Jun N-terminal kinase

K-RBP: KSHV RTA binding protein

KS: Kaposi's sarcoma

KSHV: Kaposi's sarcoma-associated herpesvirus

LC3: microtubule-associated protein 1 light chain 3

LMP: latent membrane protein

LUR: long unique region

MAPK: mitogen-activated protein kinase

MEK: mitogen-extracellular signal-responsive kinase

MCD: multicentric Castleman's disease

mTOR: mammalian target of rapamycin

OCT-1: octamer binding protein 1

ORF: open reading frame

PAA: phosphonoacetic acid

PARP: poly(ADP-ribose) polymerase

PCR: polymerase chain reaction

PEL: primary effusion lymphoma

PI3K: phosphatidylinositol 3-kinase

pRb: retinoblastoma

PVDF: polyvinylidene difluorid

RBP-Jκ: recombination signal binding protein Jκ

ROS: reactive oxygen species

RRV: rhesus rhadinovirus

RRE: RTA responsive element

RTA: replication and transcription activator

S6K1: ribosomal S6 protein kinase

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

THBS1: thrombospondin 1

TPA: 12-O-tetradecanoylphorbol-13-acetate

TR: terminal repeat

VEGF: vascular endothelial growth factor

vIL: viral interleukin

vIRF: viral interferon regulatory factor

XBP-1: x-box binding protein 1

CHAPTER 1

LITERATURE REVIEW

Introduction

Kaposi's sarcoma associated herpesvirus (KSHV) is one of several known human oncoviruses (Epstein-Barr virus, hepatitis B virus, hepatitis C virus, human papilloma virus, human T-cell lymphotropic virus, Merkel cell polyomavirus, JC virus, BK virus, and KSHV for example). Kaposi's sarcoma (KS), primary effusion lymphoma, and multicentric Castleman disease are known to be caused by KSHV (31, 39, 160). It is a member of the gamma-herpesviridae family (128). Like all herpesviruses, KSHV displays two distinct phases of infection, latency and lytic production phase. In most tumor cells, the virus persists in a latent form and expresses genes which regulate apoptosis, cell proliferation, and angiogenesis. Only small subpopulations of tumor cells support lytic replication (17, 151, 203). Therefore, the latent phase of infection plays a primary role in KSHV oncogenesis (14). However, the lytic program allows the virus to spread throughout the host and sustain the population of latently infected cells. In addition, clinical studies have shown that a block in the lytic but not latent KSHV infection in AIDS patients by ganciclovir treatment results in a dramatic decline in the incidence of new KS tumors (118). Currently, substantial evidence suggests that lytic replication is a prerequisite for KSHV pathogenesis. Replication and transcription activator (RTA), encoded by ORF50 of the KSHV genome, is necessary and sufficient for the initiation of lytic replication cycle of KSHV (184). RTA tethers transactivation function in regulation of numerous viral gene expressions and then facilitates viral lytic replication (162). To date, a number of RTA-targeted genes have been identified, and

various mechanisms by which RTA can function to regulate these targets result from the positive and negative participation of various cellular and viral proteins. Therefore, investigating how RTA creates a suitable environment for lytic cycle gene expression and lytic viral replication will lead to a better understanding of KSHV pathogenesis. This review will attempt to summarize the literature related to KSHV as well as the molecular mechanism involved in the regulation of the KSHV life cycle.

Clinical diseases and transmission of Kaposi's sarcoma-associated herpesvirus (KSHV)

KSHV-associated diseases

Human herpesvirus 8 (HHV-8) is the first known human member of the genus Rhadinovirus and was discovered by Yuan Chang and Patrick S. Moore in 1994 (39, 128). Viral DNA was identified by representational difference analysis of Kaposi's sarcoma (KS) tissues of AIDS patients (39). Accordingly, the virus was also named Kaposi's sarcoma-associated herpesvirus (KSHV). It has been determined by PCR analysis that KSHV is present in all forms of KS: classical, endemic, AIDS-associated and post-transplant KS (126). Classical KS is an indolent form and typically affects the skin of elderly men in Eastern European and the Mediterranean (Sardinia, Sicily, Israel, etc) (99). In contrast to classical KS, endemic form of KS predominantly occurs in young people and is commonly found in African countries such as Uganda, Malawi, and Kenya. Endemic KS can be indolent or aggressive based on the lesion spread areas. The aggressive endemic KS is usually found in young children who have lesions spreading to the lymph nodes (3). The most aggressive form of KS is found in AIDS patients in

developing countries, especially Africa. This form of KS can be life-threatening when KS lesions spread to internal organs (1). Post-transplant KS, the form of KS associated with immune deficiency, occurs in organ-transplant recipients who receive long-term immunosuppressive therapies to prevent reject allografts (3, 139).

In addition to KS, KSHV is also associated with two lymphoproliferative disorders, primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (31, 160). Nearly 50% of MCD patients without HIV infection have KSHV infection. All of HIV-positive MCD patients are infected with KSHV. Additionally, about 13% of MCD patients develop KS (58, 140). PEL is a B-cell lymphoma with low incidence and develops as serious effusion without any solid tumor mass. KSHV has been suggested to play an important role in PEL pathogenesis due to high seroprevalence of KSHV in PEL. However, KSHV infection may not be sufficient for PEL development and other cofactors may be required. Epstein Barr virus (EBV) infection occurs in most PEL cases (86%) suggesting EBV is a potential cofactor for PEL development (32, 62). Similarly, HIV (human immunodeficiency virus) has been suggested to be a cofactor of KSHV for KS development due to the decline in the incidence and mortality of AIDS-associated KS after the usage of anti-retroviral therapy (HAART) (86, 94, 167). In fact, it has been reported that HIV Tat (the trans-activating protein) enhances KSHV transmission into endothelial cells and also cooperates with KSHV proteins in promoting tumorigenesis (5).

KSHV transmission

To date, many studies have identified different potential routes and risk factors of KSHV transmission and they could vary depending on the geographic regions where these studies were conducted. The seroprevalence of KSHV is low in the United States and Northern Europe, intermediate in the Mediterranean, and high in sub-Saharan Africa (59, 83, 93, 185). Vertical transmission of KSHV can be found but is rare (115). Horizontal transmission from mother to child or from adult to adult is believed to occur mainly through saliva contact (19, 124, 141). In addition, sexual transmission is a candidate of KSHV transmission due to existence of KSHV DNA in semen and female genital tract samples (137, 187). Indeed, sexual transmission can be observed in homosexual men, especially with HIV co-infection, and may be the major mode of transmission (59, 119). Heterosexual transmission is still being debated (9, 10, 93, 113, 114, 153). KSHV transmission through blood transfusion is rare and is suggested to be a risk factor in Uganda and United States (11, 84). Interestingly, recent studies reveal that environmental cofactors, such as biting insects, drinking water, and crude plants (herbs) in traditional medicines, could result in the increase in the prevalence of KSHV in sub-Saharan Africa and the Mediterranean (8, 45, 46, 121, 186).

Characterization of KSHV

Based on phylogenetic analysis, KSHV belongs to the gamma herpesvirus subfamily that is closely related to herpesvirus saimiri, rhesus monkey rhadinovirus, bovine herpesvirus 4 (BHV-4) and murine gammaherpesvirus 68 (γHV68); thus, it is the only human member of the rhadinovirus (gamma-2 herpervirus) family (128, 147). KSHV has a typical herpesvirus structure containing the lipid envelope on the surface of

viral particle, the icosaherdal capsid (162 hexagonal capsomeres) encasing viral DNA-containing-protein core, and the tegument which is a protein-filled region between the envelope and capsid. KSHV possesses a linear double-stranded DNA genome of 170 kb. This large DNA genome is GC-rich (85%) and variable sequences of terminal repeat (TR) at both termini of the genome and a long unique region (LUR), that is about 138 kb in length. The TR region is responsible for tethering of the KSHV genome to chromosomal DNA and serves as the origin of latent plasmid replication (13, 127). LUR contains coding information for more than 87 open reading frames (ORFs). The specific ORFs which are not homologous to the genes in the herpesvirus saimiri genome are named with the prefix K and numbered sequentially (147). Among the KSHV genes, the K1 is highly variability and this variability is used to classify KSHV into five K1 subtypes (A, B, C, D,E) (60, 81, 208). The first tissue culture models (BC1 and BC2) for characterization of KSHV were established from body cavity-based lymphomas (AIDS-BCBL) that were derived from human immunodeficiency virus-positive patients (33). Later on, other cell lines (such as BCBL-1 and BC3) were established from other PEL samples, and they provide a good cell culture system for studying virus-host interactions (7, 25, 27, 28, 57, 146, 171). Nevertheless, viruses prepared from PEL cell lines usually have low primary-infection efficiencies even though they are infectious for different cell types, including monocytes, fibroblasts, lymphocytes, and keratinocytes. Moreover, primary-infected cells are unable to sustain long-term virus growth and also lose the virus after several passages (30, 95, 129, 133, 144). The other useful system for studying virus is a recombinant KSHV genome DNA, BAC36 (204). BAC36 has been engineered by cloning the full-length viral genome into a bacterial artificial chromosome (BAC) and viruses can be

recovered in human embryonic kidney 293 cells and human umbilical vein endothelial (HUVEC) cells (67) (204). This has the potential to allow characterization of individual viral genes.

KSHV is similar to other herpesviruses and displays two different phases in its life cycle, latency and lytic replication. Accordingly, all viral genes are classified into latent and lytic genes, and their expression patterns in latency and lytic phases have been studied in cell lines derived from PEL, de novo infection of cultured cells, and biopsies from KS, MCD, and PEL (171). According to the results of in situ hybridization of KS biopsies, the majority of tumor cells in the lesions express viral latent transcripts, but a subpopulation of tumor cells (1-3%) expresses lytic transcripts (161, 166). A number of KSHV-expressed gene products are homologues of cellular proteins which function in immunomodulatory, anti-apoptosis, and cell cycle regulation. Those viral proteins have been suggested to deregulate the cellular signaling cascades during the viral infection and thereby lead to tumorigenesis (131). In addition to expressing functional proteins, KSHV also produces non-coding but functional RNAs. Polyadenylated and exclusively nuclear (PAN) RNA has 1077 bp in size and is transcribed during the lytic cycle (164). Its function is to block the assembly of an mRNA-protein complex (mRNP) and retain intronless RNA in the nucleus (47). Recently, twelve pre-microRNAs have been discovered in the KSHV genome. Ten of these pre-microRNAs are localized in the non-coding region between K12 and K13, and two are identified within the K12 gene (24, 136, 149). All KSHV microRNAs are expressed during latency but they remain detectable throughout the lytic cycle (23, 130, 150).To date, the functions of viral microRNAs (miRNAs) are not fully understood but they have been suggested to regulate the KSHV

life cycle and viral pathogenesis. One of KSHV miRNAs, miR-K11, is an orthologue of cellular miR-155 and its expression causes the down-regulation of a set of mRNAs, including genes that regulate cell proliferation. Since cellular miR-155 is associated with B-cell transformation, the function of KSHV miR-K11 may be related to B cell lymphoproliferative disease (69). Additionally, gene expression profiling in cells stably expressing KSHV-encoded miRNAs shows that 81 cellular genes are significantly reduced. Among these genes, the protein level of THBS1 (thrombospondin 1) is decreased dramatically in the presence of KSHV miRNAs (150). THBS1 is a strong tumor suppressor and anti-angiogenic factor (169); thus, KSHV miRNAs may contribute to pathogenesis by suppression of THBS1 expression. KSHV-encoded miRNAs also regulate the KSHV life cycle. miR-K3 inhibits KSHV lytic cycle by targeting nuclear factor I/B which can activate the KSHV immediate-early gene (ORF50) promoter (108). miR-K9 limits RTA expression by targeting the sequence in the 3'untranslated region of RTA mRNA to prevent inappropriate entry into the lytic cycle (16). Conversely, miR-K5 can induce viral reactivation by repressing BCLAF1 (Bcl-2-associated transcription factor 1), an apoptosis-inducing factor (207). These results indicate that KSHV-encoded miRNAs plays role in controlling the balance between latency and lytic replication.

KSHV life cycle

Latency

KSHV establishes a lifelong latent infection in most infected cells. During a latent infection, the viral genome persists as a circular episome in the nucleus of the infected cell. The latent replication is dependent on the host DNA replication machinery, requiring

host cellular DNA polymerase and accessory factors, and initiates at *ori-P* (the origin of latent plasmid replication) followed by bidirectional replication (85). In addition, only a restricted number of viral genes (latent genes) are expressed during latency (145, 203). Due to limited viral gene expression and the absence of viral replication, no functional or infectious virions are produced during this period. This small subset of latent gene products, which can be detected in all three KSHV-associated malignancies (KS, PEL, MCD), includes a microRNA cluster and proteins, LANA (latent-associated nuclear antigen) encoded by *ORF73*, vCyc (viral cyclin) encoded by *ORF72*, vFLIP [viral FLICE (Fas-associated death domain like IL-1β-converting enzyme) inhibitory protein] encoded by *K13*, and Kaposin encoded by *K12* (116, 134). Additionally, viral interferon regulatory factor-3 (vIRF3; also named LANA-2) encoded by *K10.5* can be detected in latently infected PEL and MCD cells but not in KS cells (134).

The latent gene products presumably function to suppress host immune response, promote cell growth, possess cell survival and maintain latency. LANA is expressed abundantly in almost all latently infected tumor cells (134, 135). The major function of LANA is to maintain episomal viral DNA in the infected cells through tethering of the circularized viral DNA to host chromosomal DNA (13, 48, 198). LANA is also a transcriptional modulator which can be either an activator (91, 143, 168, 181) or a repressor (105). The most interesting example is that LANA suppresses the expression of RTA, which is essential for initiation of lytic replication (96, 97). In addition, LANA physically interacts with p53 to protect infected cells from undergoing apoptosis (65). To induce cell cycle progression, LANA inactivates pRb (Retinoblastoma) through interacting with unphosphorylated pRb (142).

LANA also limits the distribution of GSK-3β in the nucleus to allow accumulation of β-catenin to activate cell proliferation-related genes expression (66). Based on these findings, LANA has been suggested as a major viral protein for establishment and maintenance of latency and also promotes oncogenesis.

In addition to LANA, vFLIP is the other latent gene product that inhibits lytic replication via suppression of the AP-1 pathway (197, 202). vFLIP, which has a similar function as cellular FLIP in anti-apoptosis, blocks Fas-mediated apoptosis through inhibiting the maturation of procaspase-8 to protect infected cells from death (15). Besides being anti-apoptotic, many studies provide evidence about the transforming and oncogenic protential of vFLIP, which induces NFκB survival signaling through the association with the IκB kinase complex and the heat shock protein 90 (40, 61, 64, 106, 163). For cell cycle progression, vCyc, a homologue of human cyclin D2, can promote phosphorylation of pRb to induce cell cycle progression from G1 to S phase. During viral latency, 17 KSHV miRNAs derived from 12 pre-miRNAs are expressed (207). Some repress immediate-early gene expression, thus promoting the maintenance of latency (16, 108, 130).

The regulation of KSHV reactivation from latency

KSHV primarily persists in infected cells with a lifelong latency, and only a low percentage of infected cells undergo spontaneous lytic replication (151, 203). Nevertheless, lytic replication plays an important role in the maintenance of the population of latently infected cells because infectious viral progeny is only produced during lytic phase. In addition, a number of viral proteins expressed during the lytic cycle

are involved in modulating paracrine secretion of growth and angiogenic factors required for tumor growth and development (183). Thus, virus reactivation from latency becomes a critical step for KSHV pathogenesis. The KSHV RTA is a key protein responsible for the switch from latency to lytic stage of infection. Induced expression of RTA is sufficient for virus reactivation. The major function of RTA is to regulate the subsequent transcriptional cascade during the lytic cycle, which is described in "RTA function in transcription" section (54, 162). Besides transcriptional regulation of lytic genes expression, RTA is required for lytic viral DNA replication because it assembles the DNA replication complex at *ori-lyt* (179). To efficiently initiate lytic cycle, RTA also circumvents the innate antiviral defenses within host cells. RTA counteracts IFN (interferon)-mediated antiviral defenses by inhibiting IFNα and β production. RTA mediated ubiquitination and degradation of IRF7 in a proteasome-dependent fashion is crucial for interfering with IFN production (200). To activate lytic DNA replication, the structure of viral DNA genome is also critical. In latency, the viral genome is maintained as condensed heterochromatin during latency in the cells but the vial genome relaxes its compact chromatin structure to an euchromatin state, after induction of lytic cycle, which enables lytic gene expression and DNA replication. Hence, KSHV lytic reactivation can be triggered by sodium butyrate (an inhibitor of histone deacetylases), TPA (an inducer of histone acetyltransferases), and 5-azacytidine (an inhibitor of DNA methyltransferase) (41, 123, 175).

There are also a number of studies demonstrating that cellular signaling pathways are involved in the regulation of KSHV reactivation. For example, inhibiting Akt activity enhances TPA-induced lytic replication and RTA expression (138), suggesting Akt

maintains latency. Multiple mitogen-activated protein kinase (MAPK) signaling pathways, MEK/ERK, JNK and p38 pathways, are required for KSHV reactivation (44, 190, 199). Hypoxia activates HIF (hypoxia-inducible factors), a transcriptional factor, to up-regualte lytic gene expression (51, 79). A major downstream effector of the Notch signaling pathway, RBP-Jκ, mediates RTA-stimulated transactivation of lytic gene promoters, and constitutive activation of Notch1 is sufficient to reactivate KSHV from latency (98). The role of NF-κB in the context of the KSHV life cycle is compex (52). NF-κB is mainly activated by vFLIP during latency for cell survival and proliferation, and activated NF-κB can suppress RTA-mediated gene expression by interfering with RBP-Jκ interactions (87, 120). Conversely, viral lytic reactivation requires down-regulation of NF-κB (20) while some lytic gene expression (such as vGPCR) stimulates NF-κB expression (26, 117). Thus, the regulation between lytic replication and latency involves a complex set of viral and cellular proteins, and a number of cellular signaling pathways. There is a tight interplay between latency and lytic replication so that the virus can be maintained mostly in latency to enable cell transformation. The virus also needs to be reactivated periodically to spread viral progeny to neighbor cells and sustain the population of latently infected cells.

Lytic cycle

Unlike latent DNA replication, KSHV lytic DNA replication is dependent on its own DNA polymerase and other viral factors to initiate replication at *ori-lyt* (the origin of lytic replication), then proceed via a rolling circle mechanism; thereby, viral DNA is amplified rapidly during lytic phase. Lytic genes can be categorized into immediate-early

(IE), early, and late genes according to their sensitivity to cycloheximide and phosphonoacetic acid (PAA) (166, 205). After primary infection or upon reactivation from latency, the mRNAs of IE genes are transcribed earliest and the process is independent of *de novo* protein synthesis due to its resistance to inhibitor of protein synthesis, such as cycloheximide. Usually, IE gene products possess gene regulatory functions and directly activate early gene expression, which is sensitive to cycloheximide but resistant to the DNA polymerase inhibitor (PAA) (166). The expression of early genes is followed by late gene expression. The products of early genes are involved in the viral replication machinery and late genes encode proteins that comprise the virion . As a result of the lytic cycle, cell death and release of infectious viral progeny occur. In addition, KSHV productive infection correlates with the development of KS and MCD (2, 17, 151). A number of clinical studies have reported that treatment of KSHV infected-AIDS patients by a drug ganciclovir, which specifically blocks lytic replication of KSHV, results in a dramatic decrease in the incidence of KS (118). The evidence indicates that lytic cycle of KSHV plays an important role in KSHV pathogenesis. In fact, certain viral proteins encoded by lytic genes have oncogenic properties. The four viral interferon regulatory factors (vIRFs) identified in the KSHV genome induce transformation of established fibroblasts through inactivating the p21 tumor suppression and the p53 pathway (132). KSHV vIL-6 (viral interleukin 6), a homolog of the human IL-6, also possesses transforming ability to promote hematopoiesis, plasmacytosis, and angiogenesis through the induction of high VEGF (vascular endothelial growth factor) expression (4). Viral GPCR (G protein-coupled receptor) encoded by *ORF74* is the most studied lytic gene in cell transformation and has been implicated as an important

oncogene for tumorigenesis, especially in KS (12, 72, 92, 125, 154, 193). Various mitogenic and survival intracellular pathways are stimulated by vGPCR (154, 155). Thus, expression of vGPCR is able to cause transformation of different cell types, including fibroblasts and endothelial cells (6, 12, 125) and can also promote the development and progression of KS-like lesions in multiple animal model systems (89, 125, 193). Although these lytic gene products have oncogenic potential and interruption of lytic cycle can suppress disease progression, it is still unclear how viral gene expressed during productive infection play a role in cell immortalizaton when the infected-cells are destined to die.

KSHV replication and transcription activator (RTA)

RTA protein is encoded in the genomes of all gamma herpesviruses, including Epstein-Barr virus (EBV), rhesus rhadinovirus (RRV), herpesvrius saimiri (HVS), and murine herpesvirus 68 (MHV-68). Only the RTA protein produced by gamma-2-herpesviruses (KSVH, HVS, and MHV-68) acts as a major lytic-replication activator (68, 165, 189). For EBV (gamma-1-herpesvirus), regulation of lytic reactivation from latency requires two proteins, Rta and ZEBRA (63, 122). KSHV RTA has been suggested as the essential lytic switch protein for KSHV from studies that involved deletion and overexpression of RTA (112, 165, 191). A mutant recombinant KSHV genome containing RTA gene deletion is incapable of viral replication and unable to produce viral progeny upon tetradecanoyl phorbol acetate (TPA) treatment (191). However, expression of RTA alone in latently infected cells is sufficient to initiate lytic reactivation from latency (71, 112, 165).

RTA protein expression

KSHV RTA is encoded by the IE gene *ORF50*. ORF50 transcript is induced within 4 h of sodium butyrate treatment in latently infected PEL cells and is spliced from a major 3.6-kb IE transcript which contains the ORF50, K8, and K8.1 reading frames (111, 166, 205). The promoter of *ORF50* is heavily methylated in latently infected PEL cells and in biopsy samples obtained from latently infected patients (41), but RTA expression can be induced by demethylation of its promoter upon TPA treatment (41). ORF50 promoter is also responsive to hypoxia and may be activated by XBP-1 (x-box binding protein 1) under hypoxic conditions (22, 49, 79, 188). RTA is a transcriptional protein and can auto-regulate its expression through indirectly binding to its own promoter (36, 56). Cellular transcription factors, including OCT-1 (octamer binding protein 1), C/EBP-α (the CCAAT/enhancer binding protein α), HMGB1 (high-mobility group B1), Sp1, and Sp3, were shown to mediate RTA auto-regulation (80, 109, 148, 175, 201). A recent study showed that RTA expression can also be repressed by KSHV encoded miRNA, miRK9, which targets the sequence in the 3'untranslated region of the mRNA encoding RTA (16).

RTA is a 691-amino-acid nuclear protein which is composed of a basic DNA binding domain in the amino-terminus, an acidic transactivation domain in the carboxy-terminus and two potential nuclear localization signals (21, 184). The RTA protein is highly phosphorylated (111) and, the phosphorylation of RTA is mediated by the cellular Ste20-like kinase hKFC and poly(ADP-ribose) polymerase (PARP-1) (77). In addition, RTA has E3 ubiquitin ligase activity and could direct ubiquitination of itself to affect its

stability (200). Moreover, amino acids (aa) 520 to 535 of RTA protein regulate its protein stability and DNA binding efficiency (36).

RTA function in transcription

RTA activates the transcription of a number of viral lytic promoters, including its own promoter (56, 71). For example, RTA transactivates viral promoters that encode polyadenylated nuclear (PAN) RNA (156, 159), K12 (kaposin) (37), ORF57, K8 (K-bZIP) (110), K1 (18), K9 (vIRF) (42), ORF21 (thymidine kinase) (201), K5 (78), K6 (vMIP-1) (38), ORF6 (single-stranded DNA binding protein), K14 (vOX-2), ORF74 (vGPCR) (90), ORF59 (107), ORF47 (gL)(35), glycoprotein B (gB) (50, 206), K2 (vIL6) (55), ORF60 and ORF61(176). RTA also positively regulates the latency gene ORF73, suggesting a feedback mechanism for the establishment of KSHV latency (97). Besides viral genes, RTA also activates transcription of cellular genes, such as cellular interleukin 6 (IL-6) (53), STAT3 (76), CD21, CD23 (34) and HEY1 (192). The molecular mechanism by which RTA activates those target genes has been extensively investigated by electrophoretic mobility shift assay, transient transfection assays, and *in vivo* chromatin immunoprecipitation assays coupled with KSHV whole genome tiling microarray (ChIP on chip). In summary, RTA regulates downstream gene expression through direct recognition and binding to the responsive elements in the promoter region or by interaction with cellular proteins which then bind to the promoter region (38). A number of RTA responsive elements (RREs) have been identified in various target promoters but they do not seem to share a conserved common DNA binding site (37, 38, 43, 55, 110, 157, 170, 182). The RRE in PAN and K12 promoters contain homologous

sequence which can be directly bound by RTA with high affinities (37, 159, 178). The direct binding of RTA to the RRE is necessary and sufficient for activation of PAN and K12 transcription (38). The first identified potential RRE is in the ORF57 and K8 promoters and it consists of palindromic and AT-rich motifs (103, 110). This palindromic RRE is also present in the origin of lytic DNA replication (*ori-Lyt*) and binding to RTA is essential for *ori-Lyt*-dependent DNA replication (178). We and others have discovered that multiple RREs in K8 and ORF57 promoters, and they do not share the same consensus sequence (152, 177, 182). In addition, transcriptional activation by RTA requires the presence of a cellular protein RBP-Jκ□(recombination signal binding protein □□ which is the target of the Notch signaling pathway (38, 180, 182). RBP-Jκ also mediate RTA-driven transactivation of the ORF6, K6, K14, LANA, ORF59, ORF47 promoters, and the RTA itself (35, 38, 97, 100-102, 107, 180). In addition to RBP-Jκ, a number of other cellular proteins interact with RTA and synergize with RTA to up-regulate RTA-responsive promoters. These proteins include C/EBPα (the CCAAT/enhancer binding protein α), OCT-1 (octamer binding protein 1), c-Jun, CBP (CREB- binding protein)/p300, HGMB1 (high-mobility group B1), and Sp1. RTA physically interacts with C/EBPα and cooperates with K8 to up-regulate K8 promoter activity through binding to the C/EBP binding site (174). As shown in the K8 promoter, C/EBPα also binds to its specific binding sites on the PAN and ORF57 promoters, which enhances their transcriptional activities (175). However, the binding of C/EBPα and transactivation of the K8 promoter mediated by RTA may exhibit cell type specific transcriptional functions (29). Another cellular protein, OCT-1 interacts with RTA and contributes to the transactivation of the K8 promoter (29). The c-JUN protein also binds

to RTA and AP1 binding sites and this interaction stimulates RTA-mediated transactivation of the K8 and ORF57 promoters (173). The CBP/p300 protein complex binds to the amino-terminal basic domain and the carboxyl-terminal transactivation domain of RTA and modulates the transcriptional activation function of RTA (74). The interaction between RTA and CBP/p300 represses the transcriptional activity of p53 and p53-induced apoptosis (75). The HGMB1 protein has the ability to bind and bend DNA. HGMB1 promotes RTA binding to various target promoters, including ORF57, PAN, vIL-6 and K12, and also enhances RTA transactivation of these promoters (158). Sp1 binding to its binding site is essential for RTA-mediated transactivation of the thymidine kinase promoter (201). Moreover, RTA recruits chromatin remodeling complex SWI/SNF and the RNA polymerase II mediator TRAP to the viral promoter by protein-protein interaction, which ultimately stimualtes viral gene expression (73).

Conversely, a number of viral and cellular factors have been identified to suppress RTA-mediated transcription of lytic genes. These factors include KSHV K-bZIP (K8), KSHV LANA, HDAC (histone deacetylase), PARP1 [poly(ADP-ribose) polymerase 1], Ste20-like kinase hKFC (human kinase from chicken), K-RBP (KSHV RTA binding protein), HEY1, TLE2 (transducin-like enhancer of split 2), NF-κB and IRF7 (interferon regulatory factor 7) (74, 77, 82, 87, 88, 96, 104, 172, 192, 195). The expression of the early gene K8 is up-regulated by RTA. However, this gene product, K-bZIP, reduces RTA-mediated transactivation of the ORF57 and K8 promoters, but not the PAN promoter (88, 104). Thus, K-bZIP appears to have promoter-dependent effects on RTA. The KSHV latent gene product LANA directly represses the transactivation of the ORF50 gene promoter and also negatively affects RTA-mediated auto-activation. This

suppression could be due to the binding of LANA to the ORF50 gene promoter or the interaction between LANA and RTA (96). HDAC normally serves as a transcriptional repressor because it deacetylates histones and certain transacriptional factors, or directly inhibits the transactivator's function through protein-protein interaction. Similarly, HDAC down-regulates RTA transctiption activation by interacting with the RTA protein (74). PARP1and Ste20-like kinase hKFC also interact with RTA to modify the RTA protein by phosphorylation and then suppress its recruitment onto viral promoters (77). The other RTA-interacting protein, TLE2, competes with RBP-Jκ to bind to RTA and consequently inhibits RTA transactivation function (82). K-RBP interacts with the cellular co-repressor TIF1β and also directly targets GC-rich motifs on viral promoters, which ultimately suppresses RTA-mediated tansactivation (194, 195). The RTA-inducible gene, Hey1, was identified using DNA chip technology. Its promoter can be activated by RTA; however, the Hey1 protein has a negative feedback on RTA transactivity (192). IRF7 (interferon regulatory factory 7) suppresses RTA-mediated transactivation of the ORF57 gene promoter by competing with RTA for binding to RRE in the ORF57 promoter (172). NF-κB strongly reduces RTA-mediated transactivation for most RTA-responsive promoters. NF-κB interferes with the binding of RBP-Jκ to the RTA-responsive promoters (87).

RTA is a ubiquitin ligase

Besides having transcriptional activity, RTA also has E3 ubiquitin ligase activity which allows E2 ubiquitin-conjugating enzymes to transfer ubiquitin molecules to the target protein. Once the protein is modified by polyubiquitination, it is a target for 26S

proteasome degradation. The Cys-plus-His-rich region of RTA possesses ubiquitin ligase activity and directly polyubiquitinates IRF7, Hey 1 and even itself, which increases protein degradation of these respective proteins (70, 200). For polyubiquitination of K-RBP, two regions of RTA, the C-terminus located at the end of transactivation domain and Cys-plus-His-rich region at the N-terminus, are required for promoting K-RBP degradation through the proteasomal pathway (196). In addition, LANA and K-bZIP also undergo proteasome-dependent degradation in the presence of RTA (196). Interestingly, all of these proteins, which display less stability in the presence of RTA, serve as RTA repressors for inhibiting RTA-mediated transactivation (88, 96, 172, 192, 195). This result suggested that RTA may counteract the inhibitory effect caused by these repressors during RTA-mediated lytic replication.

Research aims

The objectives of this research are to understand the mechanism by which RTA regulates transcription of lytic genes and investigate the potential cellular machinery employed by RTA for reactivation of the virus from latency. The RTA responsive element (RRE) in the RTA target gene promoters plays an important role in RTA-mediated transactivation. Our previous results as well as other published studies have identified various RREs with non-conserved sequences in response to RTA. Thus, more than one binding site exists between RTA and its target gene promoters. More studies are needed to further substantiate the relationship between RTA and target gene promoter in order to understand how RTA mediate gene expression. In addition to stimulating gene expression directly, our earlier studies demonstrated that RTA promoted protein

degradation through the 26S proteasome pathway, and this function correlated with RTA-mediated transactivaiton and KSHV reactivation. There is substantial evidence demonstrating that RTA can employ cellular machinery to establish an adequate environment for KSHV lytic replication. Recently, autophagy was found to play an important role in cancer and viral pathogenesis. Besides proteasome degradation, autophagy is the other major cellular degradation pathway. Therefore, RTA may also utilize the autophagy pathway to regulate KSHV lytic replication. The overall objective of this study is to understand the molecular mechanism by which RTA mediates lytic viral replication. The specific aims are:

1) To investigate the RTA responsive elements in the ORF57 promoter that allow RTA-mediated transactivation.

2) To investigate whether RTA utilizes autophagy to regulate the KSHV life cycle.

3) To investigate the molecular mechanisms by which RTA regulates autophagy for KSHV lytic replication.

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CHAPTER 2

IDENTIFICATION AND CHARACTERIZATION OF A NEW KSHV REPLICATION AND TRANSCRIPTION ACTIVATOR/RTA RESPONSIVE ELEMENT INVOLVED IN RTA-MEDIATED TRANSACTIVATION

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[#]Dr. Veenu Minhas established the Sf9 cells expressing His-tagged RTA protein.

Abstract

KSHV RTA is well established as a key transcriptional activator which regulates KSHV life cycle from latency to lytic replication. It is expressed immediately after infection and activates a number of viral genes to lead to viral replication. The RTA responsive element (RRE) in the RTA target gene promoters is critical for RTA to mediate the transactivation. There are a number of non-conserved RREs identified in various RTA responsive promoters, and AT-rich sequences have been proposed to serve as RTA targets but no consensus RREs have so far been identified. Two non-conserved RTA responsive elements (RRE1 and RRE2) which contain AT-rich sequence have been identified previously in the one of KSHV lytic gene promoters, ORF57, which can be strongly activated by RTA. In this study, based on homology to the consensus sequence of EBV RTA RRE we identified a third RTA responsive element (RRE3) in the ORF57 promoter. This RRE consists of GC-rich sequence which can bind RTA both *in vitro* and *in vivo* and plays a role in RTA-mediated transactivation of the ORF57 promoter. The presence of two of the three RREs in close proximity to each other is required for optimal RTA-mediated transactivation of the ORF57 promoter even though the presence of only one RRE is needed for RTA binding. Our results suggest that the ability of RTA to mediate transcriptional activation is distinct from its ability to bind to its target elements.

Introduction

Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus-8 (HHV-8) was first identified from the Kaposi's sarcoma tissue of an AIDS patient in 1994 and subsequently shown to be associated with Kaposi's sarcoma (KS) and other malignancies, including primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (4, 5, 8). According to nucleotide sequence analysis, KSHV belongs to the gammaherpesvirus family (rhadinoviruses), with family members that include Epstein-Barr virus (EBV) and herpesvirus saimiri (HVS) (26). As in all herpesviruses, KSHV goes through latent and lytic phases of infection, with latency predominantly found in infected B cells (31). During latency, a small subset of viral genes is expressed in order to evade the immune response and maintain the viral genome in the host cells. These latent proteins were also found to affect the survival and proliferation of the infected cells *in vitro* (14, 24, 25, 28, 32). Viral lytic replication can be reactivated *in vitro* by treatment with chemical agents, such as sodium butyrate or tetradecanoyl phorbol acetate (TPA) (23) and *in vivo* by the KSHV-encoded transcription factor RTA (replication and transcription activator) (22). Upon reactivation from latency, lytic viral proteins are induced and expressed. These proteins are encoded by differentially expressed genes; the immediate-early (IE) genes are transcribed first, followed by the expression of early genes, and then late genes are expressed after viral DNA replication (34).

RTA is an important viral regulatory protein encoded by the immediate-early gene, ORF50. A striking feature of RTA is its ability to trigger KSHV lytic reactivation by itself in latently infected cells (15, 21, 22, 31, 33), and KSHV with the RTA gene

deletion is incapable of lytic reactivation (41). Therefore, RTA has been implicated to be an important switch to control the transition from KSHV latency to lytic replication. RTA protein is 691 amino acids in size and is a potent transcriptional factor consisting of a DNA binding domain at the amino-terminus, two putative nuclear localization signals, and a transactivation domain at the carboxyl-terminus. To date, a number of viral lytic genes, including ORF50 itself, that can be regulated by RTA have been identified (1, 6, 9, 11-13). RTA has been shown to transactivate its target promoters either by directly binding to RTA responsive element (RRE) or indirectly by interacting with cellular proteins, such as KSHV RTA binding protein (K-RBP) (37, 42), CCAAT/ enhancer-binding protein alpha (CEBP-α) (38, 39), octamer-binding protein 1(OCT-1) (27), and recombination signal binding protein-Jκ (RBP-Jκ) (18). RTA protein with its DNA binding domain recognizes the RREs in the target gene promoters, and an AT-rich or A/T-trinucleotide motif was considered to be a feature of KSHV RRE that can be found in ORF57 and K8 promoters (19, 20). However, there is a lack of a consensus RRE sequence because substantial diversity can be observed in the RREs identified in the promoters of various KSHV genes found to be responsive to RTA. They include PAN (polyadenylated nuclear RNA) (30), K12 (kaposin) (6), K2 (viral interleukin-6) (11) and K1 (1) genes.

Epstein Barr virus (EBV) Rta, a homolog of KSHV RTA, also functions as an activator of viral lytic promoters. A consensus EBV Rta responsive element (EBV RRE) GNCCN_9GGNG, where N can be any nucleotide, has been identified (16). Recently, the optimal EBV RRE sequence, GTCC(C/A)(T/C)(C/G)N(A/G)NC(A/G)(T/A)GGCG, was reported to be present in five EBV lytic-cycle promoters (10). Interestingly, a CCN_9GG-

like sequence can also be found in the KSHV ORF57 promoter (Fig. 1A). ORF57 is one of the KSHV lytic genes, which is highly responsive to RTA. It encodes a post-transcriptional activator involved in mRNA export and plays an important role in KSHV lytic replication. Previously, we and others (13, 20) have mapped a 40-bp segment of the ORF57 promoter encompassing nt 81904-81943 on the viral genome as the target site for RTA binding and up-regulation of ORF57 transcription. Two RREs (ORF57-RRE1 and ORF57-RRE2) have been identified in this 40-bp segment (Fig.1A). The ORF57-RRE1 was found to share some homology with the delayed early gene K8 promoter sequence (20, 36). ORF57-RRE2 was found to bind not only RTA but also to interferon regulatory factor 7 (IRF7), and competition between RTA and IRF7 affects the regulation of ORF57 promoter by RTA (35). Even though these two RREs are AT-rich as predicted for RRE, there is no similarity between them. However, upon inspection of ORF57 promoter, a CCN_9GG-like sequence distinct from ORF57-RRE1 and 2 can also be found. Thus, we termed this CCN_9GG-like motif in the ORF57 promoter as ORF57-RRE3. In this study we characterized this third RRE element in the ORF57 promoter and found that it plays an important role in conferring responsiveness of the promoter to KSHV RTA.

Materials and methods

Plasmids

RTA expression plasmid (pCMVtagORF50) which encodes Flag-tagged full-length RTA was described previously (36). The β-galactosidase expression plasmid pCMVβ which was used for the normalization of transfection efficiency was purchased from BD Clontech. All ORF57 reporter plasmids were cloned in the pGL3-Basic vector

(Promega), and p57p1, p57B1, p57B1M1, and p57E, have been described previously (13, 36). Plasmid p57-3RRE, p57-3RRE1, p57B2 and p57-3RRE4 contain ORF57 upstream regions spanning from nt -184 to -20, -163 to -20, 130 -68 to -184, and -151 to -20, respectively (the putative transcription start site at nt 82081 is designated as +1). Plasmid p57p1m2 contains the same ORF57 promoter region from -525 to -73 as p57p1 but has mutations in ORF57-RRE2 (the T nucleotides in poistions -141, -143, and -149 were replaced with C; -145 to -147, CTT were replaced with AGC). Plasmids p57-3RRE3 and p57-3RRE2 contain the same ORF57 promoter region as p57-3RRE but p57-3RRE2 has a substitution in the RBP-Jk binding site (nt -163 to -151) by the PstI restriction enzyme sequence, and p57-3RRE3 has a deletion in the ORF57-RRE2 (nt -151 to -138). Plasmid p57B2M contains the same ORF57 promoter region as p57B2 but has mutations in ORF57-RRE1 same as p57B1M1 construct.

Electrophoretic mobility shift assay (EMSA)

The probes were obtained by labeling the double-stranded oligonucleotides (Fig. 2A and 4A) with [α-32P]dATP (Amersham) and DNA polymerase I Klenow fragment (New England BioLabs). Labeled probes were incubated with purified His-tagged, full-length RTA protein expressed in Sf9 cells by Ni-NTA (Promega) for 30 min in a binding buffer [10mM Tris-HCl (pH7.5), 150mM KCl, 5.7mM MgCl2, 1mM EDTA, 0.1 ug of poly(dI·dC), 5 ug of bovine serum albumin, 0.38mM dithiothreitol, 0.38mM phenylmethylsulfonyl fluoride, 50mM β-mercaptoethanol, 5% glycerol]. For competition assays, the purified Sf9-RTA protein was incubated with nonradioactive competitor DNA for 30 min, and then the labeled probe was added and incubated further for 30 min. The binding mixtures were loaded onto a 4.5% polyacrylamide gel in 1x TGE buffer [5mM

Tris, 190mM glycine, 1mM EDTA (pH 8.3)]. After electrophoretic separation at 200V, gels were transferred to Whatmann 3MM paper, dried, and subjected to autoradiography with Kodak X-ray film.

Transient chromatin immunoprecipitation (ChIP)

ORF57 reporter plasmid and RTA expression plasmid were co-transfected into 293T cells or TRExBJAB-RTA cells, provided by Dr. Jae Jung, by using lipofectamine 2000 reagent (Invitrogen) or Amaxa Nucleofactor. Transfection efficiency for 293T cells was about 90% and for TRExBJAB-RTA cells it was about 70%. At 24 hours post-transfection, TRExBJAB-RTA cells were added with doxycycline to induce RTA expression. At 40-48 hours post-transfection, the cells were fixed by the addition of 37% formaldehyde to a final concentration of 1% for 10 min. The crosslinking reaction was stopped by adding glycine to a final concentration of 0.125M for 5 min. Cells were washed twice with 1X cold-PBS (Phosphate Buffered Saline) and were resuspended in cell lysis buffer [5mM PIPES (pH8.0), 85mM KCl, 0.5% NP40, 1mM PMSF (Phenylmethylsulphonylfluoride), protease inhibitor cocktail (Pierce)] for 10 min at 4°C. After centrifugation, the cell pellets were resuspended in nuclei lysis buffer (50mM Tris-Cl pH8.1, 10mM EDTA, 1% SDS) and incubated for 10 min at 4°C. The resulting solution was diluted 10-fold with dilution buffer (16.7mM Tris-Cl pH8.1, 167mM NaCl, 1.2mM EDTA, 0.01% SDS, 1.1% TritonX-100, 1mM PMSF, protease inhibitor cocktail) and sonicated. After centrifugation, the supernatant was precleared with mouse IgG (Santa Cruz) and immobilized protein A (Pierce).

Immunoprecipitation was performed by incubation at 4°C with anti-Flag M2 antibody (Stratagene) or anti-His antibody (Clontech). Immune complexes were collected

by a further incubation with immobilized protein A for 1-2 h at 4°C. The beads were washed four times with IP buffer (25mM Tris, 150mM NaCl pH7.2) and resuspended in immunoPure IgG elution buffer (Pierce). After centrifugation, the supernatant was neutralized with 1M Tris pH7.2. RNase A and NaCl were added, followed by incubation at 65°C overnight to reverse the cross-linked DNA-protein complex. Proteinase K (Stratagene) was added, followed by incubation at 55°C for 1-2 h. DNA extraction was carried out using the miniprep kit (Qiagen) and analyzed by PCR using specific primers located in reporter vector. DNA was quantified by TaqMan real-time PCR using TQMN PCR core reagents (Applied Biosystems). For calculation of relative DNA amount from quantitative real-time PCR, the Ct (threshold cycles) value of antibody-precipitated sample was normalized by the Ct value of the input, and the normalized Ct value of p57-3RRED3 was compared with the normalized Ct value of p57-3RRE. The means and standard deviations of the results for each promoter were calculated from the results of two reactions obtained from three different transfection experiments.

Cell culture, transfection, and luciferase assay

BJAB cells (KSHV negative cell line) were grown in RPMI 1640 medium (Gibco BRL) supplemented with 10% FBS and 100 ug/ml penicillin-streptomycin (Mediatech) at 37°C with 5% CO2. Transfection of BJAB cells was carried out using Amaxa Nucleofactor according to the manufacturers' recommendations. The transfection efficiency was normalized using the β-Gal expression plasmid, pCMVβ, as the internal control. Luciferase activities were determined by the Luciferase Assay System (Promega). The results were averaged from three independent experiments.

Results

The CCN$_9$GG-like consensus sequence can be identified in a number of KSHV promoters

To examine whether KSHV RTA directly binds to the RRE3 sequence of ORF57 promoter, we performed electrophoretic mobility shift assay (EMSA) using purified, His-tagged full length RTA protein expressed in recombinant baculovirus-infected Sf9 cells. The 18-bp, double-stranded oligonucleotides of ORF57-RRE3 were labeled (Fig. 2A) and incubated with binding buffer either alone or with increasing amounts of RTA. Oligonucleotides ORF57-RRE3 demonstrated a dose-dependent binding to RTA (Fig. 2B, lanes 1 to 4). To demonstrate the specificity of RTA binding to the ORF57-RRE3, excess amounts of unlabeled ORF57-RRE3 and AP1 binding sequence were used as the specific and non-specific competitors in competition experiments respectively. The interaction of RTA and ORF57-RRE3 is specific as it can be competed by homologous unlabeled oligonucleotides but not by AP1 oligonucleotides (Fig. 2B, lanes 5 to 7).

To further confirm that RTA binds to CCN$_9$GG-like motif, an 18-bp oligonucleotide fragment of K2-RRE and MIP-RRE (Fig. 2A) was used for EMSA. Similar to ORF57-RRE3, strong binding to RTA was observed, demonstrating that the K2-RRE and MIP-RRE containing CCN$_9$GG-like sequence are also as RTA targets, and this specificity was confirmed by EMSA using competitors (Fig. 2C and D). Our results indicate that CCN$_9$GG is a potentially conserved motif of KSHV RRE in addition to AT-rich sequences identified previously, and CCN$_9$GG-like motif could be a third RRE located in the ORF57 promoter.

ORF57-RRE3 is required for RTA binding to ORF57 promoter

To further confirm that ORF57-RRE3 mediates RTA binding to the ORF57 promoter *in vivo*, transient chromatin immunoprecipitation (ChIP) was carried out in 293T and TRExBJAB-RTA cells. The reporter construct containing ORF57 promoter, shown in Fig.3A, and the Flag-tagged, full-length RTA expression plasmid were cotransfected into 293T cells. The presence of ORF57-promoter DNA fragments within the immunoprecipitated complex was confirmed by PCR amplification. As shown in Fig 3A, upper panel, lane 1, a strong band was detected in the presence of RTA and p57-3RRE plasmid containing all three RREs in the ORF57 promoter. The negative controls with transfection in the absence of antibody failed to recover any PCR products (Fig. 3A, lane 2). In contrast to p57-3RRE, the transfection of p57-3RRED3, which lacks RRE3 in the ORF57 promoter element appeared to be less efficient in binding to RTA and resulted in a weaker amplified band (Fig. 3A, lower panel, lane 1). This was also confirmed by real-time PCR to quantitate the amount of precipitated DNA from 293T and TRExBJAB-RTA cells, and RRE3-lacking DNA fragment resulted in about 65% reduction in binding to RTA in 293T cells and 85% reduction in TRExBJAB-RTA cells (Fig. 3B). These results suggest that ORF57-RRE3 is required for efficient binding of RTA to the ORF57 promoter.

RTA can bind to each of three ORF57-RREs independently

In an earlier study, we have demonstrated that RTA protein expressed in *E. coli* can bind to the ORF57 promoter fragment 57R consisting of ORF57-RRE1 and RBP-Jκ binding site (Fig. 4A) (35). Results in Fig. 2 demonstrate that RTA protein expressed in baculovirus infected insect cells (Sf9) can bind to RRE3 *in vitro*. To further characterize the binding of RTA to each of the three RRE elements, EMSA was carried out with DNA

fragments containing each of these elements (Fig. 4A). Multiple high molecular weight shifted bands were observed with the 57R labeled probe (Fig. 4B). Interestingly, we also observed the interaction of RTA and RRE1 which lacks the RBP-Jκ consensus sequence, and only a single complex was detected (Fig. 4C). Similarly, only one band was detected with ORF57-RRE2 probe (Fig. 4D). Fig. 2 and 4 suggest that Sf9-RTA can directly bind to each of the three ORF57-RREs. The binding does not require the RBP-Jκ consensus sequence, but the presence of RBP-Jκ binding site may contribute to the formation of multiple complexes.

The binding of RTA to ORF57-RRE3 is weaker than ORF57-RRE1 and 2

To further investigate the differential ability of each ORF57-RRE to bind to RTA, cross-competition assays were carried out. As expected, the binding of RTA to each ORF57-RRE probe was competed efficiently by its homologous unlabeled oligonucleotide (Fig. 5, lanes 2 to 5, 17 to 20, 32 to 35). ORF57-RRE1 binding to RTA was reduced by about 50% with an excess of ORF57-RRE2 competitor (lanes 6 to 9), and ORF57-RRE2 binding was also reduced by about 80% with an excess of ORF57-RRE1 competitor (lanes 21 to 24) based on quantification of the bands. The binding of RTA to ORF57-RRE3 was competed efficiently by either the unlabeled ORF57-RRE1 or ORF57-RRE2 (lanes 36 to 43). However, ORF57-RRE3 was unable to compete for binding to RTA with either ORF57-RRE1 (lanes 10 to 13) or ORF57-RRE2 (lanes 25 to 28). Taken together, these results show that RTA binding affinity of ORF57-RRE1 is comparable to that of ORF57-RRE2, but the binding affinity of RTA to ORF57-RRE1 and ORF57-RRE2 appears to be much stronger as compared to ORF57-RRE3.

Two RREs are required for RTA-mediated transactivation of ORF57 promoter

To further verify that the binding of RTA to ORF57-RRE3 plays a role in the transactivation of ORF57 promoter, transient transfection experiments were carried out by co-transfection of the RTA expression plasmid with various reporter constructs of ORF57 promoter as shown in Fig. 6A. The p57-3RRE reporter, which contains the 40-bp segment, was induced effectively by RTA in BJAB cells (56-fold) (Fig. 6B). Surprisingly, p57-3RRE1 reporter, lacking ORF57-RRE1, was still transactivated effectively by RTA (63-fold), even though the AT-rich palindrome of ORF57-RRE1 has been considered as a critical region for binding and transactivation by RTA (20). The induction of p57B2 reporter with deletion of 48-bp segment in the C-terminal ORF57 promoter remained high (60-fold). The reporter p57B2M with mutation in ORF57-RRE1 and p57B1reporter with deleted ORF57-RRE3 still remained highly responsive to RTA as compared to p57B2. However, removal of ORF57-RRE3 and mutation of ORF57-RRE1 (p57B1M1) significantly reduced the promoter's responsiveness to RTA. These results suggest that either ORF57-RRE1 or ORF57-RRE3 is not sufficient for efficient RTA transactivation but at least one of these two elements in combination with ORF57-RRE2 is required. This was confirmed by p57E reporter containing the ORF57-RRE3 alone, which showed a dramatic reduction in transactivation (Fig. 6B).

To further confirm the role of ORF57-RRE2 in RTA-mediated transactivation, the ORF57-RRE2-deleted reporter (p57-3RRE3) was used. The transactivation of p57-3RRE3 reporter by RTA was almost abolished (Fig. 6B). To confirm that the loss of transactivation response is not due to the deletion which shortened the length of ORF57 promoter between ORF57-RRE1 and 3, we tested the p57p1m2 reporter which contains six nucleotide substitutions in the ORF57-RRE2 but the total length of the promoter

remained the same. As expected, RTA responsiveness of p57p1m2 was still significantly reduced comparing with p57p1 which is a positive control (Fig. 6C). Our results suggest that ORF57-RRE2 is essential for RTA-mediated transactivation.

RTA-mediated transactivation of ORF57 promoter requires RBP-Jκ binding site and two RREs

RBP-Jκ has been demonstrated to be a critical cellular factor for RTA-mediated transactivation of the ORF57 promoter by binding to the RBP-Jκ binding site. The two promoter reporter constructs p57-3RRE1 and p57B1, both containing the RBP-Jκ binding site, were strongly activated by RTA (Fig. 6B), suggesting that RBP-Jκ binding site in addition to the RREs is important. To further define the role of RBP-Jκ binding site and two RREs in mediating RTA transactivation, the reporter p57-3RRE4 with deletion of the upstream region of ORF57-RRE2, including RBP-Jκ binding site and ORF57-RRE1, was tested. As expected, this reporter lost its responsiveness to RTA even though both ORF57-RRE2 and ORF57-RRE3 were intact. In addition, RTA-inducibility was low with the p57-3RRE2 reporter which has a deletion in the RBP-Jκ binding site but with intact ORF57-RRE1, RRE2, and RRE3 (Fig. 6B). This is expected as RBP-Jκ is well established as a co-activator of RTA that recognizes the consensus sequence located between ORF57-RRE1 and ORF57-RRE2 to regulate ORF57 transcription (3, 17). Interestingly, mutations in RRE2, clone p57p1m2, and mutation/deletion in RRE1 and RRE3, clone p57B1M1, affected the transcriptional induction of ORF57 promoter by RTA (Figs. 6B and C). The responsiveness of these two promoter constructs to RTA was reduced substantially. Taken together, our results show that RBP-Jκ plays an essential

role in RTA-mediated ORF57 transactivation but the presence of two RREs (RRE1/RRE2 or RRE2/RRE3) is necessary.

Discussion

The role of ORF57-RREs in the RTA-mediated transactivation of ORF57 promoter

In this study, we have identified a third RRE, ORF57-RRE3, in the ORF57 promoter. It has a high G/C content which distinguishes it from the other two previously characterized RREs in the ORF57 promoter, and similar RREs with high G/C can be found in the promoter elements of several other KSHV genes which are responsive to KSHV RTA. The three ORF57-RREs are located in very close proximity to each other in the ORF57 promoter, and each has a unique sequence. Among the three RREs analyzed, RRE2 seems to be essential for RTA mediated transactivation. However, the presence of RRE2 alone is insufficient, and it requires the presence of either RRE1 or RRE3 for it to be fully responsive to RTA. It is possible that for the ORF57 promoter, RTA may require more than one RRE for it to regulate its expression and whether this is required for other RTA responsive promoters, needs to be determined. Our results also suggest that RRE1 and RRE3 may play a similar role, each can be replaced by the other in combination with RRE2 to mediate RTA transactivation, and the presence of at least two RREs elements is required for transactivation. We found that the deletion of ORF57-RRE1 alone did not affect the ORF57 transactivation mediated by RTA (Fig. 6A), which is in contrast to an earlier report demonstrating that ORF57-RRE1 was critical for RTA binding and transactivation (20). However, Lukac *et al*. tested only an isolated RRE1/RBP-Jκ element, thus making any direct comparison with our ORF57 reporter studies difficult.

We also observed that the amount of RTA bound to the promoter element containing RRE1 and RRE2 alone is less than the intact promoter with all three RREs in Fig. 3, yet their levels of transactivation by RTA are similar. It is possible that the amount of RTA bound to RRE1 and RRE2 without RRE3 is adequate to reach the threshold level of RTA needed to be recruited to the promoter element for it to mediate full transactivation. Alternatively, it is possible that two adjacent RREs are needed for the RTA complex to bind optimally since RTA has recently been reported to multimerize *in vitro* (2, 19). In fact we have observed RTA multimerization in EMSA (Fig. 4B) with multiple high molecular weight shifted bands using DNA probe containing ORF57-RRE1 and RBP-Jκ binding sites. These complexes could be due to spontaneous multimerization of RTA as described previously (2, 19), but whether such complexes exist *in vivo* and participate in binding to the two RREs and in the transactivation of ORF57 promoter need to be further investigated.

In spite of the similar roles played by ORF57-RRE1 and 3 in mediating transactivation, the binding affinities of RTA to ORF57-RRE1 and 3 are distinct, raising the possibility that the binding affinity of RTA to different RREs may not be directly proportional to the levels of RTA-mediated transactivation. Transactivation function may require the presence of additional factors other than RTA; these may include both viral and cellular factors. One such factor is RBP-Jκ which was shown to be a critical transcriptional factor interacting with RTA to bind to RBP-Jκ binding site between ORF57-RRE1 and 2 to activate ORF57 transcription (3, 17). Our data indeed reveal that RTA-mediated transcription of ORF57 promoter can proceed only in the presence of all three elements, the RBP-Jκ binding site, the ORF57-RRE2 and either ORF57-RRE1 or 3.

Taken together, ORF57-RRE1 and 3 may be playing a secondary but necessary role to enable the RTA-RBP-Jκ complex to bind to the ORF57-RRE2 and RBP-Jκ binding sites in the ORF57 promoter to mediate transactivation. We are further investigating the exact roles of ORF57-RRE1 and 3 in RTA-mediated transactivation.

The conserved sequence for KSHV RTA binding

KSHV RTA responsive elements have been extensively studied in past several years (7, 19, 43). They have been found to be diverse and lack a consensus sequence, but the exact motif mediating RTA binding and transactivation is still ambiguous. In contrast, for EBV, its RTA RRE sequence GNCCN9GGNG has been identified in several EBV lytic-cycle promoters to be responsive to EBV Rta. Our finding that CCN_9GG-like sequence can also be used by KSHV RTA to mediate transactivation of ORF57 promoter suggests that EBV and KSHV RREs may share sequence homology, and it is possible that CCN_9GG-like motif could also be found in other RTA responsive promoters. Indeed, in the KSHV PAN promoter, the major sequence in the PAN-RRE identified previously for RTA recognition and transactivation contains the $ACCN_9GG$ consensus sequence (Fig. 1B) (30). In addition, it was reported that another RTA responsive promoter MIP lost its responsiveness with a deletion in the region encompassing nt 27855 to 27876 on the viral genome (7). We found that this region contains not only the RBP-Jκ binding site but also the $ACCN_9GG$ sequence, and have confirmed that RTA can bind to the MIP $ACCN_9GG$ motif alone using EMSA. In contrast, Chang et al. failed to observe an interaction between RTA protein and a DNA probe from the MIP promoter; this is not unexpected because of an absence of the $ACCN_9GG$ motif in the DNA probe which was used in the EMSA. Interestingly, $ACCN_9GG$ motif can also be found in the K2-RRE

previously identified (Fig. 1B). It is located in a 26-bp region spanning from nucleotides 18293 to 18314 on the viral genome (11), and we have now shown that RTA binds to the K2 DNA containing ACCN$_9$GG sequence within this 26-bp region. Taken together, these results suggest that the KSHV ACCN$_9$GG motif is an important RTA target.

Studies on the EBV Rta responsive promoters which contain the recognition sequence GNCCN$_9$GGNG have shown that the differences in their binding affinities and responsiveness to RTA transcription activation could be attributed to the N$_9$ sequence variation in each promoter element (7). Similarly for KSHV, the five promoters containing the consensus sequence CACC(A/G)NTNGGN(C/G)NGG(T/C) that we have identified here displayed similar variations in the N sequences (Fig.1B), and this variation may play a role in their differential responsiveness to RTA transactivation. Indeed, it was found that PAN-RRE has a stronger binding affinity and transactivation by RTA when compared to K12 and K2-RRE even though all these RREs contain the CACC(A/G)NTNGGN(C/G)NGG(T/C) motif (29). It is possible that the PAN N$_9$ sequence is optimal for RTA binding; it is also possible that cellular factors may also play a role in the differential binding and responsiveness of these promoters to RTA. A number of cellular proteins, such as K-RBP, RBP-Jκ, OCT1, AP1, SP1, and CEBP-α, are known to regulate RTA-mediated transactivation (9, 18, 27, 38-40), and some of their binding motifs are found to be present in the vicinity of the CACC(A/G)NTNGGN(C/G)NGG(T/C) motif. Thus, the combination of RTA binding to these motifs with the presence of cellular factors may confer optimal activation of the promoter. Taken together, it is likely that the ability of RTA to mediate transactivation is distinct from its ability to bind to RREs and its binding affinity to these elements. Its

ability to uncouple these two functions may play a role in its ability to regulate lytic gene expression at different stages of the KSHV life cycle.

In summary, we have identified a new RRE sequence containing G/C-rich content in five KSHV lytic gene promoters based on sequence homology to the consensus EBV RRE. Whether this consensus RRE sequence CACC(A/G)NTNGGN(C/G)NGG(T/C) is present in other RTA target genes and whether there are optimal N sequences required needs to be further determined. This is also the first report to demonstrate that KSHV RTA-mediated transcription requires two RREs in close proximity to each other. A better understanding of the role played by RTA and various RREs will be needed to elucidate the mechanism of RTA-mediated transactivation and induction of KSHV lytic replication.

Acknowledgements

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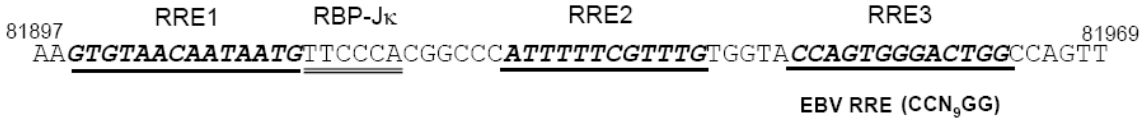
Figures and figure legends

Fig. 1

(A)



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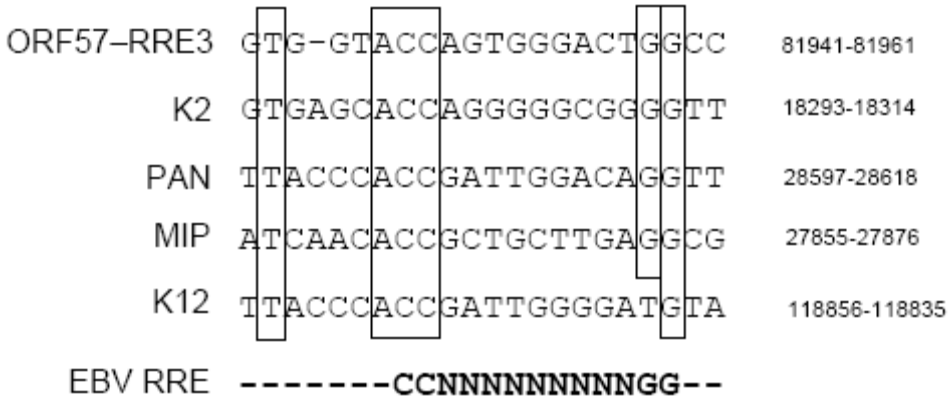


Fig. 1. Sequence comparison among RTA responsive elements (RREs).

(A) The sequence of the three potential KSHV RREs (RRE1, RRE2 and RRE3) in the ORF57 promoter is in bold, italicized, and underlined. The recombination signal binding protein-Jκ (RBP-Jκ) binding site is highlighted with double lines. (B) The alignment of potential KSHV RREs which have homology to the EBV RRE CCN_9GG in the ORF57, K2, PAN, MIP, and K12 promoters. The identical sequences are shown by open boxes. The numbers represent the nucleotide positions of the KSHV genome according to GenBank U75698. The sequence of EBV RRE is in bold and N can be any nucleotide.

Fig. 2

(A)


ORF57-RRE3 gtg-GTACCAGTGGGACTGGCC
K2-RRE gtGAGCACCAGGGGGCGGGGtt
MIP-RRE atCAACACCGCTGCTTGAGGcg

(B) **(C)** **(D)**

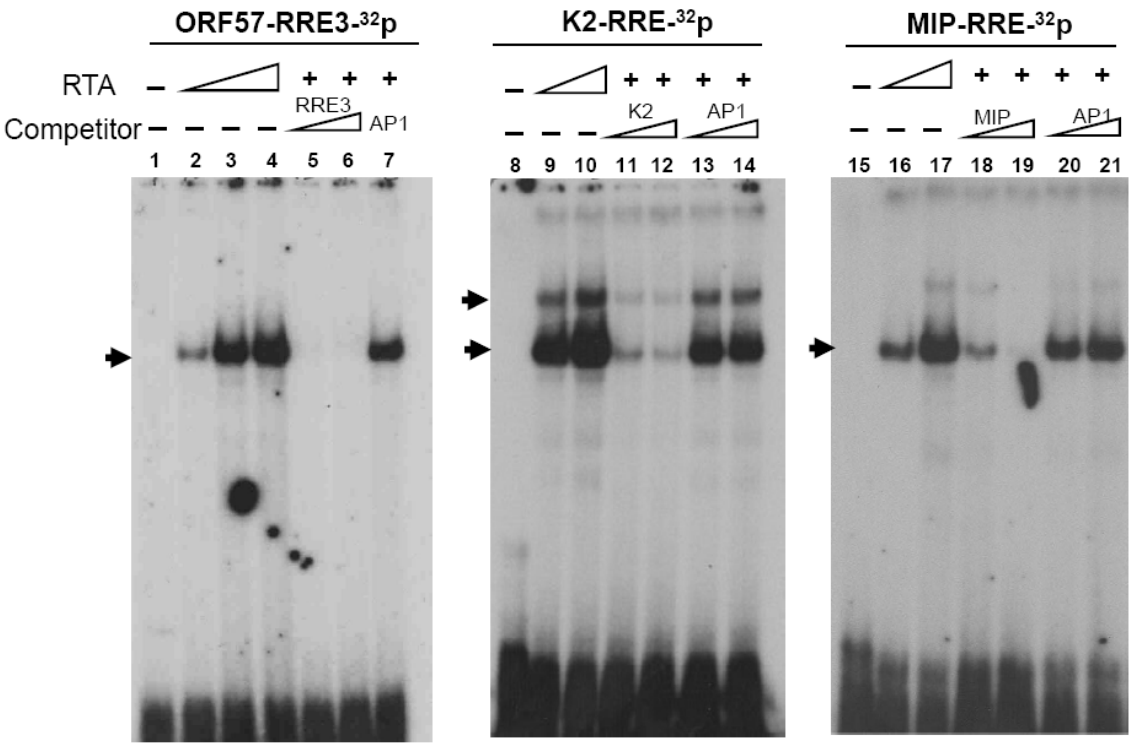
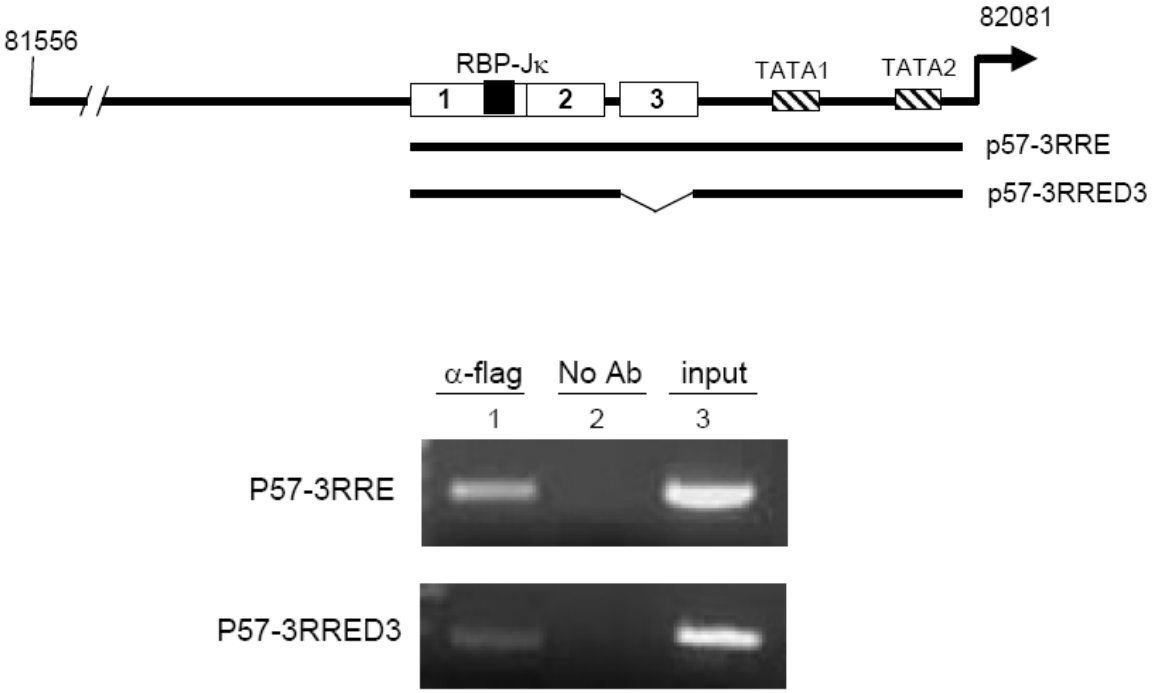

ORF57-RRE3-32p
K2-RRE-32p
MIP-RRE-32p
RTA
Competitor
RRE3
AP1
K2
MIP
1 2 3 4 5 6 7
8 9 10 11 12 13 14
15 16 17 18 19 20 21

Fig. 2. The binding of RTA to CCN_9GG core sequence *in vitro*.

(A) The alignment between ORF57-RRE3, K2-RRE, and MIP-RRE. Identical sequences are shown by vertical lines. The synthetic oligonucleotide fragments, ORF57-RRE3, K2-RRE and MIP-RRE, used for EMSA are capitalized and bolded. (B) EMSA was carried out by using the full length His-tagged RTA expressed in Sf9 cells and 32P-labeled dsDNA probes. RTA binds to ORF57-RRE3. Lanes 1 was a control with 32P-labeled ORF57-RRE3 probe alone. Increasing amounts of RTA protein were incubated with probes in lanes 2 to 4. A 50 and 100-fold excess of unlabeled specific or non-specific (AP1) competitors were incubated in the presence of purified RTA protein in lanes 5 to 7. The arrows indicate the specific RTA-DNA complexes. (C, D) RTA binds to K2-RRE and MIP-RRE. EMSA of K2-RRE and MIP-RRE was performed as described for panel b. The arrows indicate the RTA and DNA complex.

Fig. 3

(A)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82081
RBP-Jκ
TATA1
TATA2
1
2
3
p57-3RRE
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2
3
P57-3RRE
P57-3RRED3

(B)

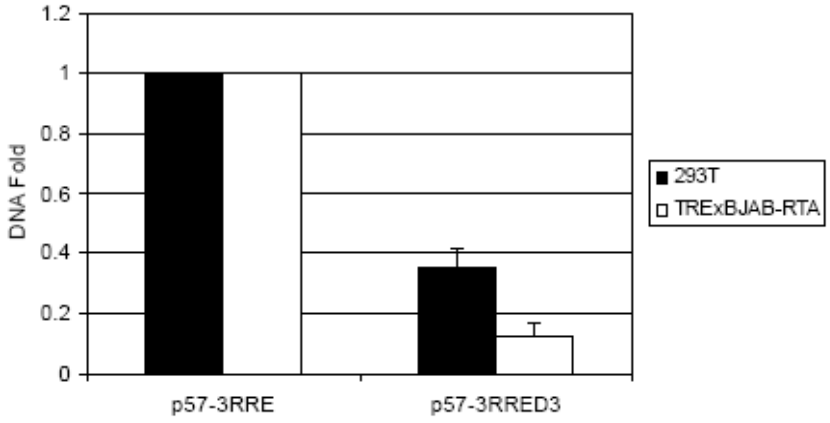

1.2
1
0.8
0.6
0.4
0.2
0
DNA Fold
293T
TREx BJAB-RTA
p57-3RRE
p57-3RRED3

Fig. 3. Binding of RTA to the ORF57 promoter RREs *in vivo.*

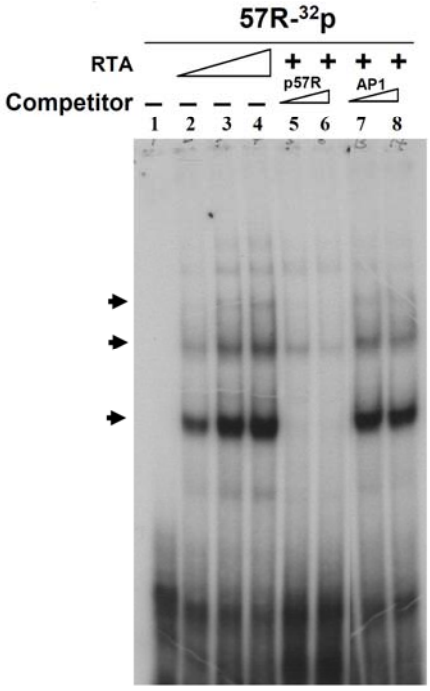
(A) Transient-ChIP assay was carried out in 293T cells transfected with reporter plasmid (p57-3RRE or p57-3RRED3) and Flag-tagged full-length RTA expression plasmid (RTA). RTA-DNA complex was specifically precipitated with or without anti-Flag antibody. Precipitated DNA was recovered by PCR with specific primers located in the backbone of the reporter vector. (B) Taqman real-time PCR was performed to quantify the immunoprecipitated promoter DNA from transfected 293T and TRExBJAB-RTA cells.

Fig. 4

(A)


RRE1
RBP-Jκ
RRE2
RRE3
GCAAGTGTAACAATAATGTTCCCACGGCCCATTTTTCGTTTGTGGTACCAGTGGGACTGGCCAGTTAATCCCACT
57R
RRE1
RRE2
RRE3

(B)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RTA
p57R
AP1
Competitor
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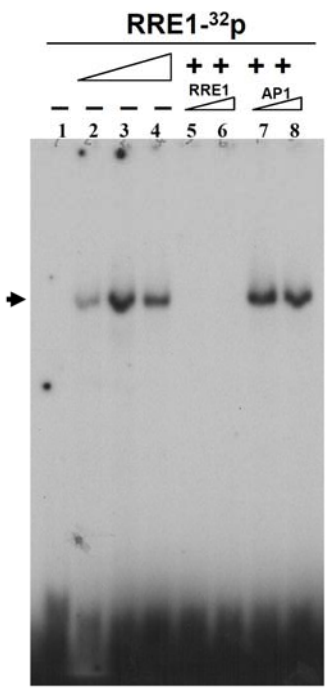
(C)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RRE1
AP1
1 2 3 4 5 6 7 8

(D)

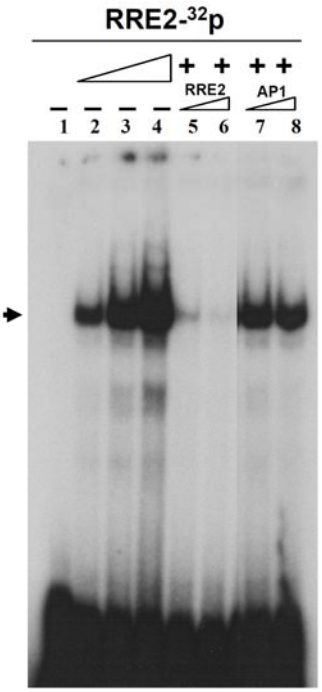

RRE2-32p
RRE2
AP1
1 2 3 4 5 6 7 8

Fig. 4. Binding of RTA to ORF57-RRE1 and RRE2 is independent of the RBP-Jk binding site *in vitro.*

(A) The solid lines show four DNA fragments (57R, RRE1, RRE2, RRE3) which were labeled with ^{32}P and used as probes for EMSA. (B, C, D) EMSA was performed with RTA and the three DNA fragments, (B) p57R, (C) RRE1, and (D) RRE2. Conditions used for EMSA were as described in Fig. 2. The unlabeled probes were used as specific competitors, and AP1 was used as a negative control. The arrows indicate the RTA and DNA complex.

Fig. 5

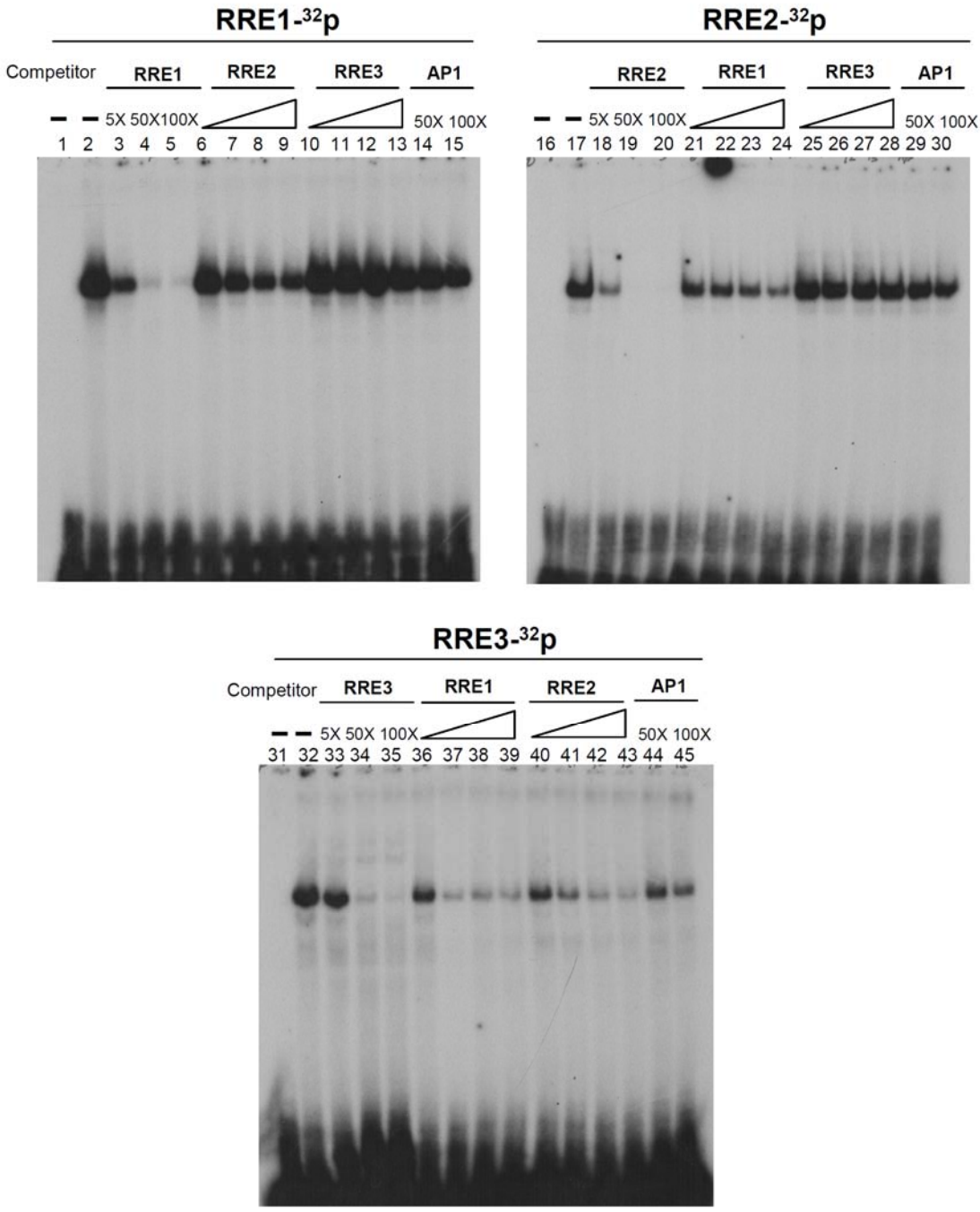
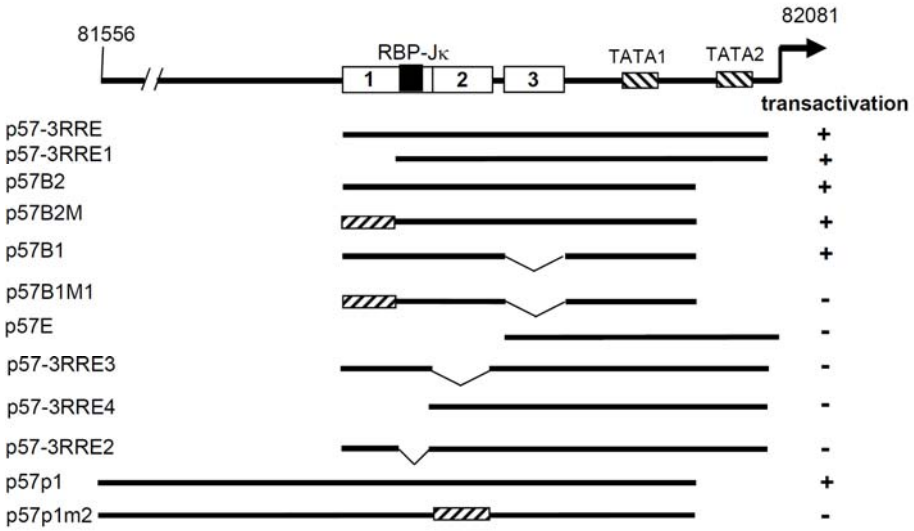

RRE1-32p
Competitor
RRE1
RRE2
RRE3
AP1
5X 50X100X
50X 100X
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
RRE2-32p
RRE2
RRE1
RRE3
AP1
5X 50X 100X
50X 100X
16 17 18 19 20 21 22 23 24 25 26 27 28 29 30
RRE3-32p
Competitor
RRE3
RRE1
RRE2
AP1
5X 50X 100X
50X 100X
31 32 33 34 35 36 37 38 39 40 41 42 43 44 45

Fig. 5. The binding affinity of RTA to ORF57-RRE1 and RRE2 is higher than to ORF57-RRE3.

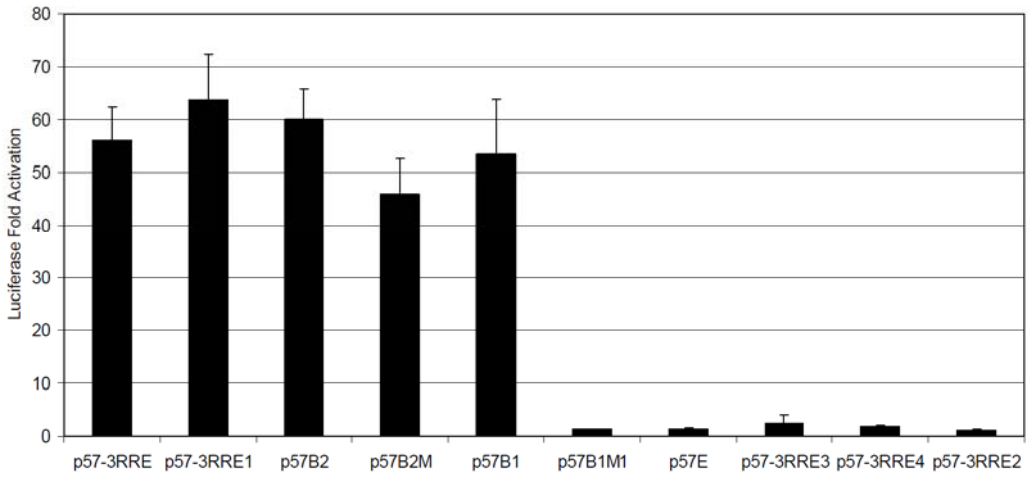
The cross-competition EMSA was used to determine the differential binding ability of RTA to the three RREs. The purified RTA protein (500 ng) was incubated with 40 fmole of labeled probes in the absence or presence of homologous or non-homologous cold competitors. An increasing amount (5x, 25x, 50x, and 100x) of various non-homologous cold competitors were added. Nonspecific competitor AP1 was used a negative control, and its fold in excess relative to labeled probes is indicated. Lanes 1, 16, and 31 represent probe alone.

Fig. 6

(A)


81556
82081
RBP-Jκ
TATA1
TATA2
1
2
3
transactivation
p57-3RRE
p57-3RRE1
p57B2
p57B2M
p57B1
p57B1M1
p57E
p57-3RRE3
p57-3RRE4
p57-3RRE2
p57p1
p57p1m2
+
+
+
+
+
-
-
-
-
-
+
-

(B)


Luciferase Fold Activation
0
10
20
30
40
50
60
70
80
p57-3RRE
p57-3RRE1
p57B2
p57B2M
p57B1
p57B1M1
p57E
p57-3RRE3
p57-3RRE4
p57-3RRE2

(C)

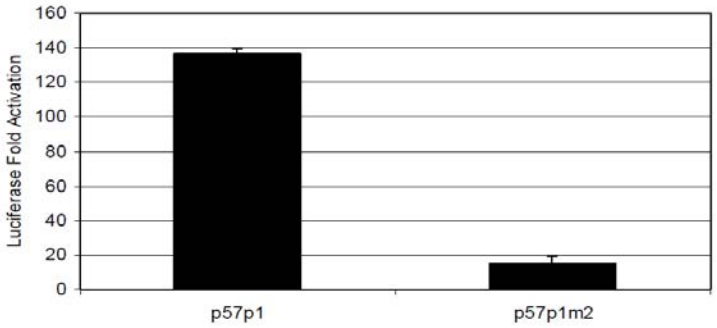

Luciferase Fold Activation
0
20
40
60
80
100
120
140
160
p57p1
p57p1m2

Fig. 6. Two RTA responsive elements and RBP-Jk binding site are required for RTA-mediated transactivation.

(A) Schematic representation of the ORF57 promoter reporter constructs and various deletion and site-directed mutagenesis clones used in transient transfection analysis. Solid lines indicate different regions of the ORF57 promoter segment with different deletions or mutagenized regions (gray boxes). The responsiveness of each reporter to RTA transactivation is indicated. (B, C) BJAB cells were used to analyze the promoter activity of different ORF57 reporter constructs. Total DNA amount used in each transfection was normalized by adding pCMVTag2A vector. Transfection of BJAB cells was carried out with 0.8 μg of promoter reporter and 1 μg of RTA expression plasmid. Luciferase activity was measured at 48 hr post-transfection.

CHPATER 3

ENHANCEMENT OF AUTOPHAGY DURING LYTIC REPLICATION BY KSHV REPLICATION AND TRANSCRIPTION ACTIVATOR/RTA

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[1]Dr. Zhilong Yang helped double-checking the results of GFP-punctate dots.

[2]Dr. You Zhou helped confocal microscopy and transmission electron microscopy.

Abstract

Autophagy is one of two major degradation systems in eukaryotic cells. The degradation mechanism of autophagy is required to maintain the balance between the biosynthetic and catabolic processes and also contributes toward defense against invading pathogens. Recent studies suggest that a number of viruses can evade or subvert host cell autophagic pathway to enhance their own replication. Here, we investigate the effect of autophagy on the KSHV (Kaposi's sarcoma-associated herpesvirus) life cycle. We found that inhibition of autophagy reduces KSHV lytic reactivation from latency, and an enhancement of autophagy can be detected during KSHV lytic replication. In addition, RTA (replication and transcription activator), an essential viral protein for KSHV lytic reactivation, is able to enhance the autophagic process leading to an increase in the number of autophagic vacuoles, an increase in the level of the lipidated LC3 protein and the formation of autolysosomes. Moreover, inhibition of autophagy affects RTA-mediated lytic gene expression and viral DNA replication. These results suggest that RTA increases autophagy activation to facilitate KSHV lytic replication. This is the first report demonstrating that autophagy is involved in lytic reactivation of KSHV.

Introduction

Autophagy is an intracellular catabolic mechanism and is principally responsible for degradation of long-lived cellular proteins and damaged organelles. The degraded products are primarily recycled to supply nutrients when cells are undergoing nutrient deficiency. The hallmark of autophagy is the formation of double-membrane cytosolic vacuoles called autophagosomes which sequester entire organelles and large protein aggregates. Eventually, autophagosomes fuse with the lysosomes to generate single-membrane vacuoles termed autolysosomes, where the contents are subsequently degraded and/or recycled by lysosomal hydrolases (34). This intracellular degradation system is tightly regulated by a family of genes, known as autophagy-related genes (*Atg*), which were initially described in yeast (27, 62). In higher eukaryotes, a number of *Atg* genes homologous to yeast *Atg* have been shown to be essential for autophagy formation in various eukaryotic systems (34). In addition to the classical homeostatic function, autophagy plays an important role in multiple biological processes including differentiation, development, anti-aging and cell death, and aberrant autophagy is implicated in a number of human diseases, such as cancer, neurodegeneration, and certain muscular myopathies (22, 35, 49).

The function of autophagy in cellular defense is to remove invading pathogens including viruses; however, some pathogens have developed strategies to adopt the host autophagic machinery for their own survival and replication (26, 33). The single-stranded DNA virus B19 parvovirus infection induces autophagy to prolong survival of the infected cells (42). The infection of the positive-stranded RNA viruses, such as poliovirus, coxsackievirus and dengue virus, induces double-membrane vesicles resembling

autophagosomes to increase viral RNA replication (21, 32, 53, 61). The induction of autophagosomes by poliovirus is also proposed to play a role in non-lytic mechanism for poliovirus release (21, 25). Autophagy is also an important innate immunity mechanism which some viruses can evade by subverting or hijacking the autophagy process. For example, Herpes simplex virus type 1 (HSV-1) ICP34.5 targets the mammalian autophagy protein Beclin 1 to block the host autophagy machinery in order to induce neurovirulence (43). Also, ICP34.5 can block autophagy through interference with phosphorylation of eIF2α (eukaryotic translation initiation factor 2 alpha) by PKR (54). Epstein-Barr virus (EBV) latent membrane protein 1 (LMP1), which is required for the proliferation of infected B cells, utilizes autophagic degradation to limit its accumulation in EBV-infected B cells (30). Human immunodeficiency virus type I (HIV-1) envelope glycoprotein-mediated killing of uninfected CD4 T cells was found to be dependent on the autophagy machinery and may be involved in the pathogenesis of AIDS (14, 15). Overall, these studies present a complex picture defining the role of autophagy in the viral pathogenesis but this effect is both viral and host strain specific.

Kaposi's sarcoma-associated herpesvirus (KSHV) belongs to the gamma-herpesvirus family and is associated with Kaposi's sarcoma and other malignancies, such as primary effusion lymphoma (PEL) and Multicentric Castleman's disease (MCD). As in all herpesviruses, KSHV exhibits two phases in its replication cycle, latent and lytic phases. During latency, KSHV is capable of evading the immune surveillance to persist in host cells without viral production; however, infectious viral particles can be produced and released after induction of lytic reactivation from latency as a result of stress or chemical stimuli, such as phorbol esters or sodium butyrate (4, 5, 39, 40, 46). In addition

to *in vitro* stimuli, an immediate early KSHV gene, ORF50, encodes the replication and transcription activator (RTA) which has the ability to initiate the entire lytic reactivation cascade (38). RTA is a typical transcriptional factor containing an N-terminal DNA-binding domain and a C-terminal activation domain. RTA can trigger KSHV lytic reactivation via the transcriptional activation of a number of viral lytic promoters (2, 6, 8, 11-13). However, in order for the virus to regulate between lytic replication and latency, the transactivation function of RTA can be suppressed by various viral and cellular repressors to limit the extent of lytic replication to return to latency (3, 17, 18, 20, 29, 58, 63-65). To overcome this suppression to facilitate lytic reactivation, viral RTA has the ability to promote degradation of the repressors by modulating the ubiquitin-proteasome pathway (16, 66, 67). Since a cellular regulatory mechanism is adopted by KSHV to switch between lytic cycle and latency and various viruses have adopted the autophagy pathway to regulate their replication, it will be of interest to determine whether KSHV replication also involves the autophagy pathway. In this study, we found that the activation of autophagy is enhanced during KSHV lytic reactivation, and the viral lytic replication inducer RTA is involved in this activation.

Materials and methods

Cells, viruses, plasmids and reagents

Human 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad CA) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 100 μg/ml penicillin-streptomycin (Mediatech) at 37°C with 5% CO_2. For starvation experiments, the cells were washed with 1x phosphate buffered saline (PBS)

three times and incubated in the 1x Earle's balanced solution (Invitrogen) for 90 min. BCBL-1 is a KSHV–positive primary effusion lymphoma cell line and BJAB is a KSHV-negative cell line derived from Burkitt's lymphoma. TRExBCBL1-RTA and TRExBJAB-RTA both carrying a tetracycline-inducible RTA gene were provided by Dr. Jae Jung (University of Southern California, Los Angeles, CA) (41). These B cells were grown in RPMI 1640 medium (Gibco BRL) supplemented with 10% FBS and 100 µg/ml penicillin-streptomycin at 37°C with 5% CO_2. Vero cells infected by rKSHV.219 virus were provided by Dr. Jeffrey Vieira (University of Washington, Seattle, WA) (57). The cells were grown in DMEM supplemented with 10% FBS, 100 µg/ml penicillin-streptomycin, and 6 µg/ml puromycin (Sigma) at 37°C with 5% CO_2. Bac50, a recombinant baculovirus expressing KSHV RTA was also provided by Dr. Jeffrey Vieira for generation of rKSHV.219 virus as described earlier (57). To establish knockdown cell line, Beclin1 (BECN 1) and scrambled negative control (N) shRNA plasmids were purchased from Origene and transfected into TRExBCBL1-RTA cells using the nucleofector system (Lonza), and the transfected cells were selected by puromycin. RTA expression plasmid (pCMVtagORF50) which encodes Flag-tagged full-length RTA has been described previously (59). mRTA contains truncated RTA (amino acid 1 to 527) which was cloned in pCMVtag-2A vector. Plasmid pGFP-LC3 encoding GFP-tagged rat LC3 gene was obtained from Dr. Mizushima (Tokyo Medical and Dental University, Japan) (23).

Bafilomycin A1, 3-methyladenine (3-MA), sodium butyrate (NaB) and 12-O-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma.

Flow cytometric analysis and cell viability assay

The rKSHV.219 infected Vero cells were infected with Bac50 virus for 4 h and treated with sodium butyrate (Sigma) for 24 h and/or 3-methyladenine (Sigma) at the indicated time points. The cells were harvested and analyzed with a FACS calibur flow cytometry using the Cell Quest software (BD Biosciences). rKSHV.219 infected Vero cells were treated with 3-MA for 24 and 40 h and their viability was measured by a Beckman Coulter with trypan blue staining.

Western blot analysis

Cells were harvested and the cell pellets were resuspended in M-PER buffer (Pierce) containing protease inhibitor cocktail (Pierce) at 4°C for 20 minutes, followed by centrifugation at 10,000 rpm for 5 minutes at 4°C. Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluorid (PVDF) membrane (GE healthcare), followed by incubation with specific antibodies as described previously (65). LC3 antibody was purchased from Abcam Inc, and BECN1 antibody against human Beclin 1 was purchased from Santa Cruz biotechnology. For protein loading control, anti-tubulin antibody and anti-GAPDH antibody were purchased from Santa Cruz biotechnology. For detection of His- and Flag-tagged proteins, the HRP-conjugated anti-6xHis antibody and mouse anti-Flag M2 monoclonal antibody were purchased from Clontech and Stratagene. Anti-K8a antibody was purchased from Novous and anti-RTA antibody was provided by Dr. Luwen Zhang (University of Nebraska-Lincoln). The band intensities were measured by using the NIH image software, image J.

Confocal microscopy and Lysotracker-red and DAPI staining

Quantitative GFP-LC3 autophagy assays were performed in 293T and TRExBJAB-RTA cells transfected with GFP-LC3 expression plasmids. The 293T cells were grown in 35 mm coverslip bottom dish (BD Biocoat) and cultured under the conditions indicated. To express RTA proteins in cells, RTA expression plasmid (pCMVtagORF50) was cotransfected into 293T cells along with GFP-LC3 expression plasmids. To induce RTA expression in TRExBJAB-RTA cells, the cells were treated with doxycycline after transfection of GFP-LC3. For TRExBJAB-RTA, cells were fixed using 4% paraformaldehyde in PBS for 15 minutes at room temperature, then washed with PBS twice and deposited onto a slide for confocal microscopy visualization. A series of optical images were obtained with an Olympus FV500 confocal system on an inverted microscope, using the 488 nm laser line (522 nm emission) for GFP detection.
The cell containing ≥ 3 GFP-LC3 dots was defined as autophagy positive cells. The number of cells with GFP-LC3 punctate dots relative to all GFP-LC3 positive cells was counted (a minimum of 200 GFP-LC3 positive cells were counted in total for each experiment) and is presented as percentages.

For lysotracker-red staining, the cells were treated with 1 μM lysoTracker Red DND-99 (Invitrogen) at 37°C for 30 min. Depending on the experiments, 293T cells were starved in Earle's balanced salts solution (EBSS; Gibco-BRL) for 90 minutes in the presence of lysotracker red dye. Then cells were fixed in 3.7% formaldehyde, and nuclei were stained with DAPI (Calbiochem) for 10 min. Three channel, optical images (DAPI, GFP, and Lysotracker) were collected using sequential scanning mode (405, 488, and 543 nm excitation; 450, 522, and 595 nm emission, respectively) of the Olympus FV500 confocal system.

Transmission electron microscopy

293T cells were transfected with pCMVtagORF50 or pCMVtag (control) plasmids and harvested at 24 h post-transfection. Cell pellets were washed with 1xPBS once and fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.4) for 1 h at room temperature. Fixed cells were dehydrated through a graduated ethanol series, and embedded in Epon 812 (Electron Microscopic Sciences, Fort Washington, PA). Thin sections (60-80 nm) were stained with uranyl acetate and lead citrate and observed under a transmission electron microscope (Hitachi H7500-I). A series of ultrastructural images were collected with a bottom-mount digital camera for confirmational analysis of autophagy.

Real-time reverse transcription (RT)-PCR

Total RNA was isolated using an RNA mini kit (Qiagen) using protocol recommended by the supplier. RNA samples were digested with DNase (Invitrogen) to remove residual DNA. Real-time RT-PCR was carried out using iScript one-step RT-PCR kit with SYBR Green. The primers used for the mRNA quantitation were ORF57 [5'- GCATATTTGGTAGCGATGGG-3' (forward) and 5'-GGGATAGTTAGGACAAAGGC-3'(reverse)] and K8.1 [5'-CGCTCCTAATCCTATGCCTT-3' (forward) and 5'-CTGATAAACCTGTCCACTCC-3' (reverse)]. All reactions were performed in duplicate. For calculation of the relative mRNA amount from quantitative real-time PCR, the Ct (threshold cycle) value of each viral gene was normalized by the Ct value of GAPDH, and the normalized Ct values from samples were compared with the control samples (untreated).

Viral DNA copy number

The intracellular viral DNA was extracted from TRExBCBL1-RTA cells using genomic DNA purification kit (Gentra). The viral DNA was quantified by real-time PCR using iQ SYBR Green supermix (Bio-Rad). To generate a standard curve for cycle thresholds versus genomic copy numbers, the pCMVtagORF50 plasmid was serially diluted to known concentrations. Primers for amplification of ORF50 gene were 5'-CAAACCCCATCCCAACAT-3' and 5'- AGTAATCACGGCCCCTT-3'. The DNA copy number was calculated using Bio-Rad iCycler software (version 3.1).

Results

An autophagy inhibitor suppresses KSHV lytic reactivation

To investigate whether autophagy affects KSHV lytic reactivation, a specific autophagy inhibitor, 3-methyladenine (3-MA), was used to suppress the autophagic pathway in rKSHV.219 latently infected Vero cells, and cells were examined by microscopy and flow cytometry (48). Vero cells were latently infected by a recombinant green fluorescent protein (GFP)-red fluorescent protein (RFP) double-labeled rKSHV.219 virus which constitutively expresses GFP. Upon induction of lytic reactivation, cells will express RFP under the control of the lytic PAN promoter (57). It had been shown that the highest number of cells expressing lytic proteins can be induced by treatment with sodium butyrate (NaB) and infection of RTA expressing baculovirus Bac50 (57). Therefore, we followed this protocol for induction of KSHV lytic reactivation in rKSHV.219 infected Vero cells. Figure 1A shows that the number of cells harboring reactivated KSHV at 40h post-induction was higher than at 24h post-induction. However, in the presence of the autophagy inhibitor 3-MA the number of KSHV-

reactivated cells was reduced when compared with untreated control. There was a 60% reduction of RFP expressing cells at 24h after 3-MA treatment, and a 33% reduction at 40h, as determined by flow cytometry (Fig. 1B). The cells expressing GFP were not affected by 3-MA treatment due to the similar percentage of cells expressing GFP in the absence and presence of 3-MA (89.2% and 90.2% respectively; data not shown). In addition, the similar cell viabilities were observed in both 3-MA treated and untreated cells, indicating that the reduction in viral reactivation was not due to the toxic effect of 3-MA (Fig. 1C). These results demonstrated that 3-MA reduces KSHV lytic reactivation, this reduction is likely due to its effect on autophagy, and the effect is most prominent during early phase of lytic reactivation.

The activation of autophagy is enhanced during KSHV lytic replication

The conversion of microtubule-associated protein 1 light chain 3 (LC3) is a hallmark of autophagy and has been used to detect autophagy activation. In quiescent cells, LC3 protein is expressed in the cytoplasm as a precursor protein known as LC3-I (18-KDa). When autophagy is activated, LC3-I can be conjugated to phosphatidylethanolamine (PE) by a ubiquitination-like reaction to generate a lipidated species termed LC3-II (16-KDa) which is associated with inner and outer membranes of autophagosomes (19, 24). Therefore, to examine the status of the autophagy activation during KSHV lytic replication, LC3 proteins were examined by Western blot analysis in KSHV-positive cells. We observed that more LC3-II molecules were accumulated in BCBL-1 cells after 12-O-tetradecanoylphorbol-13-acetate (TPA) and NaB treatments. In addition, a time course study analyzing the cells at different time points after chemical stimulation showed that there was an increase in the amount of the endogenous

autophagic protein Beclin 1, which is known to be responsible for initiation of the autophagic pathway (Fig. 2A). Therefore, the increase in the expression levels of two autophagic proteins as well as the presence of KSHV lytic gene product K8 after TPA and NaB stimulation indicate that the activation of autophagy is enhanced during KSHV lytic replication.

The KSHV immediate early gene product RTA is considered to be necessary and sufficient for KSHV lytic reactivation (38). Therefore, to determine whether the activation of autophagy is also increased during RTA-induced lytic replication, LC3 conversion was analyzed in RTA-inducible BCBL-1 cells, TRExBCBL1-RTA. In this cell line, RTA is integrated into the genome of BCBL-1 cells and its expression is regulated by doxycycline (41). We demonstrated that overexpression of RTA in TRExBCBL1-RTA efficiently triggers lytic replication as shown by the enhancement of the expression of an early gene product, K8. Simultaneously, accumulation of LC3-II was observed upon overexpression of RTA in TRExBCBL1-RTA cells (Fig. 2B). These results indicate that there is an enhancement of autophagic process during KSHV lytic reactivation upon RTA overexpression or treatment by chemicals.

RTA is able to increase the autophagic process in 293T cells

To investigate whether RTA is able to induce the autophagic process, the presence of autophagic vacuoles was monitored after co-transfection of RTA and GFP-tagged LC3 expression plasmids in 293T cells. Since LC3-II is lipidated and associated with inner and outer membrane of autophagic vacuoles, the green fluorescent signal from GFP-LC3 can be shifted from a diffuse cytosolic/nuclear staining to a punctate pattern outlining the autophagic vacuoles in the cells if autophagy is triggered. In cells

transfected with the control GFP vector and starved in salt buffer, no obvious green punctate dots were observed (Fig. 3A). In contrast, green punctate dots of GFP-LC3 were very distinct and found to be distributed in the cytosol after starvation and/or transfection of RTA expression plasmids. No such GFP punctation was seen in GFP-LC3 transfected cells in nutrient-rich media. The percentage of GFP-LC3 positive cells containing green punctate dots was then quantified and the results are shown in Fig. 3B. Very few cells (2%) transfected with pCMVtag vector were found to contain GFP-LC3 dots, and as expected nutrition deprivation of these cells showed an increase in cells containing GFP-LC3 dots to about 14%. Consistent with previous reports, we also observed that transfection of Beclin 1, which is one of autophagic proteins important for autophagic nucleation (36), moderately increased the number of GFP-LC3 dot containing cells to about 4.2%. The number of Beclin 1 transfected cells containing GFP-LC3 punctate dots markedly increases to about 19% upon starvation. Interestingly, over-expression of RTA in the cells by transient transfection of the RTA expression plasmid dramatically increased the percentage of GFP-LC3 dot containing cells (19.9%), which was further increased to about 35% upon starvation. The increased number of cells containing GFP-LC3 punctate dots was found to be dependent on the presence of RTA in a dose-dependent manner (Fig. 3C). In addition, ultrastructural analysis of transfected 293T cells by electron microscopy revealed an increased prevalence of double membrane-autophagic vacuoles in the cytoplasm of the RTA transfected cells (Fig. 4B) as compared to the vector-transfected 293T cells (Fig. 4A). To demonstrate that RTA-mediated induction of autophagy is not an overexpression phenotype, a carboxyl-terminal truncated RTA plasmid (mRTA) was overexpressed in the 293T cells for GFP dot analysis. The

expression level of mRTA was much higher than the wild type RTA (Fig. 3D left panel) but was not sufficient to enhance autophagy as indicated by the number of cells containing GFP dots (Fig. 3D right panel). To confirm that the increase in GFP-LC3 dots is due to the induction of autophagy, 3-MA was added to determine whether the presence of GFP-LC3 dots can be inhibited. Indeed, 3-MA was able to reduce GFP-LC3 dot expression even with the highest concentration of RTA added. There was a reduction of GFP-LC3 dot expressing cells from 25 to 10% (Fig. 3C). To further confirm the ability of RTA to induce the activation of autophagy, LC3 conversion was analyzed by Western blotting. As expected, LC3-II accumulation was increased either in the presence of RTA or upon starvation (Fig. 5A). Less accumulation of LC3-II was observed with the transfection of vector alone. Together, these results indicate that RTA is capable of inducing autophagic vacuole formation without involvement of other viral proteins.

RTA is capable of inducing the formation of autolysosomes

It is well established that the fusion of autophagosomes with lysosomes to generate autolysosomes is a critical step in the autophagy degradation process. Moreover, autolysosomes contain various lysosomal enzymes which are able to digest sequestered material, including LC3-II which is associated with the inner membrane of autophagosomes. Thus, to further determine whether RTA has the ability to stimulate the maturation of autophagic vacuoles into degradative organelles, LC3-II accumulation was analyzed by Western blotting in the presence of an inhibitor to prevent LC3-II degradation by lysosomal enzymes. To prevent degradation cells were treated with bafilomycin A1, a specific inhibitor of vacuolar proton-ATPases. As expected, cells transfected with control plasmid pCMVtag showed an increase in LC3-II due to the effect

of bafilomycin to prevent LC3-II degradation. However, the increase in the control cells is less than those transfected with RTA. This indicates that fusion between the autophagosomes and lysosomes were enhanced in the presence of RTA (Fig. 5A). To further confirm this result, we assessed the presence of autolysosomes in cells by using GFP-LC3 and Lysotracker red staining, which stains for acidic organelles such as lysosomes. Clearly, in the RTA expressing or starved cells, LC3 was found to be colocalized with Lysotracker suggesting the formation of the autolysosomes (Fig. 5B). Together, these results indicated that RTA is able to induce LC3 conversion and the formation of autophagosomes and autolysosomes to mediate the induction of the autophagy degradation pathway.

The enhancement of autophagy activation by RTA is cell-type independent

To further demonstrate whether RTA-induced autophagic activation can be observed in lymphoma cells, the autophagic vacuole formation and LC3 molecular conversion were estimated in RTA-inducible BJAB cell line, TRExBJAB-RTA. In this cell line, the RTA gene is integrated into the chromosomal DNA under the control of a tetracycline-inducible promoter. TRExBJAB-RTA and control TRExBJAB cells were first transfected with the GFP-LC3 plasmid, and GFP-LC3 punctate dots were analyzed at the indicated time points after doxycycline treatment. Before addition of doxycycline (at 0 h), both cell lines have similar basal number of cells containing GFP-LC3 dots, and doxycycline is unable to enhance the formation of GFP-LC3 punctate dots in the control cells TRExBJAB without the integrated RTA gene. In contrast, the percentage of cells containing GFP-LC3 punctate dots was increased upon induction of RTA expression in the TRExBJAB-RTA cells (Fig. 6A). In addition, the levels of the processed LC3-II

proteins were found to be enhanced upon RTA induction in the TRExBJAB-RTA cells but remained constant in control TRExBJAB cells (Fig. 6B). Consistent with our results obtained with 293T cells, the increase in the autophagy activation by RTA is likely to be cell-type independent.

Inhibition of autophagy affects RTA-mediated lytic replication

Our findings suggest that the autophagic process can be enhanced by RTA expression, and Inhibition of autophagy affects KSHV lytic reactivation. Therefore, inhibition of autophagy may affect RTA-induced KSHV lytic gene expression. To test this possibility, we measured the mRNA levels of early and late genes (ORF57 and K8.1) in RTA-inducible BCBL-1 cells (TRExBCBL1-RTA) in the presence or absence of an autophagy inhibitor (3-MA). In the reverse transcription (RT) quantitative-PCR analysis, induction of RTA by doxycycline was able to elevate the expression of ORF57 and K8.1 mRNA, indicating that lytic replication is triggered. However, suppression of autophagy by 3-MA treatment leads to a reduction in RTA-induced lytic gene mRNA expression of ORF57 and K8.1 (62% and 72% reduction, respectively) (Fig. 7A). To specifically inhibit autophagy, we employed gene knockdown approach using shRNA against Beclin 1 which is required for initiation of autophagy (36). After transfection of shRNA plasmid in TRExBCBL-RTA cells and puromycin selection, the reduction of Beclin 1 mRNA and protein was confirmed by RT quantitative-PCR and Western blotting (Fig. 7B). We found that RTA-mediated mRNA expression of ORF57 and K8.1 were decreased by 53% and 42% reduction respectively in the Beclin1 knockdown cells (Fig. 7C). It is well known that RTA triggers lytic gene expression to facilitate KSHV lytic replication. Thus, we also measured viral DNA copy number in the TRExBCBL-RTA cells with and without 3-

MA treatment. Figure 7D shows that inhibition of autophagy by 3-MA reduces RTA-mediated viral DNA replication (29% inhibition). Together, these results indicate that autophagy positively regulates RTA-mediated lytic replication.

Discussion

Autophagy has been implicated to be an anti-viral immune defense and a number of viruses were found to develop various strategies to block autophagy to overcome the antiviral function of autophagy (28). However, several viruses have been found to utilize autophagy to enhance viral replication. In our present study, autophagy is enhanced during KSHV lytic replication and positively regulates KSHV lytic replication. It has been shown that Infections by RNA viruses, such as poliovirus, coxsackievirus, influenza A virus, hepatitis C virus and dengue virus, can induce autophagy and this induction increases viral RNA replication and viral yield (1, 21, 32, 61, 68). Since positive-stranded RNA virus replications are associated with cytoplasmic membranes of infected cells (47), autophagosomes-like structures may serve as the membrane scaffold for viral RNA replication based on the localization of their viral proteins and viral RNA genome in the autophagosomes (21, 45, 60). In contrast to most RNA viruses, KSHV DNA replication occurs in the nucleus. Therefore, it is unlikely that the autophagic vacuoles in the cytoplasm can serve as sites for KSHV replication. It has been demonstrated that KSHV adopts various cellular machineries and recruits different cellular proteins to maintain latency or to initiate lytic replication (10, 66); therefore, autophagy could be one of the cellular mechanisms utilized by KSHV to establish an adequate environment for lytic reactivation.

To explore the possible mechanism and biological significance of the enhancement of autophagic activation during KSHV lytic replication, we tested the ability of a key viral protein RTA, which is responsible for the initiation of KSHV lytic reactivation, in the induction of autophagy. We observed that RTA possesses the ability to increase the autophagic activation and this regulation is independent of other KSHV viral proteins. Since RTA is a typical transcriptional factor located in the nucleus, it is not clear how RTA can affect this intracellular pathway which occurs in the cytoplasm. It is known that RTA up-regulate a number of intracellular and viral gene expressions (9, 37, 52, 56, 63); thus, it is possible that RTA promotes autophagic gene expression to trigger the autophagic pathway. A similar mechanism has been found for hepatitis B virus (HBV), where the HBV X protein can up-regulate Beclin 1 mRNA and protein levels to activate autophagy in hepatocytes (55). Therefore, the relationship between RTA and autophagy-related protein expression needs to be further investigated.

In the classic autophagic pathway, portions of cytoplasm are sequestered to form the double-membrane vacuoles, autophagosomes. Subsequently, autophagosomes fused with lysosomes to become autolysosomes where the degradation process occurs. However, there are two reports showing the blockage of autolysosomal maturation by Hepatitis C virus and coxsackievirus B3 even though the induction of autophagosome-like structures can be observed during their infections, suggesting that the degradation function of autophagy is blocked by Hepatitis C virus and coxsackievirus B 3 (51, 61). From our results, we found that over-expression of RTA can enhance both autophagosomes and autolysosome formation. There was an enhancement of GFP-LC3 and lysosomal staining in the presence of RTA, which is similar to those observed during starvation. In addition,

the increase in LC3-II protein level can be observed in RTA-expressing cells treated with bafilomycin A1, suggesting that RTA is able to induce the autophagy degradation process. A number of cellular and viral proteins have been shown to interfere with RTA-mediated transcription of lytic genes to maintain KSHV latency (3, 18, 20, 29, 58, 63-65); hence, the induction of autophagy by RTA to degrade these repressor proteins may be a mechanism to enable the virus to initiate lytic replication. Since RTA has been shown to promote protein degradation through the proteasome degradation pathway (16, 66, 67), it is possible that RTA also adopts the autophagy degradation machinery for clearance of cellular or viral transcriptional repressors to lead to KSHV lytic replication. Therefore, further studies need to be conducted to demonstrate this possibility.

Currently, very little information is available regarding the roles of autophagy on herpesvirus infection. Autophagy can be an antiviral defense against HSV and HCMV (human cytomegalovirus) infection (7, 43, 44) but autophagy could also be an advantage for viral infection, as in the case of EBV which use autophagy to regulate viral LMP-1 expression level (30). In our study, we have shown that KSHV RTA may play a role to induce autophagy to enhance lytic replication; nevertheless, two other KSHV proteins, vFLIP (31) and vBcl2 (50), have been reported to possess anti-autophagic function. It is possible that KSHV can either enhance or suppress autophagy during different phases of infection and dependent upon the expression of the various viral and cellular proteins involved. Thus, the roles of autophagy seem to differ among different viruses and the regulation of autophagy may involve various viral proteins and cellular proteins as in the case of KSHV. How these various KSHV proteins function to regulate autophagy in infected cells during various phases of the viral replication cycle needs to be determined.

In conclusion, our study shows that the activation of autophagy is enhanced during KSHV lytic reactivation, induced either by chemical stimuli or by RTA. RTA is able to activate the entire processes of autophagy. Inhibition of autophagy diminishes RTA-mediated lytic gene expression and reduces KSHV lytic replication; indicating that autophagy is involved in KSHV lytic replication.

Acknowledgments

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Figures and figure legends

Fig. 1

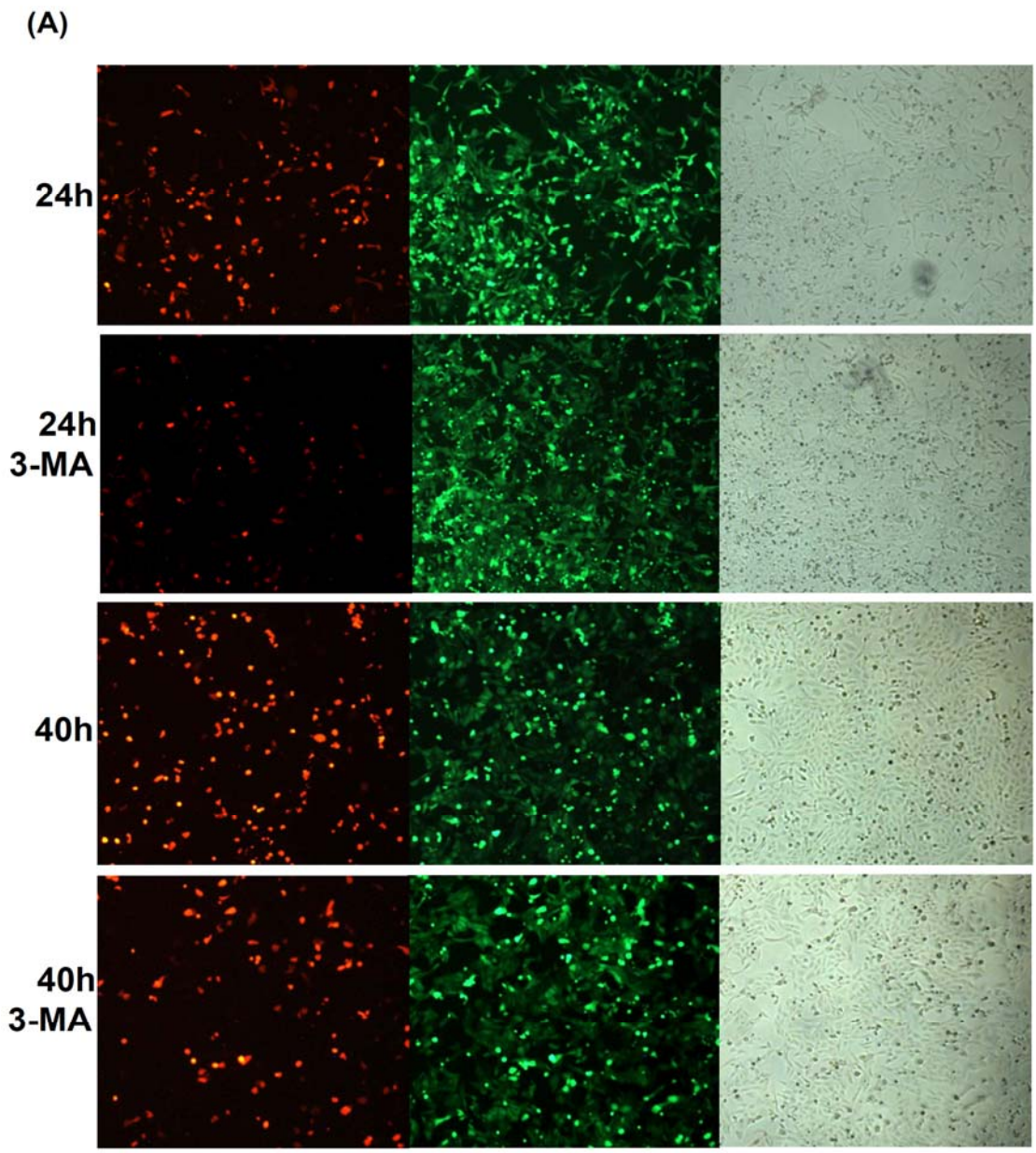

Fig. 1

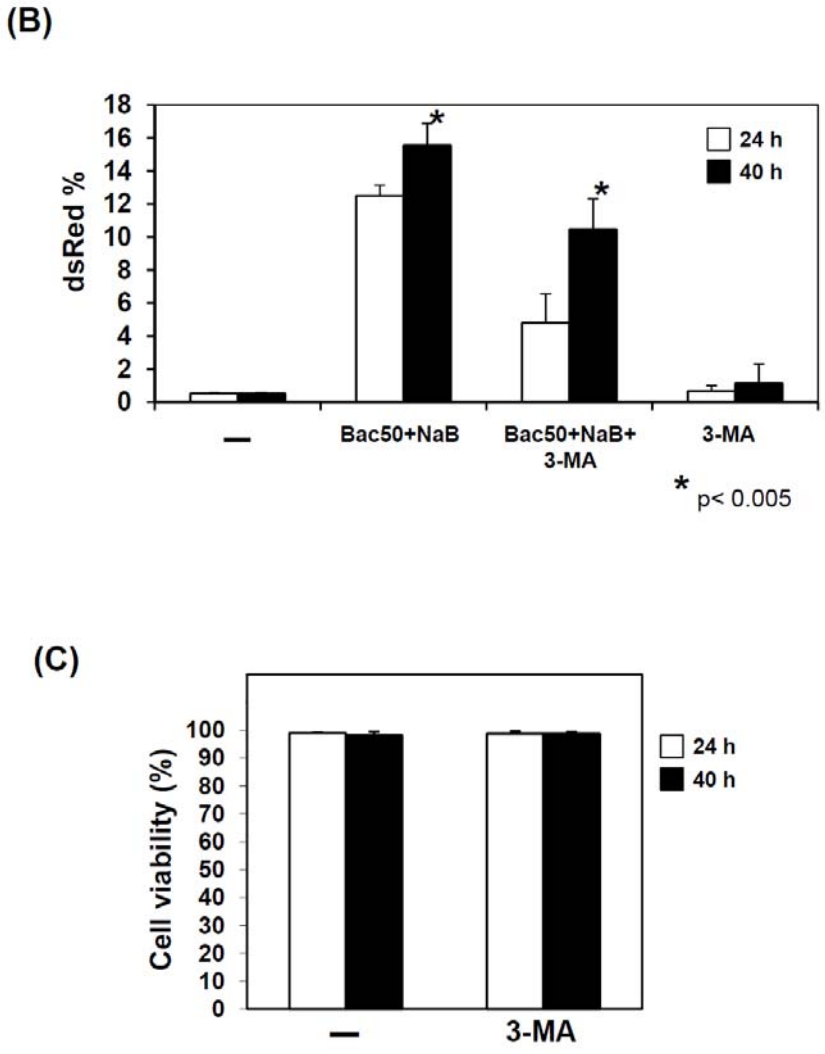
(B)
dsRed %
18
16
14
12
10
8
6
4
2
0
24 h
40 h
*
*
—
Bac50+NaB
Bac50+NaB+
3-MA
3-MA
* p< 0.005
(C)
Cell viability (%)
100
90
80
70
60
50
40
30
20
10
0
24 h
40 h
—
3-MA

Fig. 1. Defective autophagy reduces KSHV lytic reactivation.

(A) rKSHV.219 latently infected Vero cells were infected with Bac50 virus and treated with sodium butyrate (NaB) and cells with lytic replication were detected by RFP expression observed under a fluorescence microscope. Cells were also treated with or without 3-methyladenine (3-MA) for 24 or 40 h. The GFP fluorescence and phase contrast micrographs are showed in parallel. (B) The RFP signal expressed from cells as described for panel A were measured by flow cytometry. The results shown are based on the average from three separate experiments. Results are expressed as mean +/- S.D. Asterisks indicate $p < 0.05$ (student's *t* test). (C)The autophagic inhibitor 3-MA is not toxic to cells. A Beckman coulter with trypan blue staining was used to measure cell viability.

Fig. 2

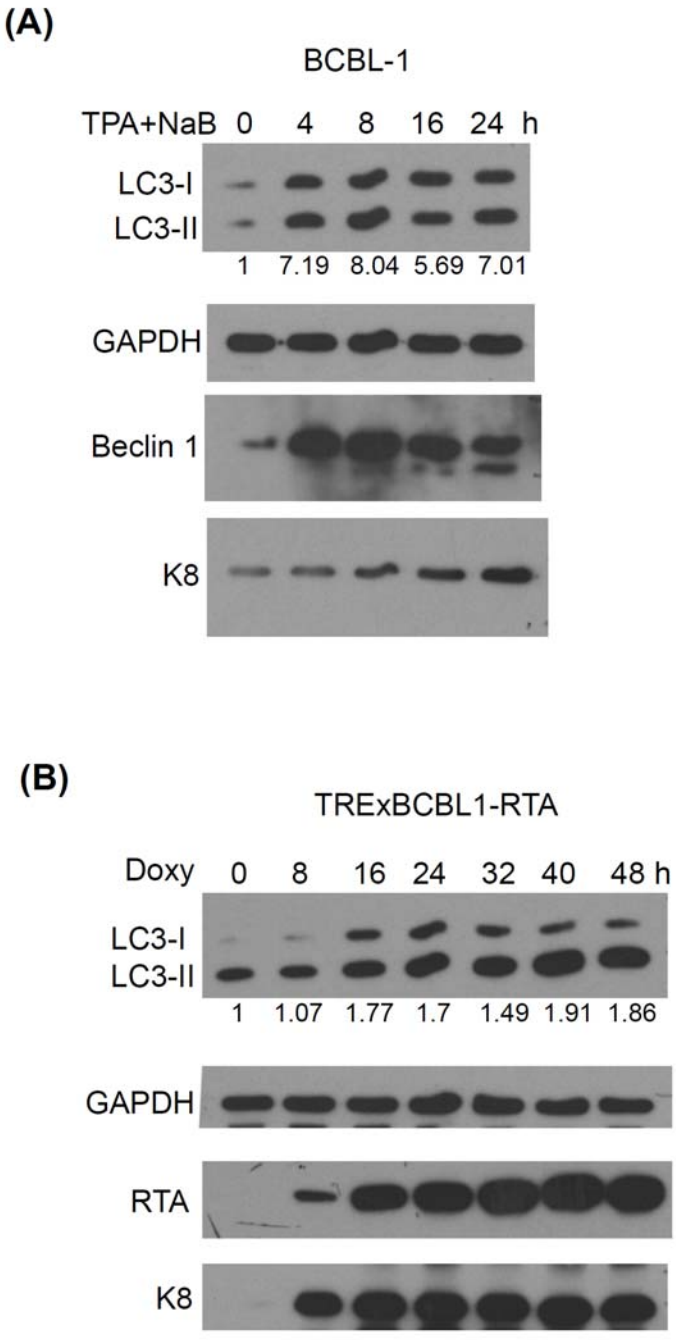
(A)
BCBL-1
TPA+NaB 0 4 8 16 24 h
LC3-I
LC3-II
1 7.19 8.04 5.69 7.01
GAPDH
Beclin 1
K8
(B)
TRExBCBL1-RTA
Doxy 0 8 16 24 32 40 48 h
LC3-I
LC3-II
1 1.07 1.77 1.7 1.49 1.91 1.86
GAPDH
RTA
K8

Fig. 2. Autophagy is enhanced during KSHV lytic reactivation.

(A) BCBL-1 cells were treated with 20 ng/ml TPA and 3 mM NaB for the indicated time. (B) TRExBCBL1-RTA cells were treated with 1μg/ml doxycycline (Doxy) for the indicated time to induce RTA expression. Western blot analysis was performed using the specific antibodies as indicated. The numbers below the blots indicate the relative amounts of LC3-II protein which were normalized by GAPDH protein.

Fig. 3

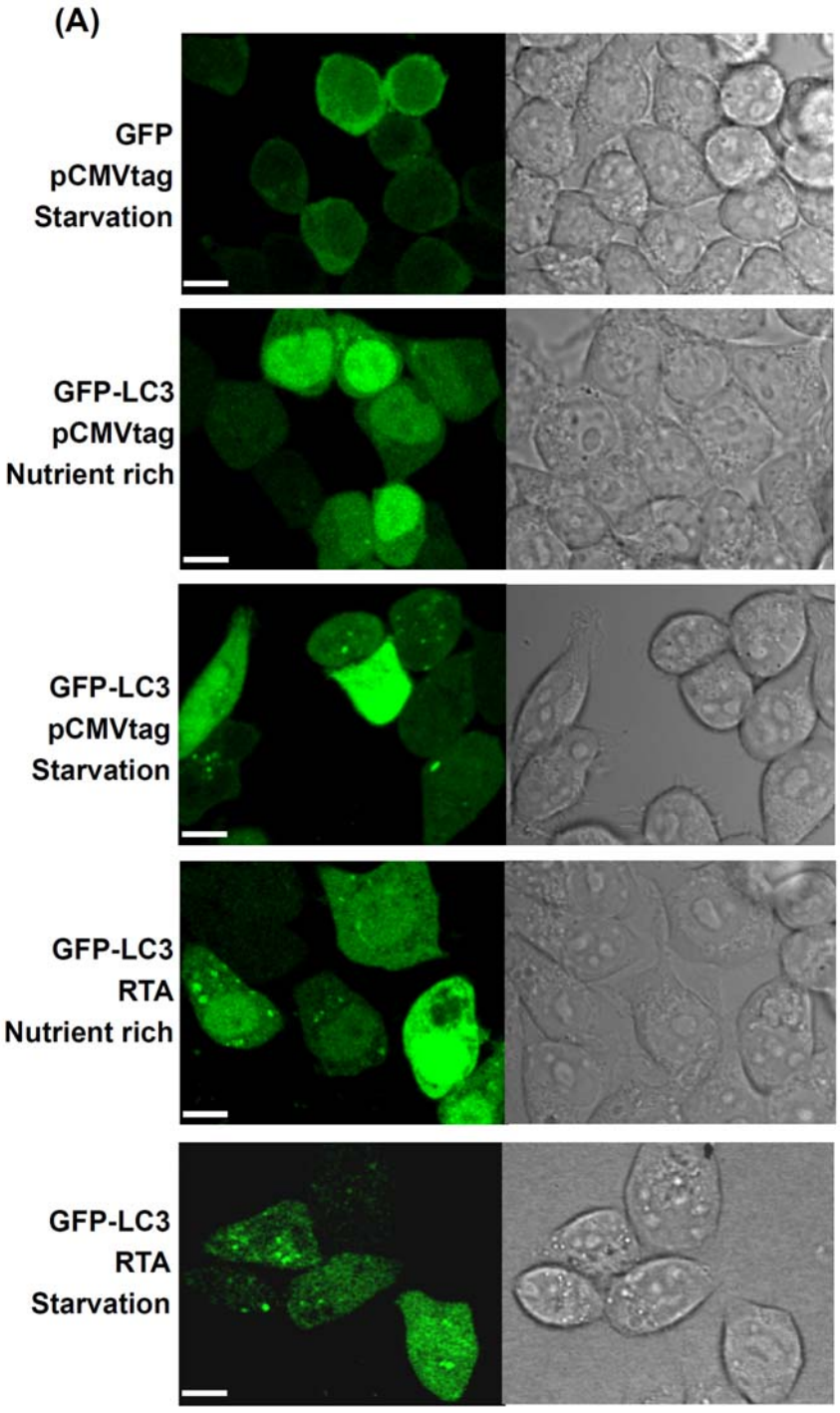
(A)
GFP
pCMVtag
Starvation
GFP-LC3
pCMVtag
Nutrient rich
GFP-LC3
pCMVtag
Starvation
GFP-LC3
RTA
Nutrient rich
GFP-LC3
RTA
Starvation

Fig. 3

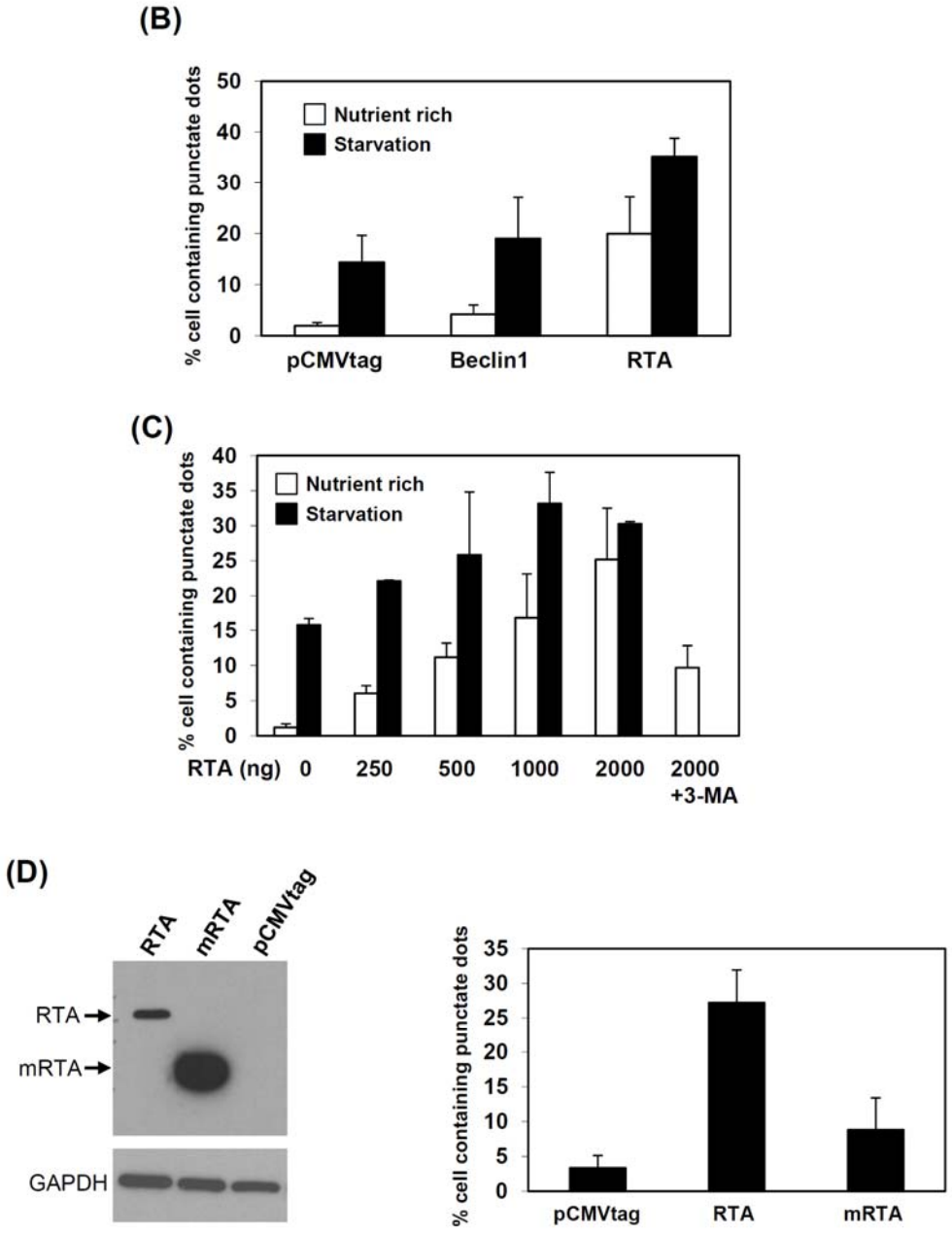
(B)
% cell containing punctate dots
Nutrient rich
Starvation
pCMVtag
Beclin1
RTA
(C)
% cell containing punctate dots
Nutrient rich
Starvation
RTA (ng) 0 250 500 1000 2000 2000 +3-MA
(D)
RTA
mRTA
pCMVtag
RTA
mRTA
GAPDH
% cell containing punctate dots
pCMVtag
RTA
mRTA

Fig. 3. Induction of autophagic vacuoles by overexpression of RTA.

(A) confocal microscopy of autophagic vacuoles. GFP or GFP-LC3 plasmid was cotransfected with pCMVtag or pCMVtagORF50 (RTA) expression plasmid into 293T cells. At 22 h after transfection, the cells were cultured either in regular medium (nutrient rich) or buffered saline, 1x Earle's balanced solution, (starvation) for another 90 min. The phase contrast micrograph is shown in parallel. Scale bars=10 μm. (B) Quantitation of the number of cells containing GFP-punctate dots in transfected 293T cells. GFP-LC3 was co-transfected with pCMVtag, Beclin 1 or pCMVtagORF50 (RTA) into 293T cells, and the cells were cultured in regular medium (nutrient rich) or buffered saline (starvation). (C) Induction of autophagy by RTA in a dose-dependent manner. An increasing concentration of RTA expression was co-transfected with GFP-LC3 into 293T cells. An autophagic inhibitor 3-MA was added into the transfected cells for 3 h. The number of cells containing green punctate dots was then quantified. (D) The increase in the autophagy by RTA is not due to protein overexpression. GFP-LC3 was co-transfected with pCMVtag, pCMVtagORF50 (RTA) or mutant RTA (mRTA) into 293T cells and the cells were cultured in regular medium. The protein expression levels of RTA and mRTA are detected by Western blotting with anti-flag antibody as shown in the left panel. The number of cells containing green dots is shown in right panel. All of results are based on the average from three independent repeats. Results are expressed as mean +/- S.D.

Fig. 4

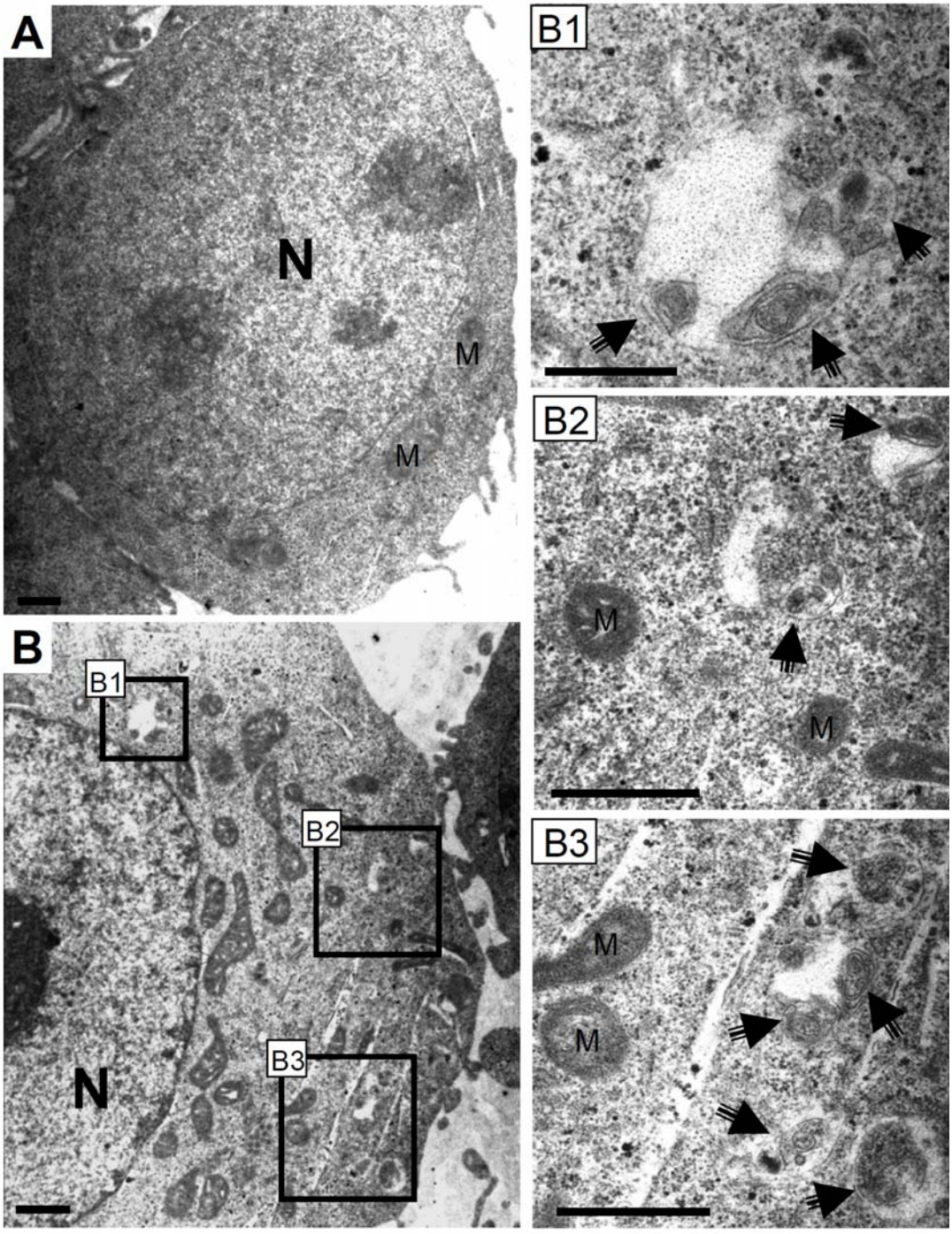

Fig. 4. TEM ultrastructural confirmation of autophagic vacuoles.

293T cells were transfected with pCMVtag (A) as a control or with pCMVtagORF50 (RTA) (B) and were cultured under nutrient rich condition. Arrows indicate autopahgic vacuoles. N indicates nucleus. Panel B1, B2 and B3 are magnified views of auotphagic vacuole structures from the boxed regions shown in panel B. M represents mitochondria. Scale bars=1 μm.

Fig. 5

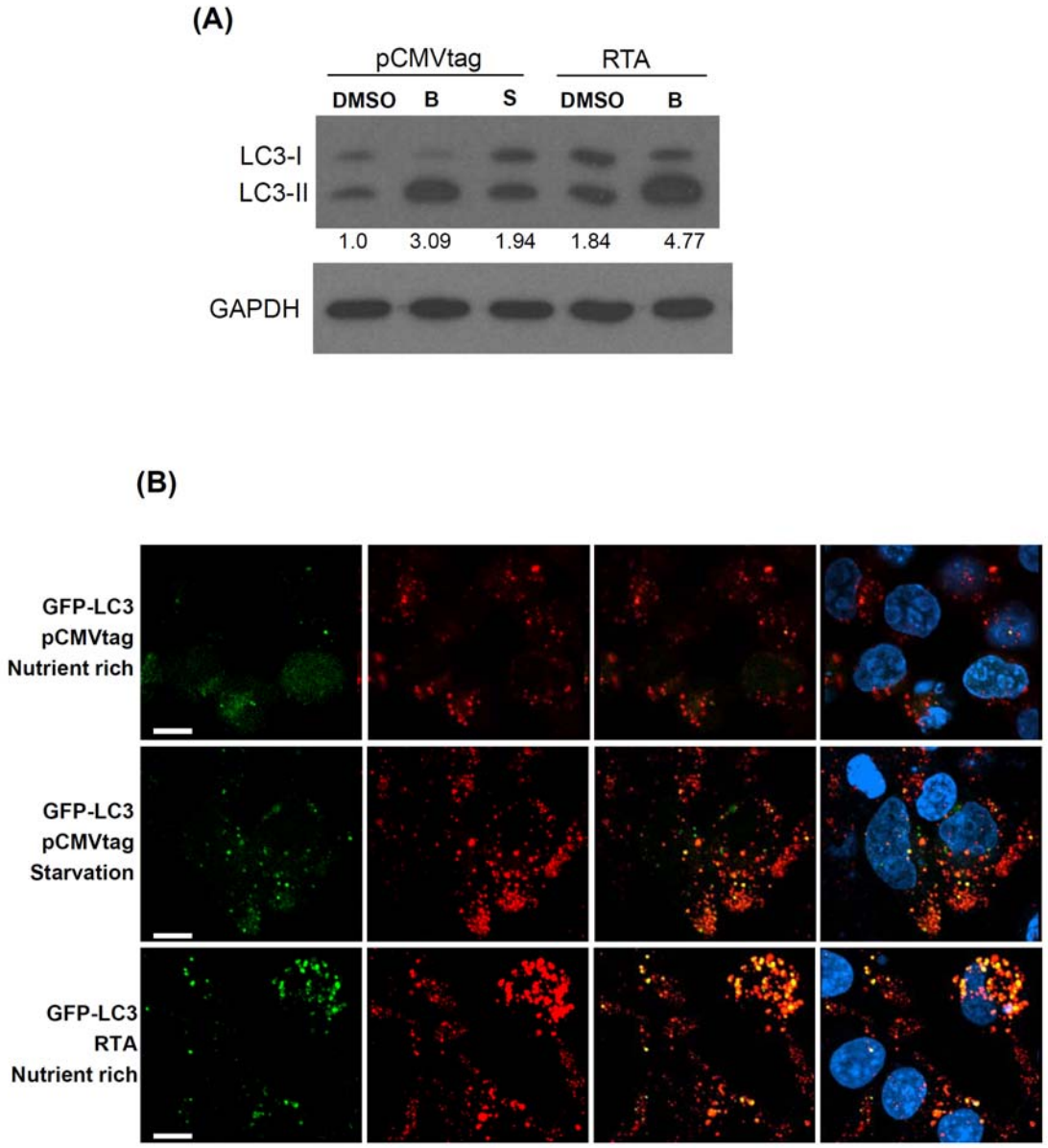
(A)
pCMVtag
RTA
DMSO
B
S
DMSO
B
LC3-I
LC3-II
1.0
3.09
1.94
1.84
4.77
GAPDH
(B)
GFP-LC3
pCMVtag
Nutrient rich
GFP-LC3
pCMVtag
Starvation
GFP-LC3
RTA
Nutrient rich

Fig. 5. Induction of autolysosomes by RTA.

(A) Western blot analysis of LC3 in 293T cells transfected with pCMVtag or pCMVtafORF50 (RTA) and treated with bafilomycin A1 (as indicated by B) or DMSO (control) for 4 h or salt buffer (as indicated by S) for 90 min. The numbers indicate the relative amounts of LC3-II protein which were normalized with the GAPDH protein. (B) Confocal microscopy of autolysosomes. The plasmids transfected into 293T cells were as indicated. For starvation, the transfected cells were starved for 90 min in salt buffer. Lysosomes were stained by Lysotracker red dye (red), autophagosomes were stained by GFP-LC3 (green), and autolysosomes were stained by Lysotracker red and GFP-LC3 showing yellow signal. The nuclei were stained with DAPI (blue). Scale bars=10 μm.

Fig. 6

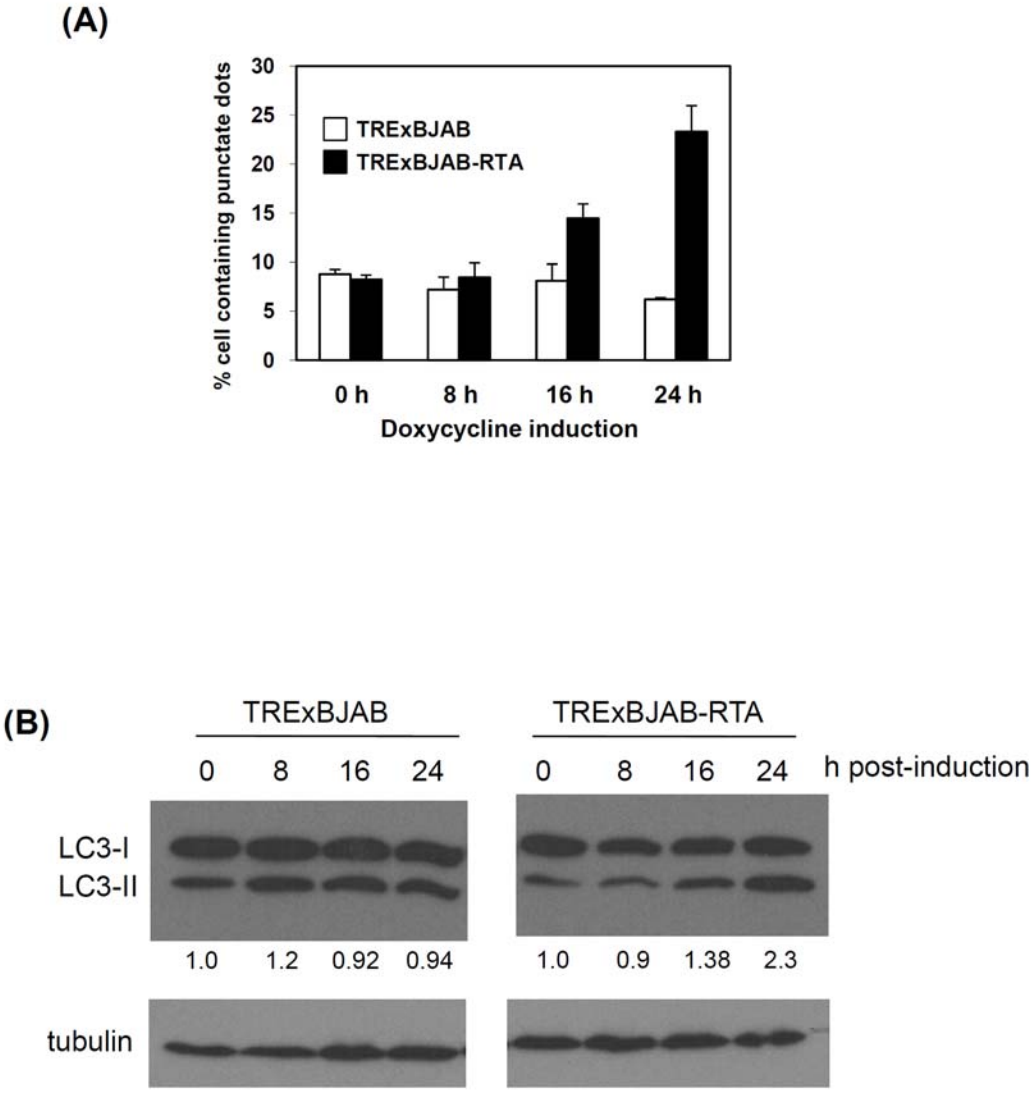
(A)
% cell containing punctate dots
30
25
20
15
10
5
0
TRExBJAB
TRExBJAB-RTA
0 h
8 h
16 h
24 h
Doxycycline induction
(B)
TRExBJAB
TRExBJAB-RTA
0 8 16 24
0 8 16 24
h post-induction
LC3-I
LC3-II
1.0 1.2 0.92 0.94
1.0 0.9 1.38 2.3
tubulin

Fig. 6. Enhancement of autophagy by RTA in B cells.

(A) Induction of autophagic vacuoles by RTA. After transfection with GFP-LC3 plasmids, TRExBJAB and TRExBJAB-RTA cells were treated with doxycycline for the indicated time. The number of cells containing green punctate dots was then quantified. The results are from the average of three independent experiments. Results are expressed as mean +/- S.D. (B) LC3 was analyzed by Western blot analysis of TRExBJAB and TRExBJAB-RTA cells treated with doxycycline for the indicated time. The numbers shown below the blots indicate the relative amounts of LC3-II protein which were normalized with the GAPDH protein.

Fig. 7

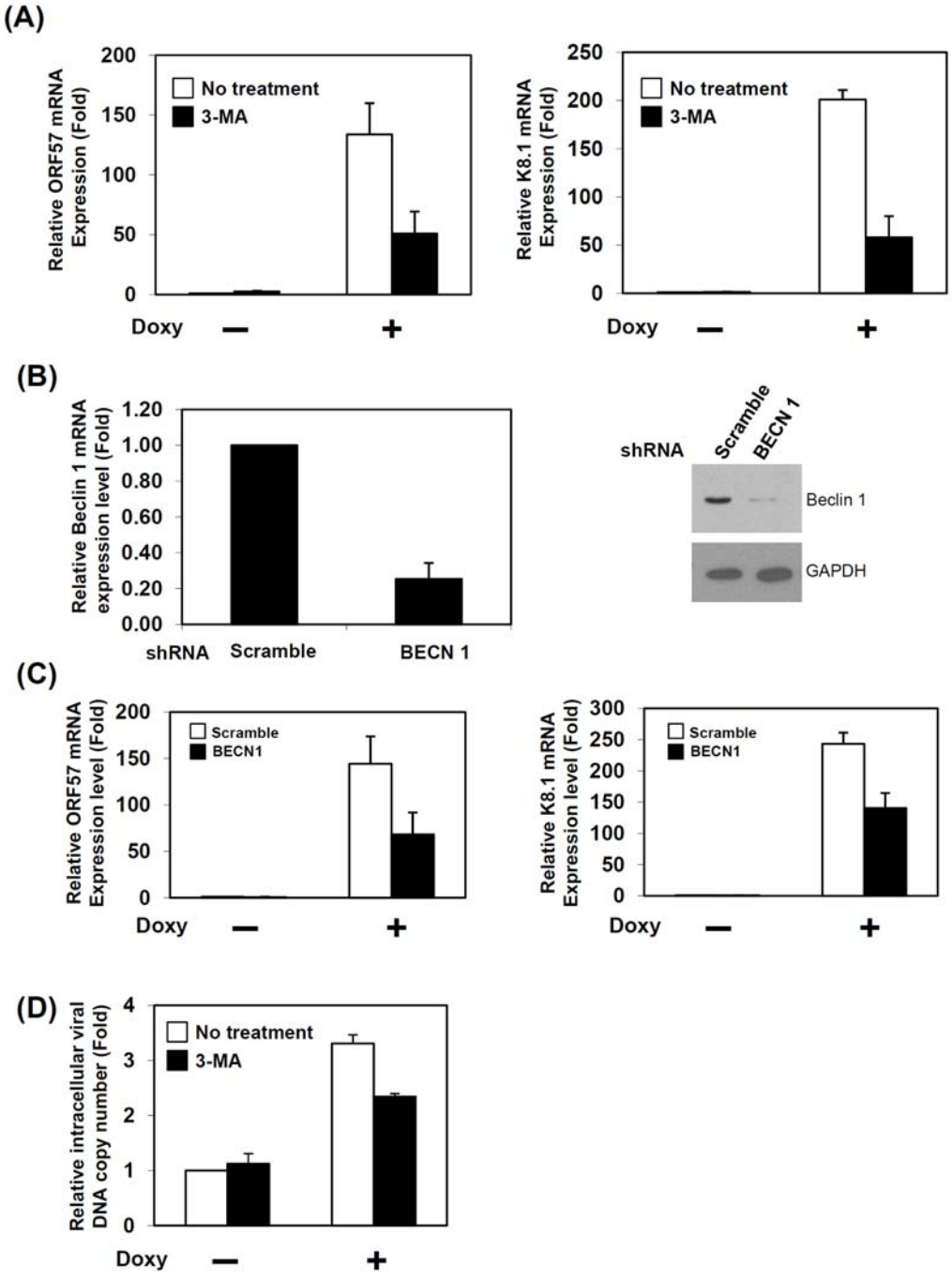
(A)
Relative ORF57 mRNA Expression (Fold)
200
150
100
50
0
No treatment
3-MA
Doxy
–
+
Relative K8.1 mRNA Expression (Fold)
250
200
150
100
50
0
No treatment
3-MA
Doxy
–
+
(B)
Relative Beclin 1 mRNA expression level (Fold)
1.20
1.00
0.80
0.60
0.40
0.20
0.00
shRNA
Scramble
BECN 1
shRNA
Scramble
BECN 1
Beclin 1
GAPDH
(C)
Relative ORF57 mRNA Expression level (Fold)
200
150
100
50
0
Scramble
BECN1
Doxy
–
+
Relative K8.1 mRNA Expression level (Fold)
300
250
200
150
100
50
0
Scramble
BECN1
Doxy
–
+
(D)
Relative intracellular viral DNA copy number (Fold)
4
3
2
1
0
No treatment
3-MA
Doxy
–
+

Fig. 7. Defective autophagy reduces RTA-mediated lytic replication.

(A) Inhibition of autophagy by 3-MA affects RTA-mediated lytic gene expression. TRExBCBL1-RTA cells were treated with different combinations of doxycycline (Doxy) and 3-MA. ORF57 and K8.1 mRNA were quantified by real-time PCR. (B) Quantitative real-time PCR and Western blot analysis detected the mRNA and protein levels of Beclin 1 from negative (N) and Beclin 1 (BECN 1) knockdown BCBL1-RTA cells. (C) Inhibition of autophagy by knockdown of Beclin 1 reduces RTA-mediated lytic gene expression. ORF57 and K8.1 mRNA levels from negative (N) and Beclin 1 (BECN 1) knockdown BCBL1-RTA cells with or without doxycycline (Doxy) treatment were quantified by real-time PCR. (D) Inhibition of autophagy reduces viral DNA replication. Cellular viral DNA copy number from TRExBCBL1-RTA cells with different combinations of 3-MA and doxycyclin (Doxy) was quantified by real-time PCR. The results are from the average of three independent experiments. Results are expressed as mean +/- S.D.

CHAPTER 4

THE MOLECULAR MECHANISM OF RTA-STIMULATED AUTOPHAGY

Abstract

KSHV (Kaposi's sarcoma-associated herpesvirus) is the etiological agent of Kaposi's sarcoma, which is a common neoplasm found in human immunodeficiency virus-infected individuals. KSHV lytic reactivation from latency is critical for KSHV pathogenesis. A viral protein, RTA (replication and transcription activator), plays a major role in regulation of KSHV reactivation. Previously, we have demonstrated that a cellular catabolic process involving degradation of the cellular components, autophagy, is involved in RTA-mediated lytic replication because RTA can stimulate autophagy. In this study, we found that Bcl-2 phosphorylation is increased in the presence of RTA and inhibition of JNK activity decreases Bcl-2 phosphorylation. This reduction also affects RTA-enhanced LC3 (microtubule-associated protein 1 light chain 3) conversion, suggesting that the mechanism by which RTA stimulates autophagy is JNK-dependent Bcl-2 phosphorylation. In addition, we determined that autophagy is not sufficient but can cooperate with RTA to activate lytic replication. Inhibition of autophagy reduces RTA-mediated transactivation of the lytic gene promoters. Collectively, these results suggest that autophagy is activated and utilized by RTA to promote RTA-target gene expression and facilitate virus lytic replication.

Introduction

KSHV (Kaposi's sarcoma associated herpesvirus) is a human oncogenic gammaherpesvirus which is the etiology agent of Kaposi's sarcoma (KS), primary effusion lymphoma (PEL) and Multicentric Castleman's disease (MCD) (2-4). It has been implicated that the development of these malignancies involves not only latent KSHV infection but intermittent periods of viral reactivation is also required (57). KSHV latency involves the expression of a restricted number of viral proteins to maintain viral latency, prevent cell death from apoptosis, inhibit immune recognition, and promote cell cycle that can lead to cell transformation. During the lytic cycle, most viral proteins are expressed and mainly function in viral replication and viral progeny production. In addition to producing infectious virions, some lytic gene products can stimulate paracrine signaling and cell transformation (57). With respect to KSHV-associated malignancies, only a small fraction of infected tumor cells undergo spontaneous lytic replication. However, clinical studies involving ganciclovir showed that interruption of lytic but not latent replication can lead to KS regression, suggesting lytic viral replication is important for tumor spread (32).

KSHV reactivation from latency is primarily controlled by an immediate early gene product, the replication and transcription activator (RTA). Ectopic expression of RTA is sufficient to induce viral lytic reactivation from latency (13, 30, 45). RTA acts as a transcriptional activator to induce the expression of numerous virus lytic genes and thereby facilitates the lytic replication program (5, 6, 8, 20, 43). To activate lytic gene transcription, RTA either binds directly to the RTA responsive elements (RREs) in its target gene promoters and/or requires the interaction with cellular transcriptional factors.

In fact, a number of cellular proteins, such as recombination signal binding protein (RBP-Jk), CCAAT/enhancer-binding protein alpha (C/EBPα), the high mobility (HMG) protein HMGB1, octamer binding factor (Oct1), KSHV-RTA binding protein (K-RBP), interferon regulatory factor 7 (IRF7), CREB-binding protein (CBP), poly(ADP-ribose) polymerase 1 (PARP1), and Hey1, have been demonstrated to be involved in RTA-mediated transcription (14-16, 50, 53, 56, 60, 62). However, among these transcriptional factors, some repress RTA-mediated transcriptional activation by inhibiting the recruitment of RTA onto the promoters of its target genes (14, 15, 50, 60, 62). Conversely, RTA employs proteasome degradation as a mechanism to overcome the suppression and these targets includ K-RBP, IRF7, and Hey1 (63, 64) (12). Therefore, to achieve RTA-mediated lytic replication, the interplay between RTA and cellular factors or other cellular pathways is critical. Previously, we have demonstrated another cellular degradation process, autophagy, is involved in RTA-mediated lytic replication (56).

Autophagy is a self-digestion process which not only degrades proteins but also digests unwanted organelles. Autophagy can be activated in response to various stimuli, including nutrient deprivation, environmental stress (hypoxia, oxidative stress), microbial infection, and organelle damage. Accordingly, this self-degradative process is involved in cell survival, development, anti-aging, and innate or adaptive immunity (27). Conversely, dysfunction of autophagy contributes to multiple human diseases including neurodegenerative disorders, inflammatory bowel, and cancers (21, 28). The typical hallmark in the autophagic process is the formation of autophagic vacuoles (autophagosomes and autolysosomes). Autophagosomes are lipid-rich and protein-poor vesicles with double membrane structure. The cytoplasmic constituents are invaginated

and sequestered by the membrane when the isolated membrane is elongated to form an autophagosome. When the outer membrane of the autophagosome fuses to the lysosome to become an autolysosome, the internal content is delivered into the lumen of the degradative compartment. Subsequently, the inner membrane of the autophagosome and internal materials are degraded by lysosomal enzymes (10). Autophagy is a multistep process which can be divided into three regulatory levels: The first level involves the cellular signaling pathways upstream of the molecular machinery of autophagy; the second level is molecular machinery which is related to the protein-protein interactions and the activity of autophagy-related gene (Atg) products. This level is mainly responsible for the formation of the autophagosomes. The third regulatory level is involved in the maturation and fusion of the autophagosomes with the lysosomes (9).

A number of cellular signaling pathways in the first regulatory level have been identified and can be categorized on the basis of mTOR involvement (17). mTOR (mammalian target of rapamycin), a serine/threonine protein kinase, regulates cell proliferation in response to nutrients, growth factors and cellular energy (ATP). Conversely, mTOR can be inactivated by rapamycin leading to activation of autophagy, suggesting that mTOR exerts an inhibitory effect on autophagy (61). It has been demonstrated that amino acids- and insulin-induced signaling activate mTOR through Rag proteins (Ras-related small GTPases) and class I PI3K (phosphatidylinositol 3-kinase)-Akt pathway, respectively, to inhibit autophagy (1, 23). In contrast, low energy (ATP) level, ROS (reactive oxygen species), ER stress, and hypoxia inhibit mTOR activity through phosphorylation and activation of the TSC1/2 (tuberous sclerosis

complex1 and 2) complex by AMPK (5' adenosin monophosphate-activated protein kinase) that are able to induce autophagy (17, 19, 37).

In addition to the mTOR-dependent pathway, ER stress and hypoxia can also stimulate autophagy through the mTOR-independent pathway which is related to Beclin 1. Beclin 1 is an ortholog of yeast Atg6 and plays an important role in the second regulatory level of the autophagic process. Beclin 1 is part of a class III PI3K complex and promotes the catalytic activity of class III PI3K for synthesis of a lipid, phosphatidylinositol 3-phosphate, which is essential for the elongation of the preautophagosomal membrane (phagophore) and recruitment of other autophagy proteins to the phagophore (49). In normal conditions, this complex is interfered by an anti-apoptotic protein Bcl-2 (B-cell leukemia/lymphoma 2) which directly interacts with Beclin 1 (39). However, ER stress activates DAPK (death-associated protein kinase) to phosphorylate Beclin 1 that causes the dissociation of Beclin 1 from Bcl-2 and induces autophagy (65). Hypoxia induces the expression of BNIP3L (Bcl-2 adenovirus E1a nineteen kDa interacting protein 3-like protein) to compete with Beclin 1 for binding to Bcl-2, which in turn stimulates autophagy (48). Moreover, the dissociation of Bcl-2 from Beclin 1 resulting in the induction of autophagy can also be triggered by nutrient starvation or ceramide treatment. It was demonstrated that JNK 1 (c-Jun N-terminal kinase 1) is activated in response to nutrient starvation or ceramides to disrupt the interaction between Beclin 1 and Bcle-2 by phosphorylation of Bcl-2, thereby allowing autophagy to proceed (38, 54).

We have previously shown that RTA is able to enhance the autophagy pathway during KSHV lytic replication. In the current study, we further demonstrate that

autophagy serves to enhance lytic replication initiated by RTA and plays an important role in RTA-mediated transcription of lytic genes. In addition, we found that JNK-dependent phosphorylation of Bcl-2 is involved in RTA-stimulated autophagy.

Materials and methods

Cells, plasmids and reagents

Human 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 μg/ml penicillin-streptomycin (Mediatech) at 37°C with 5% CO2. TRExBCBL1-RTA cell line carrying a tetracycline-inducible RTA gene was provided by Dr. Jae Jung (University of Southern California, Los Angeles, CA) (33). B cells were grown in RPMI 1640 medium (Gibco BRL) supplemented with 10% FBS and 100 μg/ml penicillin-streptomycin at 37°C with 5% CO2. To establish knockdown 293T cell lines, Beclin1 (BECN 1), Atg5, and scrambled negative control (Scramble) shRNA plasmids were purchased from Origene and transfected into 293T cells using lipofectamine 2000 (invitrogen), and transfected cells were selected by puromycin.

RTA expression plasmid (pCMVtagORF50) which encodes Flag-tagged full-length RTA has been described previously (51). RTA Mutant plasmid pCMVtagRTA678, pCMVtagRTA621, pCMVtagRTA579, and pCMVtagRTA527, which encode Flag-tagged RTA amino acids 1 to 678, 1 to 621, 1 to 579, and 1 to 527, respectively, were generated by inserting the PCR-generated DNA fragments into suitable sites of pCMV-tag2A. Plasmid pGFP-LC3 encoding GFP-tagged rat LC3 gene was obtained from Dr. Mizushima (Tokyo Medical and Dental University, Japan) (22). The β-galactosidase

expression plasmid pCMVβ which was used for the normalization of transfection efficiency was purchased from BD Clontech. Reporter plasmids pGL3-ORF57-3RRE (containing ORF57 promoter), PanPluc (containing PAN promoter), and pHIVLTR-luc (containing HIV long terminal repeat region) have been described previously (52, 55). Plasmids pCDNA-Tat and pCMVtag-Beclin 1 express the full-length HIV Tat protein and Flag-tagged full-length Beclin 1 protein, respectively.

Bafilomycin A1, doxycycline, and SP600125 were purchased from Sigma. Rapamycin was purchased from Santa Cruz biotechnology.

Western blot analysis

Cells were harvested and the cell pellets were resuspended in M-PER buffer (Thermo Scientific) containing protease inhibitor cocktail and Halt phosphatase inhibitors (Thermo Scientific) at 4°C for 20 minutes, followed by centrifugation at 10,000 rpm for 5 minutes at 4°C. Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membrane (GE Osmonics), followed by incubation with specific antibodies as described previously (62). LC3 antibody was purchased from Abcam Inc. BECN1 antibody against human Beclin 1, anti-Bcl-2 antibody, anti-tubulin antibody, and anti-GAPDH antibody were purchased from Santa Cruz biotechnology. For detection of His- and Flag-tagged proteins, the HRP-conjugated anti-6xHis antibody and mouse anti-Flag M2 monoclonal antibody were purchased from Clontech and Stratagene, respectively. Anti-JNK, anti-phosphorylated JNK, anti-phosphorylated Bcl-2, anti-mTOR, anti-phosphorylated mTOR, anti-p70S6K, anti-phosphorylated p70S6K antibodies were purchased from Cell Signaling Technology. The band intensities were measured by using the NIH image software, image J.

Real-time reverse transcription (RT)-PCR

Real-time reverse transcription PCR was performed as described previously (56). Total RNA was isolated using an RNA mini kit (Qiagen) using protocol recommended by the supplier. RNA samples were digested with DNase (Invitrogen) to remove residual DNA. Real-time RT-PCR was carried out using iScript one-step RT-PCR kit with SYBR Green. The primers used for the mRNA quantitation of K8 were [5'-GCCGAAGTATGTGATCAGTC- 3' (forward) and 5'- ATTCGCATCAGCATGTCG-3' (reverse)]. The primers for ORF57 and K8.1 have been described previously (56). All reactions were performed in duplicate. For calculation of the relative mRNA amount from quantitative real-time PCR, the Ct (threshold cycle) value of each viral gene was normalized by the Ct value of GAPDH, and the normalized Ct values from samples were compared with the control samples (untreated).

Transfection and luciferase assay

Transfection of 293T cells was carried out by using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's recommendations. Luciferase assay was performed as described previously (55). Luciferase activities were determined by the Luciferase Assay System (Promega). The transfection efficiency was normalized using the β-Gal expression plasmid, pCMVβ, as the internal control.

Quantitative GFP-LC3 autophagy assay

Quantitative GFP-LC3 autophagy assay was performed in 293T cells as described (56). The 293T cells were grown in 35 mm coverslip bottom dish (BD Biocoat), and cells were transfected with GFP-LC3 expression plasmid and plasmids expressing full-length RTA or various RTA mutants. A series of optical images were obtained with an Olympus

FV500 confocal system on an inverted microscope, using the 488 nm laser line (522 nm emission) for GFP detection. The cell containing ≥ 3 GFP-LC3 dots was defined as autophagy positive cells. The number of cells with GFP-LC3 punctate dots relative to all GFP-LC3 positive cells was counted (a minimum of 200 GFP-LC3 positive cells were counted in total for each experiment) and were presented as percentages.

Immunoprecipitation assay

Endogenous Beclin 1/Bcl-2 immunoprecipitation was performed in 293T cells by lysing cells with lysis buffer (50mM Tris pH 7.9, 150 mM NaCl, 1mM EDTA, 1% Triton-X100, protease inhibitor cocktail and Halt phosphatase inhibitors (Thermo Scientific) at 4°C for 1 hour. Immunoprecipitation was performed with a monoclonal anti-Bcl-2 antibody-conjugated to agarose (Santa Cruz biotechnology).

Results

Autophagy is not sufficient for initiation of KSHV lytic replication

We have demonstrated that autophagy is involved in RTA-mediated lytic replication. To determine whether autophagy alone is sufficient to trigger KSHV lytic replication, an autophagy inducer rapamycin was tested on RTA-inducible BCBL1 cells, TRExBCBL1-RTA. In this cell line, RTA is integrated into the genome of KSHV infected B cells, BCBL1, and its expression is regulated by doxycycline (33). To induce autophagy machinery, the cells were treated with rapamycin for 48 hours and LC3 conversion was detected by Western blotting. LC3, microtubule-associated protein 1 light chain 3, has been widely used as an indicator of autophagy activation. LC3 is constitutively expressed in the cytoplasm as a precursor protein known as LC3-I (18

KDa). When autophagy is activated, LC3-I is conjugated to phosphatidylethanolamine (PE) becoming a lipidated molecule termed LC3-II (16 KDa). Hence, the change in the molecular weight of LC3 protein indicates the presence of autophagy (18, 25). As shown in figure 1A, an increase in LC3-II protein was observed in the presence of rapamycin, indicating that rapamycin enhances the autophagic process. No significant changes in mRNA levels of two early genes (ORF57 and K8) and one late gene (K8.1) were observed in the cells treated with rapamycin alone, as measured by quantitative real-time PCR. Nevertheless, RTA expression induced by doxycycline efficiently elevated mRNA levels of those lytic genes, and addition of rapamycin could enhance RTA-mediated lytic gene expression (Fig. 1B, C and D). These results suggested that rapamycin-induced autophagy alone was unable to trigger KSHV lytic replication, but cooperated with RTA to stimulate lytic gene expression.

Autophagy affects RTA transactivation function

In our previous study, we demonstrated that inhibiton of autophagy by knocking down Beclin 1 RNA levels decreased RTA-mediated lytic genes expression (56). To further substantiate that autophagy directly affected RTA-dependent transactivation of the KSHV promoters, we established several gene knockdown 293T cell lines using shRNA that targeted Atg5 or Beclin 1 (BECN1) to test whether autophagy altered the ability of RTA to transactivate KSHV gene promoters. Two RTA targeted viral gene promoters, ORF57 and PAN, as expected, were highly responsive to RTA in the negative control cells transfected with a scrambled shRNA. However, luciferase activity was decreased when the same cell line was transfected with shRNA directed against Atg5 or Beclin 1 (Fig. 2A and B). In contrast, HIV Tat-mediated transactivation of the HIV LTR

(long terminal repeat) promoter was not affected by any of the shRNAs tested (Fig. 2C). This study suggested that the enhancement of transactivation by RTA was stimulated by autophagy, and not the result of reduced general transcription activity. In addition, RTA expression levels were comparable among negative control and gene knockdown cells, indicating that the decrease in response of the two KSHV promoters to RTA in gene knockdown cells was not due to changes in protein levels of RTA (Fig. 2D). Taken together, autophagy was able to stimulate RTA-mediated transcription of gene promoters.

The transactivation domain of RTA is required for RTA-induced autophagy

To explore the potential mechanism by which RTA activates autophagy, we determined whether the transactivation domain of the RTA protein was involved in the induction of autophagy. A series of RTA mutant constructs with deletions in the C-terminus were expressed in 293T cells and their protein expression levels were similar (Fig. 3A). These RTA transfected cells were further co-transfected with GFP-LC3 expression plasmid for visualization of autophagic vacuoles. In nutrient-rich condition, LC3 protein is distributed in cytoplasm but is re-located to the inner and outer membrane of autophagic vacuoles as a result of C-terminal conjugation to phosphatidylethanolamine in response to stress, such as starvation; hence, the green dots can be observed if LC3 is tagged with green fluorescence protein (GFP) (22). The results from GFP punctate dot assay show that the RTA deletion mutant, RTA678 with a C-terminal deletion outside of the transactivation domain induced autophagy with the same efficiency as wild type RTA (Fig. 3B). However, the RTA527 mutant lacking 164 amino acids at the C-terminus of RTA, which encompasses most of the transactivation domain, induced autophagy with only about 32 % efficiency relative to wild type RTA. The deletion mutant RTA579,

which lacks its C-terminus 112 amino acids, and RTA621, which lacks 70 amino acids, were less effective than wild type RTA. RTA579 and RTA621 induced less autophagy with 68% and 48% reduction respectively when compared to wild type RTA (Fig. 3B). Our results suggest that the transactivation domain of RTA was important for induction of autophagy.

To examine the transactivation ability of these RTA mutants, the activation of two KSHV lytic gene promoters (ORF57 and PAN) were carried out using luciferase assay. Like wild type RTA, mutant RTA678, possesses similar transactivation activity of both target gene promoters. In contrast, dramatic reduction in luciferase activity was observed with the two promoters when transfected with the RTA621, RTA579, or RTA527 mutants (Fig. 3C). Taken together, these results demonstrate that a partial deletion (from a.a. 621 to 678) within the transactivation domain of RTA affects both RTA functions in regulation of transcription of target promoters and its ability to induce autophagy. It is possible that RTA may induce autophagy via up-regulating the expression of cellular genes which are involved in autophagy.

RTA does not affect mTOR signaling

A well recognized pathway that regulates autophagy is the mammalian target of rapamycin (mTOR) signaling, which negatively regulates autophagy. To determine whether RTA induces autophagy through the inhibition of mTOR signaling, we examined the kinase activity of mTOR in the presence and absence of RTA expression. The activity of mTOR can be determined by measuring the level of phosphorylation of itself or a substrate, ribosomal S6 protein kinase (S6K1, also known as p70S6K). We used an autophagy inducer rapamycin as a positive control which blocks mTOR kinase activity to

up-regulate autophagy, and the expression of Beclin 1 as a negative control which activates autophagy independent of mTOR signaling (41). As expected, the addition of rapamycin inhibited the phosphorylation of mTOR, thus increasing the frequency of autophagy. Also as expected, expression of Beclin 1 has no effect on mTOR phosphorylation but will induce autophagy independent of the mTOR pathway. For RTA, we observed that the phosphorylation of mTOR and p70S6K was not affected by overexpression of RTA, indicating that RTA may be similar to Beclin 1 and involves mTOR-independent mechanism to activate autophagy (Fig. 4).

RTA disrupts the interaction between Beclin 1 and Bcl-2

Since RTA is incapable of inhibiting mTOR kinase activity for induction of autophagy, it was possible that RTA may stimulate autophagy by directly regulating the activity of the Atg machinery involved in autophagosome formation. Beclin 1 is one of the Atg proteins responsible for initiating autophagosome formation and its effect on autophagy is modulated by formation of complexes with various cellular proteins, such as Bcl-2 which suppresses Beclin 1-mediated induction of autophagy (39). Thus, it is possible that RTA may affect the association of Beclin 1 with this protein to mediate autophagosome formation. By using immunoprecipitation assay, we found that the interaction between Beclin 1 and Bcl-2 was reduced in cells transfected with RTA (Fig. 5). The expression levels of Beclin 1 and Bcl-2 were similar in empty vector and RTA-transfected cells; however, less Beclin 1 was associated with Bcl-2 in the RTA-transfected cells (Fig. 5). Since dissociation of the Beclin 1-Bcl-2 complex to stimulate autophagy has been demonstrated under nutrient starvation and in response to BH3

mimetic molecules (31, 54). We suggest that RTA may induce autophagy by interfering with the interaction between Beclin 1 and Bcl-2.

RTA stimulates phosphorylation of Bcl-2

Post-translational modification of Bcl-2 by phosphorylation interferes with the interaction between Beclin 1 and Bcl-2 during the induction of autophagy by starvation and ceramides (38, 54). To test whether RTA induced the Bcl-2 phosphorylation, we analyzed the phosphorylation status of Bcl-2 by immunoblotting with a specific antibody which recognizes phosphorylation at Ser70. We found a slight increase in the phosphorylation of Bcl-2 in the RTA transfected-293T cells but the expression levels of Bcl-2 remained the same either in the absence or presence of RTA (Fig. 6A). In addition, phosphorylation of Bcl-2 was also found to be elevated upon RTA expression in KSHV-infected TRExBCBL1-RTA cells (Fig. 6B). These results indicate that RTA is able to induce the Bcl-2 phosphorylation.

JNK signaling is involved in RTA-induced phosphorylation of Bcl-2 and autophagy activation

It has been shown that Bcl-2 phosphorylation induces dissociation of Beclin 1 and Bcl-2 and is regulated by the c-JUN N-terminal kinase 1(JNK 1) (54), and the phosphorylation of JNK-1 is needed to activate its kinase activity prior to phosphorylation of Bcl-2. Interestingly, an increase in posphorylation of JNK 1 (p-JNK1) was observed after RTA induction in TRExBCBL-RTA cells (Fig. 6B).

To demonstrate whether RTA employs JNK signaling for Bcl-2 phosphorylation and autophagy activation, a JNK specific inhibitor, SP600125, was used to study its effect on Bcl-2 phosphorylation and LC3 conversion with and without RTA expression.

As shown in Figure 7, Bcl-2 phosphorylation elevated by RTA can be suppressed by the JNK inhibitor in the 293T (Fig. 7A) and TRExBCBL-RTA cells (Fig. 7B). In addition, the increase in LC3-II was observed in the presence of RTA, but addition of the JNK inhibitor reduced RTA-induced LC3-II expression, suggesting that RTA-mediated autophagy was suppressed by interfering with JNK signaling. We therefore concluded that RTA stimulates autophagy through JNK-dependent phosphorylation of Bcl-2.

Discussion

Autophagy has been implicated as an antiviral defense mechanism of infected cells. Autophagy can directly engulf virion particles and/or viral components for lysosomal degradation, or facilitate viral antigens presentation process via MHC (major histocompatibility complex) presentation. However, some viruses can evade or counteract the execution of autophagy, and some viruses can utilize the autophagy machinery to facilitate their replication and enhance their pathogenic potential (24). Our previous study found that the autophagy pathway is enhanced by RTA and is involved in KSHV lytic replication upon stimulation by RTA (56). This current study extended the observation to show that the induction of autophagy alone is not sufficient to initiate KSHV lytic replication but can potentiate lytic replication in the presence of RTA. A proposed model on how RTA utilizes autophagy for virus lytic replication is shown in Fig. 8. RTA induces Bcl-2 phosphoylation through the JNK signaling pathway to cause the dissociation of Beclin 1 and Bcl-2. Beclin 1 which is released from Bcl-2 can bind to class III PI3K complex to initiate the autophagic process. The induction of autophagy enhances RTA-mediated transactivation of the virus lytic promoters and thereby

facilitates virus lytic replication. The involvement of autophagy in KSHV replication supports findings from other herpesviruses, which also involve autophagy during their infection. Another member of the gammaherpersvirus family, Epstein-Barr virus (EBV), induces autophagy and the latent membrane protein 1 (LMP1) plays a role in this process. Induction of autophagy is needed to limit accumulation of the LMP1 protein as well as modulate B cell transformation (26). The infection by varicella zoster virus (VZV), an alphaherpesvirus, can also induce autophagy but whether autophagy activation is related to viral DNA replication or viral pathogenesis remained unknown (46). Our findings in this study present the first example for a herpesvirus that activates autophagy to facilitate its viral replication.

Previous studies in our laboratory demonstrated that the viral transactivator RTA promotes ubiquitin modification of its transcriptional repressors to enable the cellular proteasome degradation pathway to target and degrade the ubiquitinated-transcriptional repressors. Consequently, RTA can stimulate lytic gene expression and virus lytic replication (63). In the current study, we found that inhibition of autophagy reduced the response of RTA to the target promoters of viral lytic genes, suggesting autophagy cooperates with RTA to promote reactivation from latency. Since autophagy is a lysosomal degradation pathway which also targets ubiquitinated proteins for degradation (7), we suggest that autophagy can also degrade transcriptional repressors to facilitate RTA-responsive gene expression. This possibility need to be further explored.

We have demonstrated that RTA increases Bcl-2 phosphorylation to release Beclin 1 from Bcl-2 to activate the autophagy pathway; however, we cannot rule out the possibility that the competitive binding between Beclin 1 and Bcl-2 can also be mediated

by other proteins. It has been shown that the BH3-only proteins (Bad or Bim), the pharmacologic BH3 mimetic ABT737, and the BNIP3L protein induced by hypoxia can disrupt the interaction between Beclin 1 and Bcl-2 (or Bcl-X_L) competitively and thereby induce autophagy (31, 35, 48). We were attempting to address whether the expression of pro-apoptotic protein Bad is up-regulated by RTA for autophagy induction since RTA has been suggested as an apoptotic inducer in uninfected cells (34). However, we did not observe a significant increase in Bad expression when RTA is expressed (data not shown). Hence, additional studies are needed to further investigate whether RTA up-regulates the expression of Bim, BNIP3L, or other BH3-only protein in order to release Beclin 1 from Bcl-2 for the initiation of autophagy.

Based on our findings, we purpose that RTA induces autophagy through dissociation of Beclin 1 and Bcl-2 via Bcl-2 phophorylation, which then facilitates virus reactivation from latency, and this process involves the JNK1 kinase pathway. The JNK signaling pathway is one of the mitogen-activated protein kinase (MAPK) pathways in response to stress stimuli, and is required for KSHV primary infection and reactivation of KSHV from latency (11, 36, 59). Xie et al. showed that MAPK pathways are involved in the early stages of TPA (12-*O*-tetradecanoyl-phorbol-13-acetate)-induced KSHV reactivation from latency and suggested that AP-1 (activator protein 1), a downstream target of MAPK pathways, is a key transcriptional factor mediating lytic gene expression (59). In this study, we observed that autophagy is a downstream target of the JNK signaling pathway and further substantiates the importance of the JNK signaling pathway during KSHV reactivation from latency. Taken together, the JNK signaling pathway has

wide-ranging effects that are important for KSHV reactivation, including mediating autophagy.

During lytic replication, however, the induction of autophagy by RTA may also be inhibited by KSHV Bcl-2 homolog (vBcl-2), which is inferred as a viral lytic gene product due to the observation of its mRNA expression at late stages of productive infection (42). The vBcl-2 protein was shown to have anti-autophagic activity through its direct interaction with Beclin 1 because the binding affinity of vBcl-2 to Beclin 1 was higher than that of the cellular Bcl-2 (29, 39). Moreover, vBcl-2 does not have the phosphorylation sites of Bcl-2 (38). Thus, RTA may not interrupt the interaction between vBcl-2 and Beclin 1 to initiate autophagy through JNK1 kinase pathway when vBcl-2 is expressed. It is likely that autophagy may be transiently activated by RTA only at the very early stage of viral lytic infection but then counteracted by the expression of vBcl-2 at the late stage of viral lytic infection, reflecting an intricate mechanism that the virus regulates viral gene expression at different phases of infection. Interestingly, it has been shown the vBcl-2 protein was only detected in the spindle cells from late-stage KS lesions, but not in stimulated PEL cell lines even though its mRNA is transcribed during the lytic phase in the PEL cell line (42, 58). Therefore, whether vBcl-2 is able to inhibit RTA-stimulated autophagy as suggested during viral lytic phase remained to be determined.

It is also possible that RTA may use an alternative mechanism for inducing autophagy in addition to interfering with the interaction between Beclin 1 and Bcl-2. RTA may directly up-regulate the expression of genes which are involved in the autophagic machinery since RTA is a transcriptional activator. In fact, a number of

studies have shown that an increase in the expression level of the Atg proteins, such as Beclin 1, LC3, and Atg5, can activate the autophagic process (40, 44, 47, 66). We have in fact detected the expression of mRNA of autophagy-related genes, including Beclin 1, Atg5, Atg7, and LC3 during KSHV lytic reactivation, but no obvious changes in those mRNA levels were detected when RTA was expressed (data not shown), suggesting that RTA does not induce autophagy by directly activating the expression of these autophagy-related genes. It nevertheless remains possible that there may be other autophagy-related genes activated by RTA.

In conclusion, we provide evidence that RTA stimulates autophagy through JNK-dependent Bcl-2 phosphorylation. Furthermore, KSHV utilizes the autophagy pathway to mediate the transactivation function of RTA and enhance KSHV lytic replication. Overall, those findings further reveal that KSHV can hijack multiple cellular machineries, including autophagy, for its viral DNA replication.

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Figures and figure legends

Fig. 1

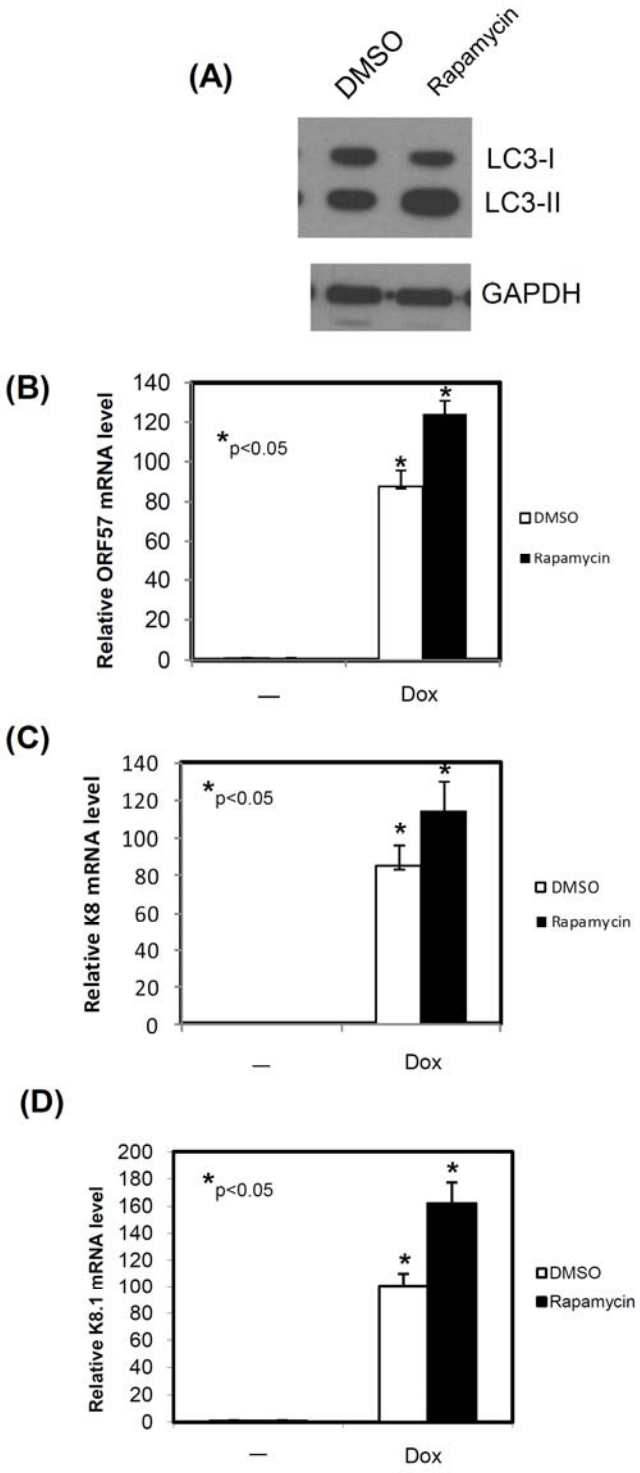


Fig. 1. Autophagy is not sufficient to trigger KSHV lytic reactivation.

(A) Rapamycin treatment induces LC3 conversion. TRExBCBL-RTA cells were treated with Rapamycin or DMSO for 48 hours. LC3 was analyzed by Western blot. (B) ORF57, (C) K8, and (D) K8.1 mRNA levels from TRExBCBL-RTA treated with DMSO or Rapamycin and/or Doxycycling (Dox) were quantified by real-time PCR. The results shown are based on the average from three separate experiments. Results are expressed as mean +/- S.D. Asterisks indicate $p < 0.05$ (student's *t* test).

Fig. 2

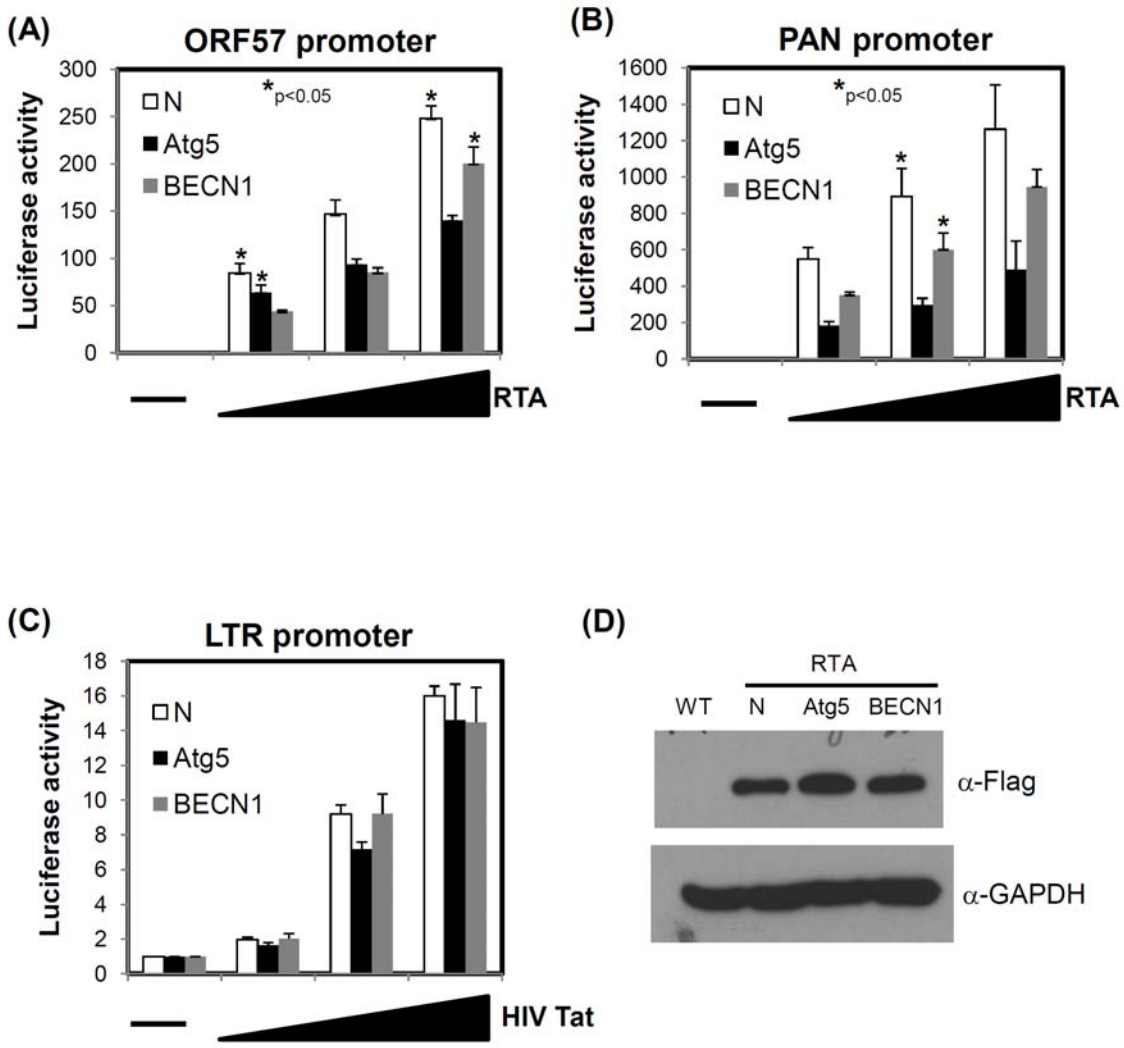
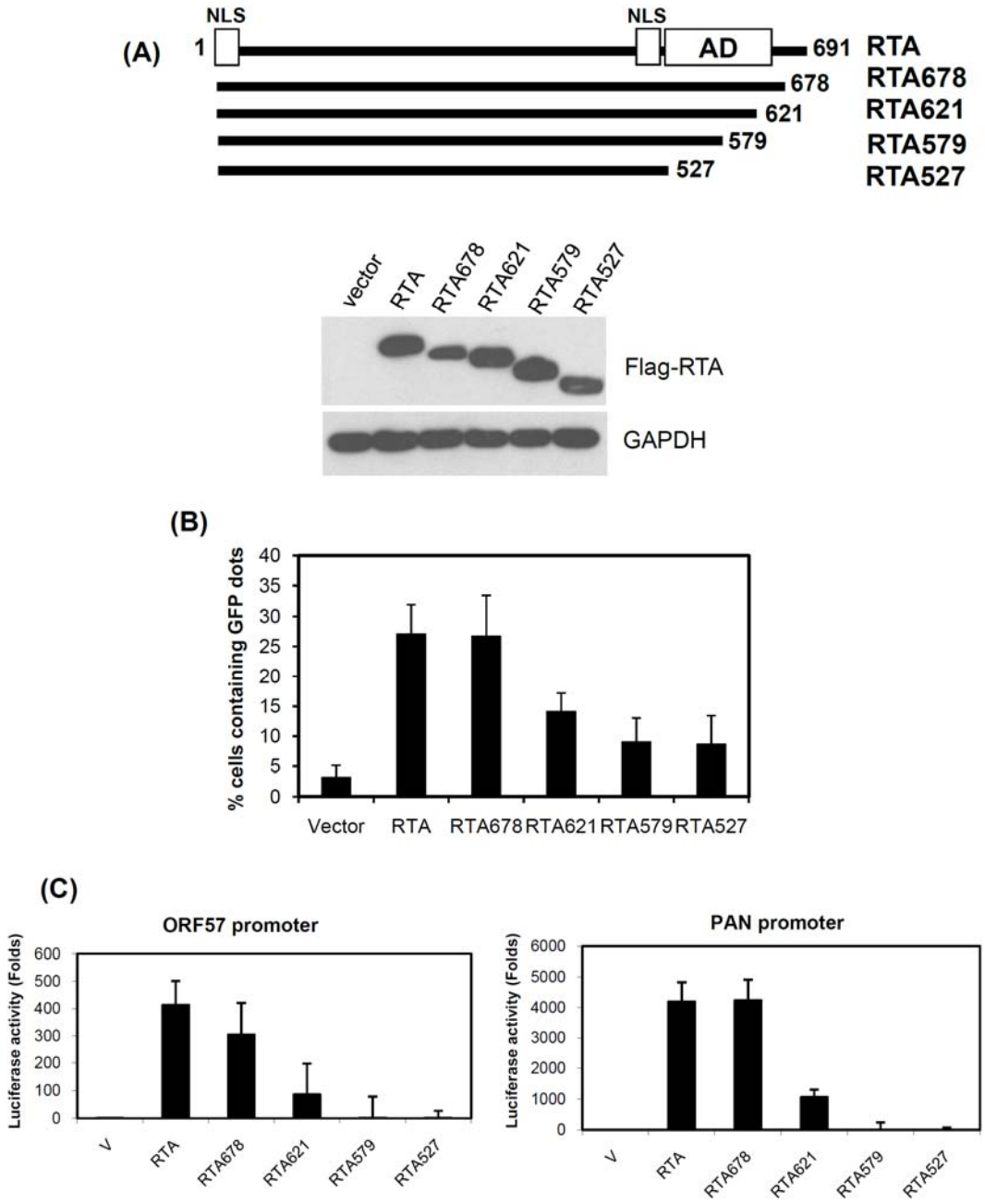

(A)
ORF57 promoter
Luciferase activity
300
250
200
150
100
50
0
N
Atg5
BECN1
*p<0.05
RTA
(B)
PAN promoter
Luciferase activity
1600
1400
1200
1000
800
600
400
200
0
N
Atg5
BECN1
*p<0.05
RTA
(C)
LTR promoter
Luciferase activity
18
16
14
12
10
8
6
4
2
0
N
Atg5
BECN1
HIV Tat
(D)
RTA
WT
N
Atg5
BECN1
α-Flag
α-GAPDH

Fig. 2. Defective autophagy affects RTA-mediated transactivation of gene promoters. (A) ORF57, (B)PAN, and (C)LTR promoter reporters were co-transfected with (A,B) RTA or (C) HIV Tat expression plasmid into various knockdown 293T cells expressing Beclin 1 (BECN), Atg5, or scramble (N) shRNA. Luciferase activity was measured at 24 hr post-transfection. All results shown are based on the average from three independent repeats. Results are shown as mean +/- S.D. (D) The RTA expression levels in wild type and various knockdown 293T cells were analyzed by Western blot with anti-Flag antibody.

Fig. 3


(A)
NLS
NLS
1
AD
691
RTA
678
RTA678
621
RTA621
579
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527
RTA527
vector
RTA
RTA678
RTA621
RTA579
RTA527
Flag-RTA
GAPDH
(B)
% cells containing GFP dots
40
35
30
25
20
15
10
5
0
Vector
RTA
RTA678
RTA621
RTA579
RTA527
(C)
ORF57 promoter
Luciferase activity (Folds)
600
500
400
300
200
100
0
V
RTA
RTA678
RTA621
RTA579
RTA527
PAN promoter
Luciferase activity (Folds)
6000
5000
4000
3000
2000
1000
0
V
RTA
RTA678
RTA621
RTA579
RTA527

Fig. 3. The transactivation domain of RTA is required for RTA-induced autophagy. (A) Schematic representation of full-length and truncated RTA constructs. The numbers indicate amino-acid positions. NLS and AD indicate nuclear localization signal and activation domain, respectively. Various RTA expression constructs and pCMVtag2A (vector) were expressed in the 293T cells and their expression levels were analyzed by Western blot with anti-Flag antibody. (B) Quantitation of the number of cells containing GFP-LC3 punctate dots in transfected 293T cells. GFP-LC3 was co-transfected with vector or various RTA deletion constructs into 293T cells for 22 hours and the cells with GFP dots were visualized using a fluorescent microscope. (C, D) The responsiveness of (C) ORF57 and (D) PAN promoters to wild-type and various RTA deletion mutants. The promoter reporter was co-transfected with various RTA expression plasmids into 293T cells. Luciferase activity was measured at 24 hr post-transfection. All results shown are based on the average from three independent repeats. Results are expressed as mean +/- S.D.

Fig. 4

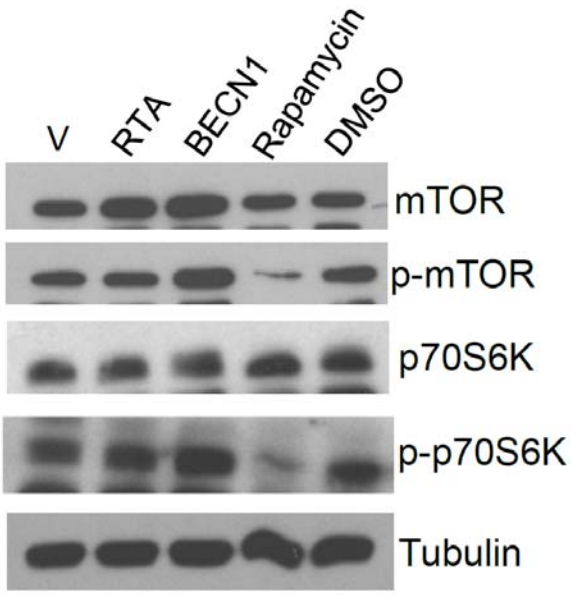
V
RTA
BECN1
Rapamycin
DMSO
mTOR
p-mTOR
p70S6K
p-p70S6K
Tubulin

Fig. 4. RTA does not affect mTOR kinase activity.

293T cells were treated with rapamycin or DMSO, or transfected with pCMVtag2A (V), RTA, or Beclin 1 (BECN1) expression plasmid. The expression levels of mTOR, p-mTOR, p70S6K, P-70S6K, and tubulin were analyzed by Western blot with specific antibodies.

Fig. 5

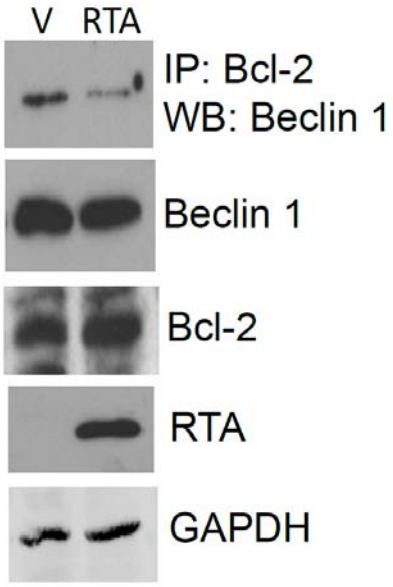

Fig. 5. RTA interrupts the interaction of Beclin 1 and Bcl-2.

pCMVtag2A (V) or RTA expression plasmid was transfected into 293T cells. The immunoprecipitation (IP) was carried out by anti-Bcl-2 antibody-conjugated agarose. The expression levels of Beclin 1, Bcl-2, RTA and GAPDH were analyzed by Western blot (WB) with specific antibodies.

Fig. 6

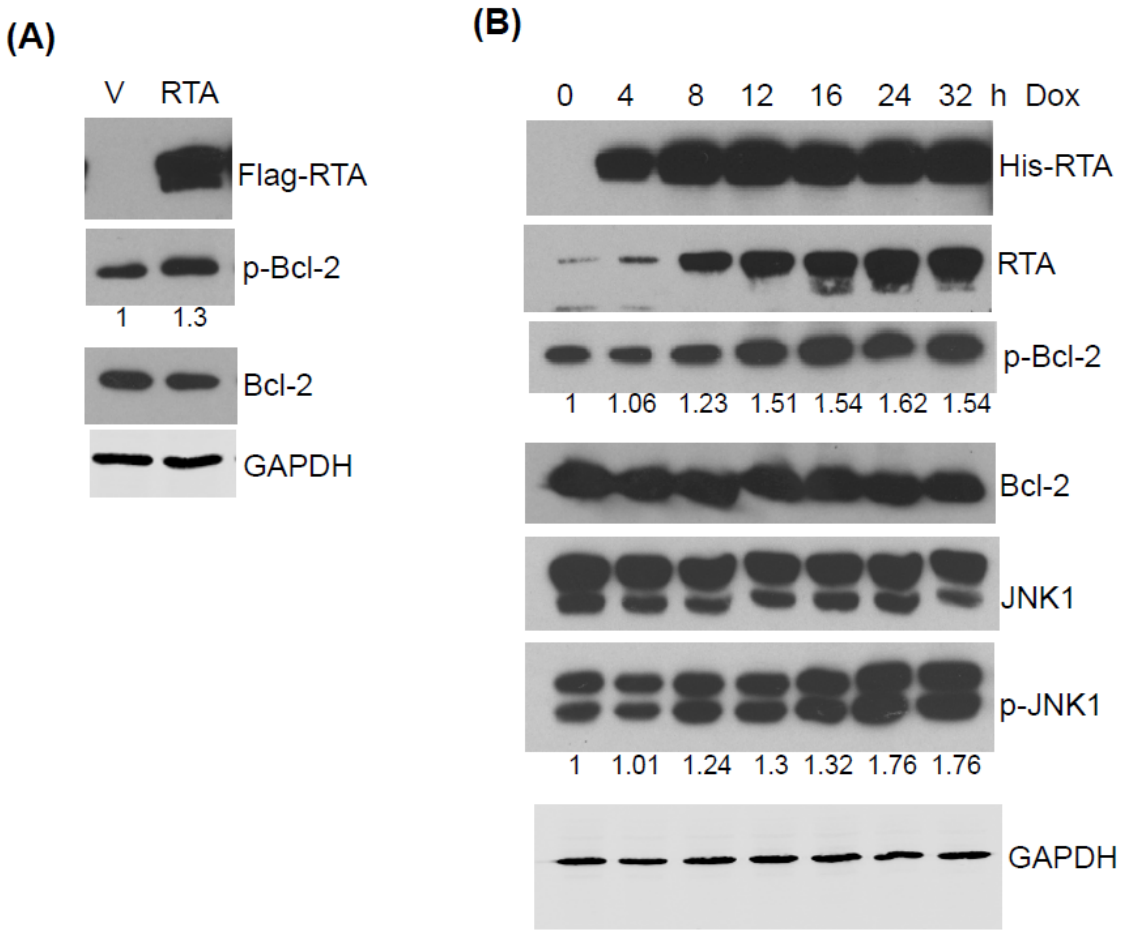
(A)
V
RTA
Flag-RTA
p-Bcl-2
1
1.3
Bcl-2
GAPDH
(B)
0
4
8
12
16
24
32
h
Dox
His-RTA
RTA
p-Bcl-2
1
1.06
1.23
1.51
1.54
1.62
1.54
Bcl-2
JNK1
p-JNK1
1
1.01
1.24
1.3
1.32
1.76
1.76
GAPDH

Fig. 6. RTA stimulates phosphorylation of Bcl-2.

(A) pCMVtag2A (V) and RTA expression plasmid was transfected into 293T cells. The expression levels of protein as indicated were analyzed by Western blot. (B) To induce RTA expression, TRExBCBL-RTA cells were treated with doxycyline (Dox) for the indicated time. Western blot analysis was performed using the specific antibodies as indicated. The numbers indicate the relative amount of the p-Bcl-2 and p-JNK1 proteins that were normalized with the GAPDH protein.

Fig. 7

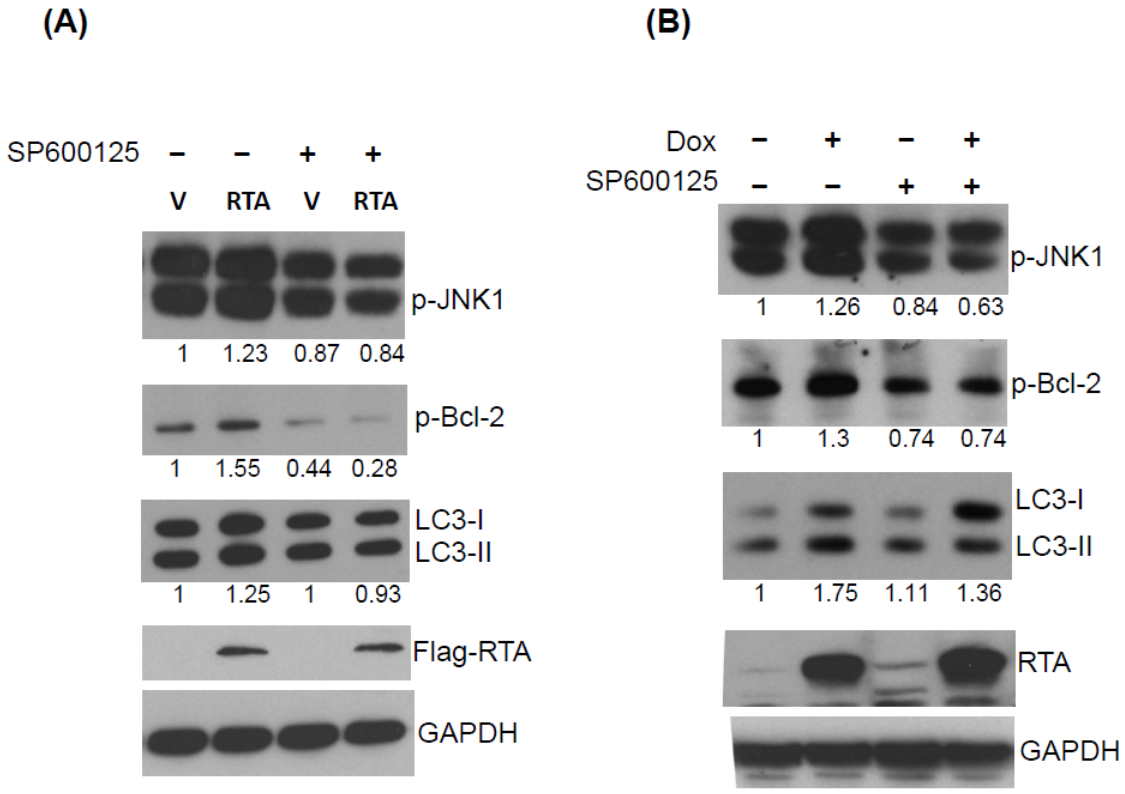
(A)
SP600125 – – + +
V RTA V RTA
p-JNK1
1 1.23 0.87 0.84
p-Bcl-2
1 1.55 0.44 0.28
LC3-I
LC3-II
1 1.25 1 0.93
Flag-RTA
GAPDH
(B)
Dox – + – +
SP600125 – – + +
p-JNK1
1 1.26 0.84 0.63
p-Bcl-2
1 1.3 0.74 0.74
LC3-I
LC3-II
1 1.75 1.11 1.36
RTA
GAPDH

Fig. 7. Inhibition of JNK activity reduces RTA-stimulated Bcl-2 phosphorylation and LC3 conversion.

(A) Vector (V) or RTA expression plasmid was transfected into 293T cells. (B) TRExBCBL-RTA cells were treated with doxycyline (Dox) to induce RTA expression. The transfected 293T cells and the induced TRExBCBL-RTA cells were treated with SP600125 for 3 hr, and Western blot analysis was performed using the specific antibodies as indicated. The numbers indicate the relative amount of the p-JNK1, p-Bcl-2 and, LC3-II proteins that were normalized with the GAPDH protein.

Fig. 8

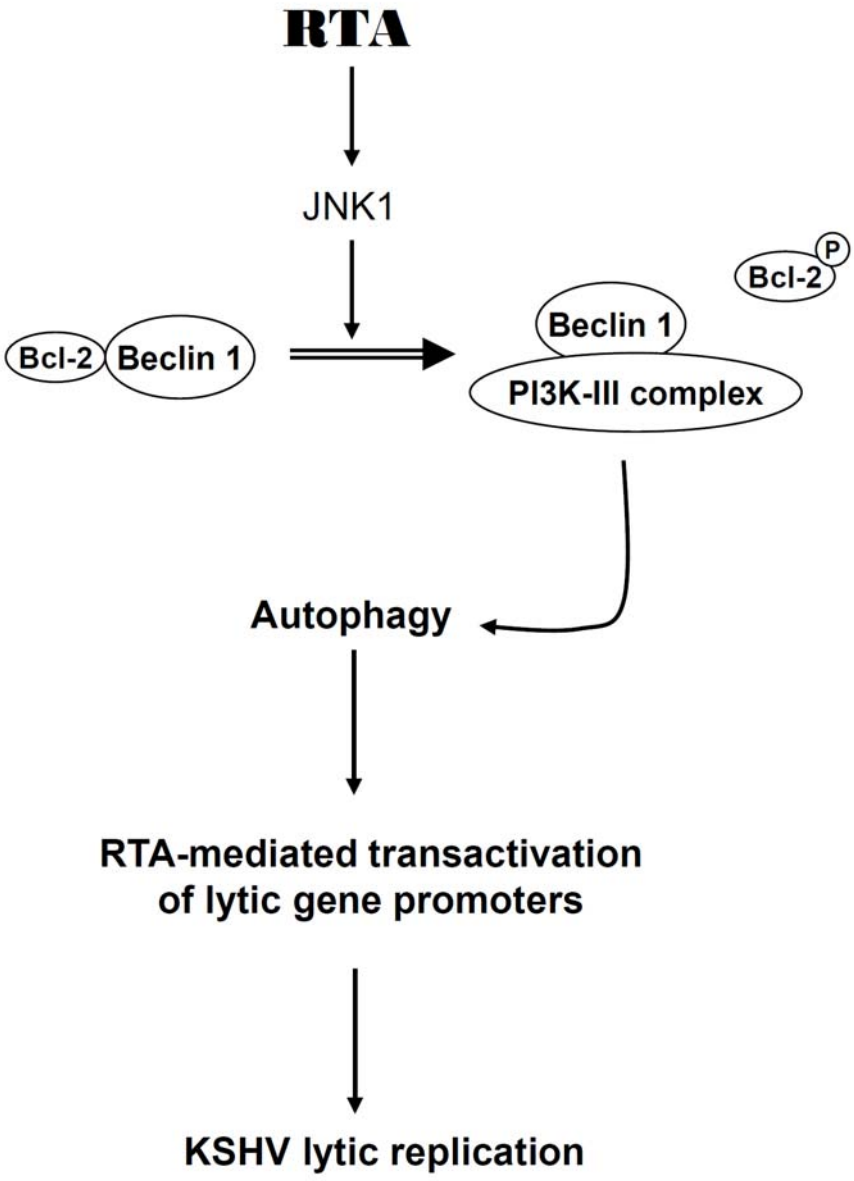
RTA
JNK1
Bcl-2
Beclin 1
Bcl-2
P
Beclin 1
PI3K-III complex
Autophagy
RTA-mediated transactivation
of lytic gene promoters
KSHV lytic replication

Fig. 8. Proposed model of autophagy stimulated by RTA for KSHV lytic replication. RTA induces the dissociation of Beclin 1 and Bcl-2 through JNK-dependent Bcl-2 phosphorylation to stimulate autophagic process. Based on our present and previous results (51), RTA-activated autophagy is essential for RTA-mediated transactivation of lytic gene promoters and lytic replication.

CHPATER 5

CONCLUDING REMARKS

KSHV RTA is a key regulator for initiation of KSHV lytic replication. This research attempts to demonstrate the molecular mechanism utilized by RTA to up-regulate lytic gene expression and facilitate virus lytic activation. From our studies, the following conclusions can be made.

GC-rich DNA sequence in the RTA responsive element (RRE) and multiple RREs are required for RTA-mediated transactivation of its target gene promoters, such as the ORF57 promoter

The sequence of the KSHV RTA responsive element (RRE) was initially identified to be AT rich or A/T trinucleotide (1, 3, 4). We identified a new RRE consisting of GC-rich sequence ($ACCN_9GG$) based on homology to the consensus sequence of the EBV RRE. This sequence can be found in a number of RTA-responsive lytic gene promoters, including ORF57, PAN, K12, K2, and MIP. Importantly, the variation of N_9 sequence in each promoter element may contribute to differences in the binding affinity and transactivation responsiveness to RTA. We focused on one of the lytic gene promoter ORF57, which is highly responsive to RTA. Previous studies in our laboratory have identified two RREs (RRE1 and RRE2) in the ORF57 promoter, and both contain two dissimilar AT-rich sequences (1, 8). In the current study, a newly identified RRE with high GC content was named RRE3. The three RREs are located in very close proximity to each other in the ORF57 promoter. All of them can be bound by the RTA

protein independently, but their binding affinities to RTA are distinct. For RTA-mediated transactivation of the ORF57 promoter, RRE2 is essential, but not sufficient. Two of the RREs (RRE1/RRE2 or RRE3/RRE2) and the RBP-Jκ binding site are required to obtain optimal transcriptional activation in response to RTA. These results suggest that the ability of RTA to mediate transactivation is distinct from its ability to binding to its responsive elements. This study also suggests a novel mechanism that multiple RREs are utilized by RTA for lytic gene expression.

A cellular pathway, autophagy, employed by RTA for virus reactivation

The results of this study showed that autophagy is enhanced during KSHV lytic replication that is induced by either chemical stimuli or RTA, and suppression of the autophagy pathway reduced RTA-mediated lytic gene expression and viral DNA replication. It indicates that autophagy, in conjunction with RTA, positively regulates the KSHV lytic cycle. However, autophagy alone is not sufficient to activate viral replication. Inhibition of autophagy prominently reduced the efficiency of early phases of viral reactivation. Our current studies also demonstrated that RTA activated the autophagy pathway independent of other viral proteins. Taken together, autophagy is activated and utilized by RTA to reactivate viral lytic replication. To date, HBV (hepatitis B virus) which is another DNA virus, in addition to KSHV, was reported to induce autophagy for its DNA replication (7). However, it is not clear how autophagy regulates HBV DNA replication. A number of RNA viruses such as poliovirus, coxsackievirus, hepatitis C virus, and dengue virus have been implicated to induce autophagy and appear to utilize the membrane of autophagic vacuoles as a site for their viral genome replication (6).

Since KSHV DNA replication occurs in the nucleus, it is unlikely that KSHV could take advantage of autophagic vacuoles as a site of its DNA replication. In fact, our results showed that defective autophagy causes less transactivation of lytic gene promoters in response to RTA, indicating that autophagy is employed by RTA to optimize transcriptional activation of the target promoters and then facilitate lytic reactivation. Autophagy could be a factor but may not the major cellular machinery used by RTA to regulate its transactivation function and lytic replication. Previous studies in our laboratory have demonstrated that another cellular degradation pathway, the ubiquitin-proteasome pathway, regulates RTA-mediated transactivation and lytic replication by degrading the ubiquitinated repressors and then enables RTA to activate its target gene promoters (11). Since autophagy is a lysosomal degradation pathway, it is highly likely that autophagy is involved in the degradation of cellular transcriptional repressors.

The overall conclusions of this dissertation can be summarized as Fig. 1. RTA induces Bcl-2 phosphorylation through JNK1 activity to interfere with the interaction of Beclin 1 and Bcl-2. Beclin 1 released from Bcl-2 can then interact with the class III PI3K complex and initiates the autophagy pathway. Autophagy supports RTA to stimulate lytic gene expression, and autophagy-modulated RTA transactivation may involve the autophagy degradation process. It has been demonstrated that RTA possesses E3-like ubiquitin ligase activity which ubiquitinates cellular transcriptional repressors for proteasome degradation (2, 11, 12). Alternatively, autophagy may target ubiquitinated or un-ubiquitinated transcriptional repressors for lysosomal degradation. Once repressors are released from KSHV promoters and degradated by the cellular degradation systems, RTA or RTA-coactivator complexes can bind to RRE in the promoter (for example, RTA

and RBP-Jκ binds to multiple RREs and RBP-Jκ binding sites in the ORF57 promoter) to induce gene expression and then facilitate virus lytic replication. Moreover, RTA-induced autophagy may facilitate virus lytic replication through an unknown mechanism in addition to assisting RTA transactivation function. This possibility needs to be further investigated.

Future direction

To substantiate whether RTA-induced autophagy is responsible for degradation of the transcriptional repressors

It has been reported that a number of viruses can activate autophagy for their genome replication; however, some of those viruses, such as Hepatitis C virus and coxsackievirus B 3, induce autophagosome formation only but not autolysosome maturation, suggesting that the degradation function of autophagy is blocked (5, 9). In our current studies, we observed that RTA is able to promote the formation of autophagosomes and autolysosomes, and degradation of LC3-II bound to the inner membrane of autophagosome can be inhibited by bafilomycin A1, an inhibitor of autolysosome maturation. Therefore, RTA-induced autophagy may include the degradation process, and RTA may utilize this feature of autophagy to overcome the transcriptional inhibition derived from the transcriptional repressors. For example, previous studies in our laboratory showed that NF-κB p65 was degraded in the presence of RTA, and this degradation was not suppressed when the 26S proteasome degradation pathway was inhibited (11). It suggests that another potential degradation mechanism,

such as autophagy, is involved in RTA-induced degradation of NF-κB p65. More experiments will be needed to address these questions.

To further investigate the alternative mechanism of RTA-stimulated autophagy.

In current studies, we have demonstrated that RTA stimulates the autophagy pathway through JNK1-dependent Bcl-2 phosphorylation. However, how RTA is able to trigger the JNK signaling pathway which occurs in the cytoplasm will require further investigations. In addition to Bcl-2 phosphorylation by JNK1, it is also possible that RTA could induce the expression of proteins, such as BH3-only protein, Bim, and BNIP3L, to interrupt the interaction between Beclin 1 and Bcl-2 for autophagy initiation, or RTA could directly up-regulates autophagy-related protein to trigger the autophagy pathway since RTA is a transcriptional activator. These possibilities will also need to be further studied in future.

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Figure and figure legend

Fig. 1

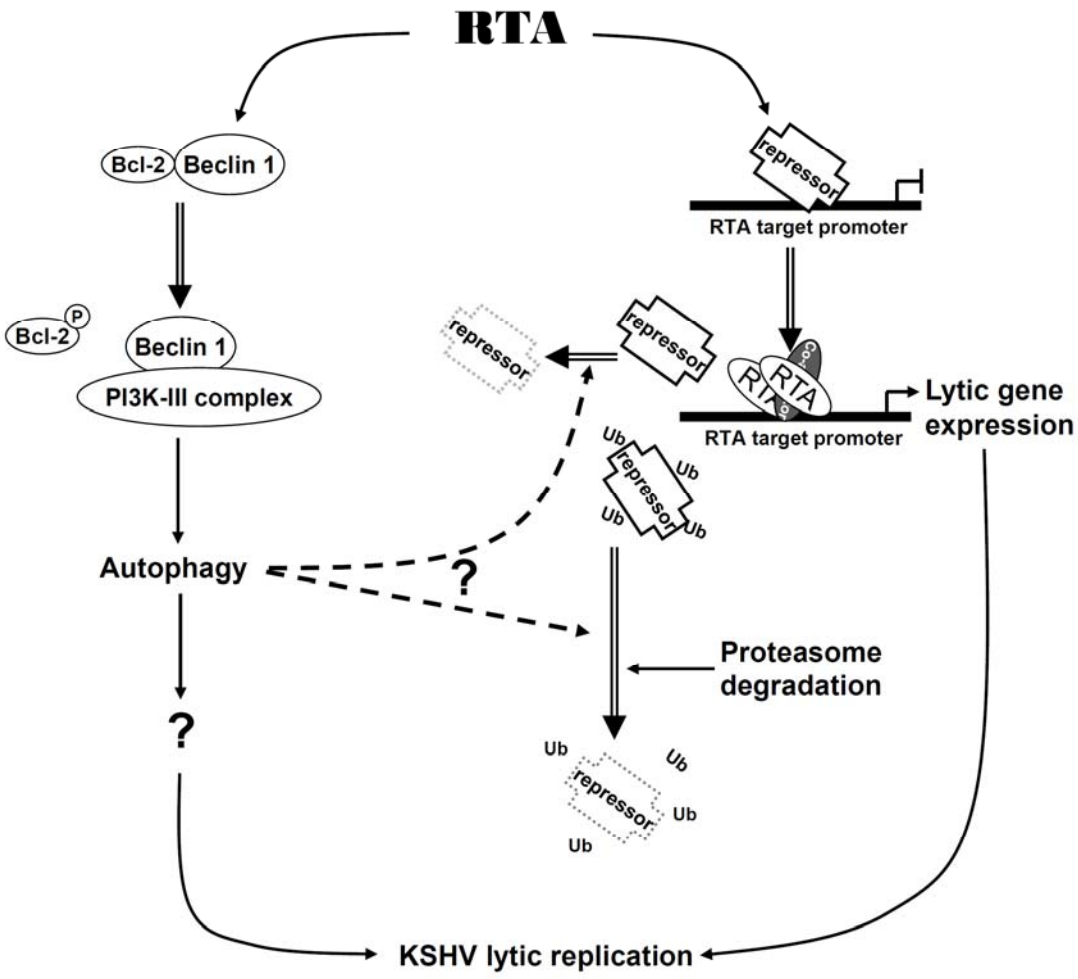

Fig. 1. Proposed mechanism utilized by RTA for KSHV lytic replication.

RTA exerts its E3-like ubiquitin ligase activity to promote the ubiquitination (Ub) of it repressors and allows the ubiquitinated repressors to undergo the 26S proteasome degradation pathway. Consequently, RTA is able to activate transactivation of lytic gene promoters and facilitates KSHV lytic replication (10, 11). Current study demonstrated that RTA induces the dissociation of Beclin 1 and Bcl-2 through JNK-dependent Bcl-2 phosphorylation and then stimulates the autophagic process. Additionally, autophagy positively regulates RTA-mediated transactivation of target gene promoters and facilitates KSHV lytic replication. The question marks indicate the potential mechanisms (repressor degradation) by which autophagy modulate RTA transactivation function and the unknown mechanism by which autophagy regulates KSHV lytic replication.