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The Epstein-Barr Virus LF2 Protein Inhibits Viral Replication[▽]

Michael A. Calderwood, Amy M. Holthaus, and Eric Johannsen*

The Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, Massachusetts 02115

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The switch from Epstein-Barr virus (EBV) latent infection to lytic replication is governed by two transcriptional regulators, Zta and Rta. We previously reported that the EBV protein encoded by the LF2 gene binds to Rta and can inhibit Rta activity in reporter gene assays. We now report that LF2 associates with Rta in the context of EBV-infected cells induced for lytic replication. LF2 inhibition of Rta occurs in both epithelial and B cells, and this downregulation is promoter specific: LF2 decreases Rta activation of the BALF2, BMLF1, and BMRF1 promoters by 60 to 90% but does not significantly decrease Rta activation of its own promoter (Rp). LF2 decreases Rta activation by at least two mechanisms: decreased DNA binding and interference with transcriptional activation by the Rta acidic activation domain. Coexpression of LF2 also specifically induces modification of Rta by the small ubiquitin-like modifiers SUMO2 and SUMO3. We further demonstrate that LF2 overexpression blocks lytic activation in EBV-infected cells induced with Rta or Zta. Our results demonstrate that LF2, a gene deleted from the EBV reference strain B95-8, encodes a potent inhibitor of EBV replication, and they suggest that future studies of EBV replication need to account for the potential effects of LF2 on Rta activity.

Epstein-Barr virus (EBV), the prototypical human gammaherpesvirus, exists in two states in infected cells. In latent infection, a limited subset of viral genes is expressed and infectious virions are not produced. During lytic infection, nearly all viral genes are transcribed, the genome is replicated by viral enzymes, and infectious virions are produced. EBV causes infectious mononucleosis in healthy individuals, B-cell lymphoproliferative disease in immunosuppressed individuals, and, rarely, B-cell lymphomas, Hodgkin lymphoma, and nasopharyngeal carcinoma in otherwise healthy persons (for a review, see reference 45). In these cancer cells, EBV infection is latent and EBV latency proteins activate critical growth and survival signaling pathways. Inhibition of virus replication is not efficacious in treating EBV-associated malignancies. Instead, activation of EBV replication is potentially therapeutic, because virus replication can directly kill EBV-infected tumor cells, sensitize them to nucleoside analogues, and stimulate immune-mediated killing via increased virus antigen expression in tumor cells (24, 25, 56).

EBV replication is regulated by two immediate-early genes, BZLF1 and BRLF1, encoding the transcriptional activators Z (Zta) and R (Rta) (45, 55, 65). Both Zta and Rta are essential for EBV replication; genomes from which either BZLF1 or BRLF1 is deleted are not competent for viral DNA replication or virion production (23). Classically, early genes that encode proteins required for DNA replication are upregulated first. After viral DNA replication occurs, late genes encoding structural proteins are expressed. EBV lytic genes vary in their responsiveness to Zta and Rta (55). Some lytic genes are activated primarily by either Rta or Zta (23, 51, 62). A third group is activated synergistically in response to both activators, while a fourth group is activated by Rta but repressed by Zta (16, 22, 26, 31, 40, 43, 50, 58). In most EBV-positive cell lines, expression of either Zta or Rta induces the expression of the other protein and disrupts latency (60, 68). The signals responsible for the induction of Rta and Zta in vivo are unknown, but in vitro their promoters are responsive to B-cell receptor cross-linking, phorbol esters, butyrate, and ionophores.

Zta is a bZIP DNA binding protein that activates promoters containing AP1 sites (TGASTCA) and related sequences called Zta-responsive elements. Rta is a 605-amino-acid (aa) acidic transactivator protein that is not homologous to any cell DNA binding proteins. Rta homologues are present in all gammaherpesviruses and exhibit the greatest homology in their N-terminal DNA binding domains (DBDs) (~40% similarity between EBV and Kaposi's sarcoma-associated herpesvirus [KSHV]). Rta activates many promoters through a direct mechanism by binding to Rta response elements (RREs), conforming to the consensus $GNCCN_9GGNG$ (14, 33–35). Rta activates other promoters that lack RREs through an indirect mechanism(s). These include its own promoter (Rp), which it activates through Sp1/Sp3 binding sites (61); the Zta promoter (Zp), via the ZII cyclic AMP response element (65); and the BALF5 DNA polymerase gene, through USF and E2F binding sites (26, 49). For Rp, it has been proposed that Rta is targeted indirectly to Sp1/Sp3 sites through an interaction with the Sp1-associated factor MCAF1 (9). This mechanism closely mirrors that of KSHV Rta, which activates promoters indirectly via an interaction with the RBP-Jκ DNA binding protein (8, 46, 47). Rta can also activate promoters indirectly through activation of mitogen-activated protein kinase and phosphatidylinositol-3 kinase, resulting in phosphorylation of ATF2 bound to the ZII element and activation of Zta expression (2, 17); however, the mechanism by which Rta activates these cytoplasmic signaling pathways is unknown. The observation that EBV Rta lacking a functional nuclear localization signal

* Corresponding author. Mailing address: Channing Laboratory, Brigham and Women's Hospital, Harvard Medical School, 181 Longwood Avenue, Boston, MA 02115-5804. Phone: (617) 525-4266. Fax: (617) 525-4251. E-mail: ejohannsen@partners.org.

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was still able to activate Rp and Zp suggests that the indirect Rta activation pathway does not always require the presence of Rta at the promoter (39).

Recently, we used a high-throughput yeast two-hybrid screen designed to identify all protein-protein interactions between EBV proteins (7). The only EBV protein found to interact with Rta in this assay was encoded by LF2 (also called Raji LF2 and BI′LF4), a gene of unknown function that is one of three genes (LF1, LF2, and LF3) deleted from the B95-8 EBV reference strain (37, 57, 59). EBV LF2 encodes a 429-aa protein with homologues in all the gammaherpesviruses sequenced to date. Early studies using P3HR1 superinfection of Raji cells to study EBV replication suggested that LF2 may be an immediate-early gene and could activate its own promoter but not EBV replication (4, 53). More-recent studies using Akata cells identified LF2 as an early gene (51, 67). However, the potential role of LF2 has received little attention, largely because the replication-competent B95-8 strain lacks this gene. LF2 shares weak homology with LF1, and both genes are thought to have arisen from duplication of the gene encoding the dUTPase protein (BLLF3 in EBV) (18). The region of homology between the LF2 protein and the dUTPase is a domain unique to herpesvirus dUTPases and is not thought be involved in catalysis. Instead, the domain appears to have arisen as a consequence of herpesvirus dUTPase evolving into a monomeric enzyme from the trimeric mammalian dUTPase. The entire LF2 open reading frame is well conserved (~46% between EBV and KSHV), and LF2 homologues are known to encode virion proteins in murine gammaherpesvirus 68 (MHV68) and KSHV (5, 70). In MHV68, deletion of the LF2 homologue (ORF11) resulted in a replication-competent genome, but the effect of this deletion mutant in vivo has not been reported (64). While LF2 is dispensable for viral replication in cell culture, its continued presence within the gammaherpesvirus family suggests that it plays an important, as yet undiscovered role in the viral life cycle.

We have previously reported (7) that LF2 had no direct effect on the activities of four lytic promoters (BMLF1, BMRF1, BALF2, and DL/DR) when expressed in the BJAB B-cell line but significantly impaired Rta activation of these promoters. LF2 was most potent at the BALF2 promoter, reducing Rta activation to near-baseline levels without decreasing the level of Rta expression. The discovery of an EBV-encoded inhibitor of Rta activation potentially has important implications for studies of lytic replication, which have generally treated B95-8 as equivalent to wild-type EBV. In this work, we sought to determine if LF2 can associate at physiologic levels of expression with Rta, to understand the mechanism by which LF2 acts to inhibit Rta activation, and to determine if LF2 can block EBV replication in infected cells.

MATERIALS AND METHODS

Cell culture. 293T is a human embryonic kidney cell line. 293T/B95-8 cell lines were produced by stable infection of 293T cells with an EBV B95-8 bacterial artificial chromosome (BAC) (13) and puromycin selection. All cell lines were cultured in Dulbecco's modified Eagle's medium or RPMI 1640 medium (both from Gibco) supplemented with L-glutamine, penicillin-streptomycin, and 10% Fetalplex (Gemini Bio-Products). The B95-8 Z-HT/R-HT cell line, containing hydroxytamoxifen-responsive estrogen receptor hormone binding domain (HT) fusions to Zta and Rta (Z-HT and R-HT, respectively), was constructed by transfecting B95-8 Z-HT cells (42) with pCEP-R-HT, followed by hygromycin selection. The P3HR1 Z-HT cell line was constructed by transfection of P3HR1 cells with linearized plasmid pcDNA3-Z-HT and G418 selection. P3HR1 Z-HT/R-HT cells were produced by stable integration of linearized plasmid pCEP-R-HT into the parental P3HR1 Z-HT cell line and hygromycin selection.

Plasmids. pGL3-Rp, pGL3-BALF2, pGL3-BMRF1, pGL3-BMLF1, pcDNA-Rta, pFLAG-LF2, p2xHA-LF2, pEXP-DsRed-HA-LF2, and pGal4-VP16 have been described previously (7, 15). pcDNA-Rta 1-550 was constructed by placing a stop codon at position 551 by QuikChange (Stratagene) using primer CCTC GTGGCCATTTGTAGCAGCTGACCACAACACTAGAG and its reverse complement. Lysine mutations K19R, K213R, and K517R were made in pcDNA-Rta by QuikChange using the following primers and their reverse complements: GCTAA CTCCTGAAATCCGGAAGCAGCTGGGCTCTC, GACTATGGTCTCGTCCC GTACGGGCCATCTGACGGACG, and GAAACCAGTCAGGCCGTGCGCG CCCTAAGGGAGATGGC. Plasmid pGal4-Rta 11-605 was constructed by cloning the genomic XmnI/NheI fragment containing all but the first 10 codons of the BRLF1 open reading frame into the SmaI/XbaI site of the pM plasmid (Clontech). C-terminal truncations were constructed by placing stop codons, using QuikChange, at the appropriate positions. N-terminal deletions were produced by inserting EcoRI sites at the appropriate sites by QuikChange and then excising the N-terminal sequence by collapsing out the sequence between the new EcoRI site and an in-frame EcoRI site immediately downstream of the Gal4 DBD. Plasmid pSG5-R-HT was constructed by cloning Rta aa 1 to 592 as an EcoRI/NsiI fragment into the pSG5-flag EcoRI/PstI sites, which eliminated the Flag tag. The HT domain was amplified from pBS-ERTM (48) by using primers CTAGAACTAGTGATATCAC GAAATGAAATGGGTGCTTC and CGAATTCACTAGTGCGGCCGCTCAG ATCGTGTTGGGGAAG and was cloned as an in-frame C-terminal fusion using the EcoRV/NotI sites. pCEP-R-HT was produced by replacing the E3C insert from pCEP-E3C (54) with the R-HT insert from pSG5-R-HT as an SfiI/NotI fragment. Plasmids pcDNA-3 HA-SUMO1, HA-SUMO2, and HA-SUMO3, encoding processed forms of hemagglutinin (HA)-tagged SUMO1, SUMO2, and SUMO3, were kindly provided by Ron Hay (University of St. Andrews, Scotland). In all QuikChange reactions, mutated inserts were recloned into the parent vector, and the resulting mutant plasmid was verified by DNA sequencing.

Antibodies. The following antibodies were used for Western blotting, immunofluorescence, and immunoprecipitations: a mouse monoclonal antibody against HA (HA-11; Covance), mouse monoclonal anti-Flag (M2; Sigma), rabbit polyclonal anti-Gal4 DBD (sc-577; Santa Cruz), a mouse monoclonal antibody to EBV Rta (8C12; Argene), a mouse monoclonal antibody against EBV Zta (AZ-69; Argene), mouse monoclonal anti-BMRF1 (EA-D-p52/50, R3; Chemicon), a mouse monoclonal antibody to EBV gp350 (72A1) (38), and a mouse monoclonal antibody to EBV sCP (BFRF3/VCA-p18, a kind gift from Jaap Middeldorp, VU University Medical Center, Amsterdam, The Netherlands). A polyclonal anti-LF2 rabbit serum was raised against glutathione *S*-transferase–LF2 (aa 1 to 429) (Pocono Rabbit Farms and Laboratory).

Western blot analysis. Total-cell lysates or immunoprecipitated proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, blotted onto a nitrocellulose membrane, and probed with appropriate antibodies. After extensive washing, horseradish peroxidase-conjugated secondary antibodies were applied, and the gel was developed with a chemiluminescence reagent (Perkin-Elmer) and visualized on a Kodak Image Station 4000R (Kodak Molecular Imaging Systems).

Immunofluorescence and FACS analysis. Induced cells were incubated with a mouse anti-gp350 antibody (72A1) (38) followed by an anti-mouse secondary antibody conjugated to Alexa Fluor 488 (Molecular Probes). Cells were analyzed with a fluorescence-activated cell sorter (FACS) (FACSCalibur; Becton Dickinson) or counterstained with Hoechst stain (Molecular Probes) and visualized by fluorescent microscopy (Zeiss Axiovert 200).

Immunoprecipitation. Transfected 293T or induced B95-8 Z-HT/R-HT or P3HR1 Z-HT/R-HT cells were lysed in NP-40 lysis buffer (1% Nonidet P40, 50 mM Tris-HCl [pH 7.4], 2 mM EDTA, 150 mM NaCl) and cleared by centrifugation at 13,000 × *g* for 10 min. Supernatants were incubated for 2 to 3 h at 4°C with appropriate antibody-conjugated agarose beads (anti-HA [F7] agarose [Santa Cruz]; anti-Flag M2 agarose Sigma) or with a primary antibody with protein G-agarose (Amersham Pharmacia). The beads were washed extensively with lysis buffer, and the proteins were eluted with SDS sample buffer.

Reporter assays. As much as 1.3 μg of total DNA was transfected with Effectene (Qiagen) into 293T cells. After 48 h, cells were lysed in reporter lysis buffer (Promega) and clarified by centrifugation. Luciferase (Luciferase assay system; Promega) and β-galactosidase (Galacto-Light; Tropix) levels were measured using an Optocomp I luminometer (MGM Instruments). Luciferase assays were corrected for transfection efficiency based on β-galactosidase activity.

SUMOylation assays. 293T cell monolayers at 60 to 80% confluence in 6-well plates were transfected with 1 μg pcDNA-Rta, with or without 1 μg pSG5-LF2,

and 1 μg of pcDNA3 HA-SUMO-1, -2, or -3 or the empty vector. The SUMOylation assay was performed as described elsewhere (6). Briefly, after 48 h, the cells were dislodged with ice-cold phosphate-buffered saline (PBS), cell pellets were resuspended in 100 μl of PBS, and cells were disrupted by addition of 100 μl of 2% SDS in Tris-buffered saline (pH 7.5). Cell lysates were then boiled for 10 min, followed by the addition of 5 volumes of 0.5% NP-40 in Tris-buffered saline with Complete protease inhibitor (Roche). Diluted lysates were sonicated for 30 s, precleared with protein G-agarose beads, and immunoprecipitated with anti-HA agarose.

EMSA. Probes were prepared from annealed oligonucleotides, including BMLF1 RRE (GATCGGCCCAGATGTCCCTCTATCATGGCGCAGACAT TCTC and GATCGAGAATGTCTGCGCCATGATAGAGGGACATCTGGG CC), Gal4-wt (GATCTCCGGATGACTGTCCTCCGGC and GATCGCCGGA GGACAGTCATCCGGA), and Gal4-mut (GATCTCC**t**GATGACTGTCCTC **a**GGC and GATCGCC**t**GAGGACAGTCATC**a**GGA) (lowercase indicates mutated residues). End labeling was carried out by Klenow fill-in using [α-^{32}P]dGTP. Electrophoretic mobility shift assays (EMSAs) were performed as described previously (41). Briefly, 2 μg of nuclear extract from transfected 293T cells was added to EMSA buffer [10 mM HEPES (pH 7.9), 5% glycerol, 0.5 mM EDTA, 1 mM dithiothreitol, 125 mM phenylmethylsulfonyl fluoride, 100 mg/ml bovine serum albumin, 40 mg/ml poly(dI-dC), 50 mM KCl] with 1 to 2 ng of probe, and the mixture was incubated for 15 min at room temperature. For supershift assays, a mouse monoclonal antibody against Rta (8C12; Argene) was preincubated with nuclear extract in reaction buffer on ice for 10 min. Samples were separated on a 5% polyacrylamide gel in 0.53 Tris-borate-EDTA (TBE), after which the gel was dried and visualized on a PhosphorImager (Molecular Dynamics).

Gardella gel electrophoresis and Southern blotting. 293T/B95-8 cells were transiently transfected with 1 μg of pcDNA-Rta, pSG5-Zta, or p2xHA-LF2 individually and in combination. Forty-eight hours posttransfection, the cells were washed in PBS and harvested. Linear DNA molecules were detected using the Gardella gel electrophoresis protocol, as described previously (27). Briefly, horizontal agarose gels were prepared in two steps. Initially, a 0.75% agarose gel in TBE buffer was prepared. Once the gel was solidified, ~5 cm of the gel was removed above the wells and replaced with 0.8% agarose containing 2% SDS and 1 mg/ml pronase (Sigma). Cell pellets were resuspended in sample buffer (15% Ficoll, 0.01% bromophenol blue, 1× TBE) and separated at 4°C for 4 to 5 h at 15 V and then for 18 h at 120 V. After electrophoresis, the gel was depurinated for 20 min (0.2 M HCl), denatured for 1 h (1.5 M NaCl–0.5 M NaOH), neutralized (with 1 M Tris [pH 8.0] and 1.5 M NaCl for 1 h), transferred to a GeneScreen nylon membrane (Perkin-Elmer), and probed with the BamHI W fragment of the EBV genome.

RESULTS

Association of Rta and LF2. EBV LF2 protein was previously identified as an Rta binding protein in a comprehensive yeast two-hybrid screen testing all potential EBV-EBV protein interactions (7). To determine the extent of Rta and LF2 association in mammalian cells, Flag-Rta and HA-LF2 were coexpressed in human embryonic kidney 293T cells. Immunoprecipitation with Flag agarose retrieved ~10% of Flag-tagged Rta and about 2% of the coexpressed HA-LF2 (Fig. 1a). In the reciprocal experiment, HA agarose precipitated >10% of the HA-LF2 and about 1% of the coexpressed Flag-Rta. Neither protein was precipitated with protein G alone. These results are consistent with a strong, stable association between LF2 and Rta in the 293T epithelial-cell line.

In order to map the LF2 binding domain within Rta, a series of Rta C-terminal truncations were tested for their abilities to associate with LF2. Rta deletion mutants (aa 11 to 605, 11 to 479, and 11 to 456) fused to the Gal4 DBD were transfected into 293T cells along with HA-tagged LF2. HA immunoprecipitations were performed using HA agarose from the cell lysates 48 h posttransfection, and immunoprecipitates were blotted for the Gal4 DBD. Rta 11-605 interacted strongly with LF2 (Fig. 1b). Rta 11-479 demonstrated a weak but consistent

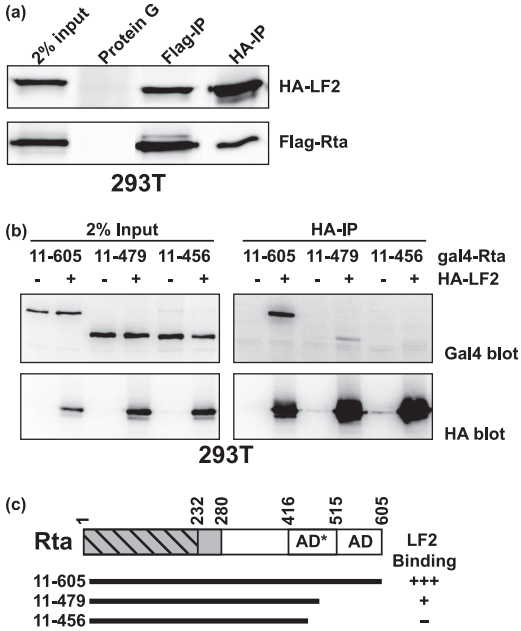

FIG. 1. LF2 associates with Rta in epithelial cells. (a) 293T cells were cotransfected with HA-LF2 and Flag-Rta, and immunoprecipitations (IP) were performed with either Flag or HA agarose. Samples were separated by SDS-polyacrylamide gel electrophoresis and probed by Western blotting with anti-HA and anti-Flag antibodies (as indicated). As a negative control, lysates were immunoprecipitated with protein G agarose alone. Two percent of the input lysate is shown for comparison. (b) 293T cells were transfected with Gal4-Rta fusion proteins (aa 11 to 605, 11 to 479, and 11 to 456) in the presence or absence of HA-LF2. Proteins immunoprecipitated by anti-HA agarose from cell lysates were identified by Western blotting (right panels) with anti-Gal4 DBD and anti-HA antibodies. In each case, 2% of the input lysate is shown for comparison (left panels). (c) Schematic of Rta demonstrating overlapping N-terminal dimerization (aa 1 to 232) and DNA binding (aa 1 to 280) domains, the essential acidic activation domain (AD) (aa 515 to 605), and an accessory activation domain (AD*) (aa 416 to 515) required for full activity. Results from LF2 coimmunoprecipitations are summarized as follows: −, no binding; +, weak binding; +++, strong binding.

interaction, whereas Rta 11-456 did not bind to LF2. This finding suggests that the C-terminal 149 aa of the Rta activation domain are required for interaction with LF2.

To determine if LF2 associates significantly with Rta during EBV replication, endogenous LF2 was immunoprecipitated from P3HR1 cells induced for replication. For these experiments, Z-HT and R-HT were stably expressed in P3HR1 cells. Upon the addition of 4-hydroxytamoxifen (4HT) to the medium, ~25% of P3HR1 Z-HT/R-HT cells become positive for gp350 immunofluorescence staining (Fig. 2a and b). Polyclonal anti-LF2 rabbit serum efficiently coimmunoprecipitated both stably expressed R-HT and endogenously expressed Rta (Fig. 2c, left), whereas preimmune serum did not. Control experiments using the LF2-negative B95-8 Z-HT/R-HT cell line demonstrated that the polyclonal anti-LF2 serum does not immunoprecipitate Rta in the absence of LF2 (Fig. 2c, right). In control experiments, the anti-LF2 serum efficiently immunoprecipitated DsRed-HA-tagged LF2 expressed in 293T cells, whereas preimmune serum did not (Fig. 2d), confirming the specificity of the polyclonal anti-LF2 serum. These experi-

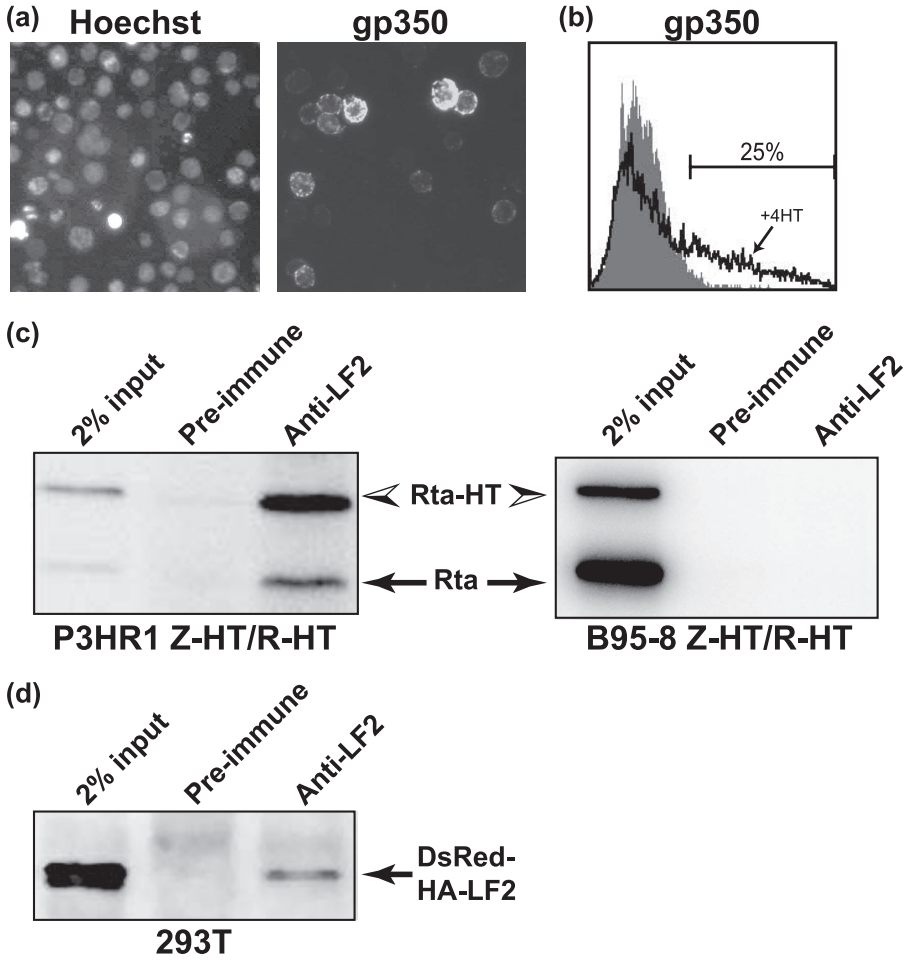

FIG. 2. LF2 associates with Rta in B cells. (a) Immunofluorescence staining for gp350 surface expression (right) and Hoechst DNA counterstaining (left) of 4HT-induced P3HR1 Z-HT/R-HT cells. (b) FACS profile of gp350 surface staining of uninduced (gray) and 4HT-induced (white) P3HR1 Z-HT/R-HT cells. (c) Rta Western blots of immunoprecipitates obtained with a polyclonal rabbit anti-LF2 antibody or preimmune serum from P3HR1 Z-HT/R-HT (left) or B95-8 Z-HT/R-HT (right) cell lysates. Cell lysates were prepared 48 h after induction of replication by addition of 4HT, and 2% of the input lysate is shown for comparison. (d) HA Western blot of immunoprecipitates obtained with a polyclonal rabbit anti-LF2 serum or preimmune serum from 293T cells expressing DsRed-HA-tagged LF2 protein.

ments demonstrate that endogenously expressed LF2 efficiently associates with Rta in EBV-infected cells induced for viral replication.

LF2 inhibits Rta transactivation of lytic promoters in epithelial cells. In the BJAB B-cell lymphoma line, LF2 impaired the ability of Rta to activate early lytic promoters, including BALF2, BMLF1, and BMRF1, but did not reduce Rta protein levels (7). Because epithelial cells are an important site of EBV replication in vivo, we tested whether LF2 could also inhibit Rta activity in 293T cells. Using luciferase reporter gene assays, we found that Rta activated the Rp, BALF2, BMRF1, and BMLF1 promoters 7- to 45-fold (Fig. 3, third bar in each graph). Expression of LF2 alone had no significant effect on any of the promoters tested (Fig. 3, second bar in each graph). LF2 cotransfection reduced Rta-induced activation in three out of four promoters studied—BALF2, BMRF1, and BMLF1—by 85, 80, and 66%, respectively (Fig. 3, fourth bar in graphs i to iii). Levels of Rta in the presence of LF2 were equal to or higher than those in the absence of LF2, suggesting that LF2 does not act by reducing Rta protein levels but rather by inhibiting the activity of Rta. By contrast, Rta activation of its own promoter (Rp) was not significantly reduced by LF2 coexpression (Fig. 3, graph iv). These data demonstrate that LF2 can inhibit Rta activation of some lytic promoters in an epithelial-cell background. The LF2-regulated promoters (BALF2, BMRF1, and BMLF1) all contain RREs, whereas Rp does not. The resistance of Rp to LF2 downmodulation suggests that LF2 may inhibit lytic promoter activation by blocking Rta binding to $GNCCN_9GGNG$ sites.

LF2 inhibits Rta DNA binding. To determine if LF2 can disrupt Rta binding to cognate DNA, EMSAs were conducted using Rta aa 1 to 550, a construct that exhibits robust binding to $GNCCN_9GGNG$ sites (14). Nuclear extracts were prepared from 293T cells transfected with Rta aa 1 to 550 alone or with increasing amounts of LF2. In addition to cell binding activities, a single Rta binding activity was observed (Fig. 4, compare the top band in lane 4 with lane 2 or 3). The specificity of this activity was confirmed by supershifting with anti-Rta antibody (Fig. 4, lane 9, SS). At the lowest level of LF2 coexpression, Rta binding activity was decreased by more than 90% (Fig. 4,

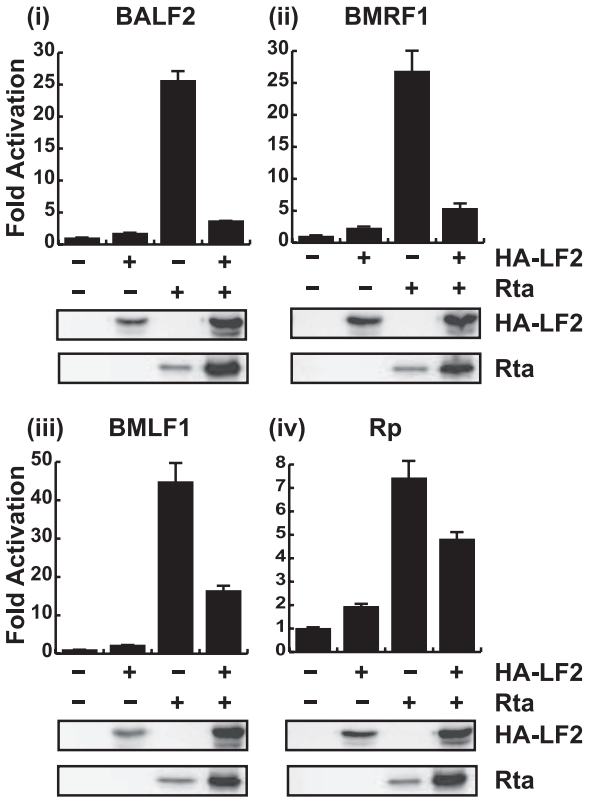

FIG. 3. LF2 inhibits Rta activation of the BALF2, BMLF1, and BMRF1 lytic promoters. Reporter assay results from 293T cells transfected with luciferase reporter constructs from the indicated promoters with or without Rta and LF2 are shown. Luciferase activities are shown as *n*-fold activation compared to that with the reporter alone and were normalized for transfection efficiency as determined by β-galactosidase activity. Data are averages for six transfections from three independent experiments. Error bars indicate the standard errors of the means. Western blots of the cell lysates with anti-HA and anti-Rta antibodies are shown below each graph to demonstrate LF2 and Rta protein expression levels.

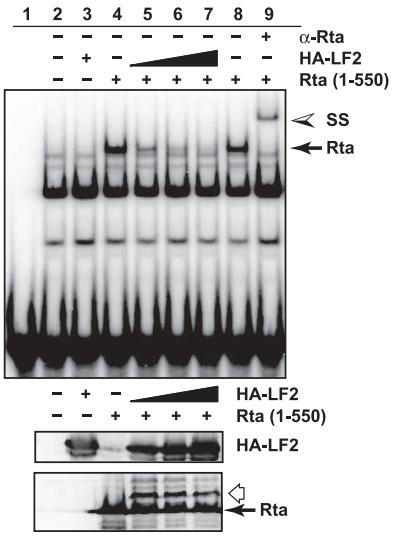

FIG. 4. LF2 inhibits Rta binding to response elements. Shown are the results of an EMSA demonstrating the effect of cotransfected LF2 on Rta DNA binding. Rta binding activity is indicated (arrow) and confirmed by supershifting (SS). Nuclear extracts for the EMSA were prepared from 293T cells transiently transfected with plasmids expressing Rta aa 1 to 550 and increasing amounts of HA-LF2 proteins as indicated. Western blots of nuclear extracts for Rta and LF2 protein levels are shown for each lane below the EMSA results. A higher-molecular-weight form of Rta (open arrow) is observed in the presence of cotransfected LF2.

compare lanes 4 and 5). With increasing amounts of LF2, Rta binding activity became undetectable (Fig. 4, lanes 5 to 7). In separate EMSA experiments, the Rta gel shift was competed by a 100-fold excess of cold probe but not of a mutant probe (mutated from GTCCN$_9$GGCG to tgaaN$_9$GGCG) (lowercase indicates mutated residues) lacking an Rta binding site (data not shown). Western blotting of nuclear extracts demonstrated that LF2 coexpression did not significantly affect Rta protein levels but did correlate with the appearance of higher-molecular-weight forms of Rta. Western blotting also confirmed the presence of LF2 protein in our nuclear extracts. Thus, LF2 appears to inhibit Rta binding to RREs. Based on our results, this does not appear to be due to competition between LF2 and Rta for RRE binding, because no LF2-specific binding activities were observed (Fig. 4, compare lanes 2 and 3). Neither does LF2 appear to inhibit Rta binding by relocalizing Rta (e.g., by retention in the cytoplasm), since Rta was still present in the nuclear extracts. Rather, the presence of LF2 impairs Rta's ability to bind DNA.

LF2, when bound to Rta, may block DNA binding through steric hindrance, or possibly through an allosteric effect on the DBD. Alternatively, LF2-induced modification of Rta may be responsible for the observed change in Rta binding activity. We speculated that the higher-molecular-weight forms of Rta induced by LF2 might represent Rta modification by the small ubiquitin-like modifier (SUMO) proteins. This is based in part on the propensity of SUMO modification to inhibit transcription factor activity (29, 30, 36, 66). Moreover, the fact that Rta levels were normal or often increased in the presence of LF2 is consistent with the known ability of SUMO modification to stabilize proteins through inhibition of ubiquitin-mediated protein degradation (21, 28).

LF2 promotes the accumulation and SUMOylation of Rta. To determine if LF2 induces SUMO modification of Rta, 293T cells were transfected with Rta and HA-tagged SUMO1, SUMO2, or SUMO3, with or without Flag-tagged LF2. A denaturing immunoprecipitation procedure was used to inactivate SUMO proteases and to avoid the coprecipitation of SUMO binding proteins. When LF2 was cotransfected with Rta, Rta protein levels increased and a higher-molecular-weight band appeared. This Rta species coimmunoprecipitated with either HA-SUMO2 or HA-SUMO3, consistent with covalent modification of Rta by SUMO2 or SUMO3 (SUMO2/3) under these conditions (Fig. 5, upper right panel). The higher-molecular-weight Rta species did not immunoprecipitate with HA-SUMO1, suggesting that Rta is not significantly modified by SUMO1 under these conditions. Rather, the higher-molecular-weight form of Rta seen in the input lysate is due to the modification of Rta by endogenous, non-HA-tagged SUMO2/3. This interpretation is supported by the presence of a nonprecipitating higher-molecular-weight Rta species in the vector control input lysate (Fig. 5, upper right panel, second lane). By contrast,

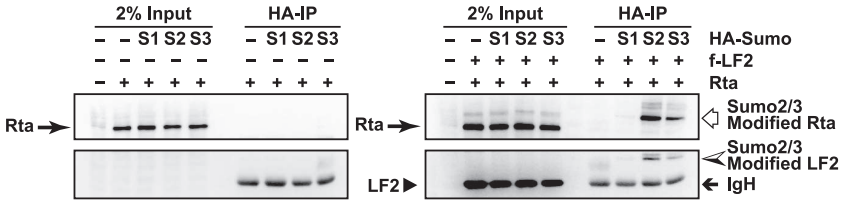

FIG. 5. SUMO2/3 modification of Rta is promoted by LF2. HA immunoprecipitation (IP) from 239T cells transfected with full-length Rta with or without Flag-LF2 (f-LF2) and HA-SUMO1 (S1), HA-SUMO2 (S2), or HA-SUMO3 (S3) was conducted under partially denaturing conditions. Western blotting with anti-Rta antibodies (upper panels) demonstrated the presence of SUMO2- and SUMO3-modified Rta in the presence (right) but not in the absence (left) of cotransfected LF2. Input lysates (2%) for IPs are shown in the left half of each panel. Western blotting using anti-Flag antibodies revealed bands corresponding to HA-SUMO2- and HA-SUMO3-modified LF2 (lower right panel). In the anti-Flag blots, a background band corresponding to heavy-chain immunoglobulin (IgH) from IPs comigrates with unmodified LF2.

SUMO-modified Rta could not be detected in the absence of cotransfected LF2 (Fig. 5, upper left panel). Western blotting for LF2 revealed that it, too, was modified by SUMO2 and SUMO3 but not by SUMO1 (Fig. 5, lower right panel). Thus, LF2 coexpression specifically induces Rta modification by SUMO2/3.

LF2 inhibits activation by Rta fused to a heterologous DBD. Because LF2 induces Rta SUMOylation, its repressive effects may extend beyond inhibition of DNA binding. In order to test this possibility, we cloned Rta aa 11 to 605 downstream of the Gal4 DBD (Gal4-Rta). Gal4-Rta activated a 4× Gal4 reporter 3,000-fold (Fig. 6a, left graph, third bar). Interestingly, LF2 coexpression strongly repressed Gal4-Rta activation (Fig. 6a, left graph, fourth bar) but, as occurred with Rta, did not appear to reduce Gal4-Rta protein levels. As a control, the effect of LF2 on the Gal4 DBD fused to the VP16 activation domain (Gal4-VP16) was tested. Gal4-VP16 activated the same reporter approximately 900-fold and was not affected by LF2 coexpression (Fig. 6a, right graph). To confirm that this effect was not dependent on the Rta DBD, the Gal4-Rta_233-605 and Gal4-Rta_416-605 constructs, containing the indicated Rta residues cloned downstream of the Gal4 DBD, were tested. These constructs activated the Gal4 reporter 30,000- to 65,000-fold (Fig. 6b, first and third bars) and were markedly repressed by LF2 coexpression (Fig. 6b, second and fourth bars). To determine whether the Gal4-Rta fusions were able to bind DNA in the presence of LF2, EMSAs were performed using a probe containing a single Gal4 binding site. As with EMSAs of the BMLF1 RRE (Fig. 4), the Rta C-terminal 55 aa were deleted because of their potential inhibitory effect in gel shift assays (14). Nuclear extracts were prepared from 293T cells transfected with Gal4-Rta_233-550 or Gal4-Rta_416-550 or with the Gal4 DBD vector alone, in the presence or absence of LF2. Gal4 binding activities were observed for Gal4-Rta_233-550 and Gal4-Rta_416-550 (Fig. 6c, lanes 3 and 5) and were confirmed by supershifting with an anti-Rta antibody (data not shown). Coexpression of LF2 did not impair the DNA binding of Gal4-Rta (Fig. 6c, compare lanes 3 and 4 and lanes 5 and 6). No supershifting of the Gal4-Rta complex was observed in the presence of LF2, as might be expected if Rta-LF2 complexes are stably associated on DNA. Additionally, LF2 had no effect on the ability of the Gal4 DBD alone to bind by EMSA (Fig. 6c, compare lanes 7 and 8). Western blotting of nuclear extracts confirmed the expression of the transfected Gal4-Rta fusion proteins and demonstrated no effect of LF2 coexpression on Gal4-Rta expression levels (Fig. 6c, bottom panels). Thus, LF2 appears to inhibit activation by Gal4-Rta fusion proteins without altering Gal4-Rta expression or DNA binding. The Rta activation domain, even when targeted to a promoter by a heterologous DBD, appears to be impaired for transcriptional activation by the presence of LF2.

LF2 inhibits Rta-induced EBV replication. The ability of LF2 to repress Rta activation of lytic promoters in reporter assays suggested that LF2 may be capable of blocking EBV replication. To test this directly, the abilities of Rta and Zta to induce viral lytic replication were assessed in the presence or absence of LF2 in 293T cells infected with EBV B95-8. In the absence of Rta and Zta expression, these cells are latently infected by EBV, as judged by the absence of gp350 staining and the lack of DNA replication, detected as linear DNA by a Gardella gel assay (Fig. 7a, lane 1). Rta expression induced DNA replication and gp350 surface expression (Fig. 7a, lane 2). When coexpressed with LF2, Rta failed to induce DNA replication or gp350 expression (Fig. 7a, lane 4). LF2 coexpression also abrogated the ability of Zta to induce DNA replication or gp350 expression (Fig. 7a, compare lanes 3 and 5). This is presumably due to LF2 inhibition of endogenous Rta activity, because both Zta and Rta are essential for DNA replication, and Zta cannot induce gp350 expression in the absence of Rta (23). Consistent with this interpretation, the amount of Rta expressed from the endogenous genome in response to Zta was not significantly decreased by LF2 coexpression (Fig. 7b, Rta blot, compare lanes 3 and 5). Cotransfection of Zta and Rta was synergistic for DNA replication and gp350 surface expression (Fig. 7a, lane 6). Coexpression of Zta, Rta, and LF2 resulted in a low level of DNA replication, several orders of magnitude less than that seen in the absence of LF2. gp350 staining was also significantly decreased, to levels approximating those induced by Zta expression alone.

To examine the effects of LF2 on the expression of other EBV replication proteins, Western blotting of transfected 293T/B95-8 cell lysates was performed. The DNA polymerase accessory protein encoded by BMRF1 (also called EA-D) was expressed in response to Rta only in the absence of LF2 (Fig. 7a, compare lanes 2 and 4). Zta and Rta coexpression resulted in synergistic BMRF1 upregulation that was only partly blocked by LF2 (Fig. 7a, compare lanes 6 and 7). Zta activation of BMRF1 was also partially blocked by LF2 (Fig. 7a, compare lanes 3 and 5). The smallest capsid protein (sCP, also called VCA-p18), encoded by BFRF3, was weakly induced by Rta or Zta expression and strongly induced by Rta and Zta coexpres-

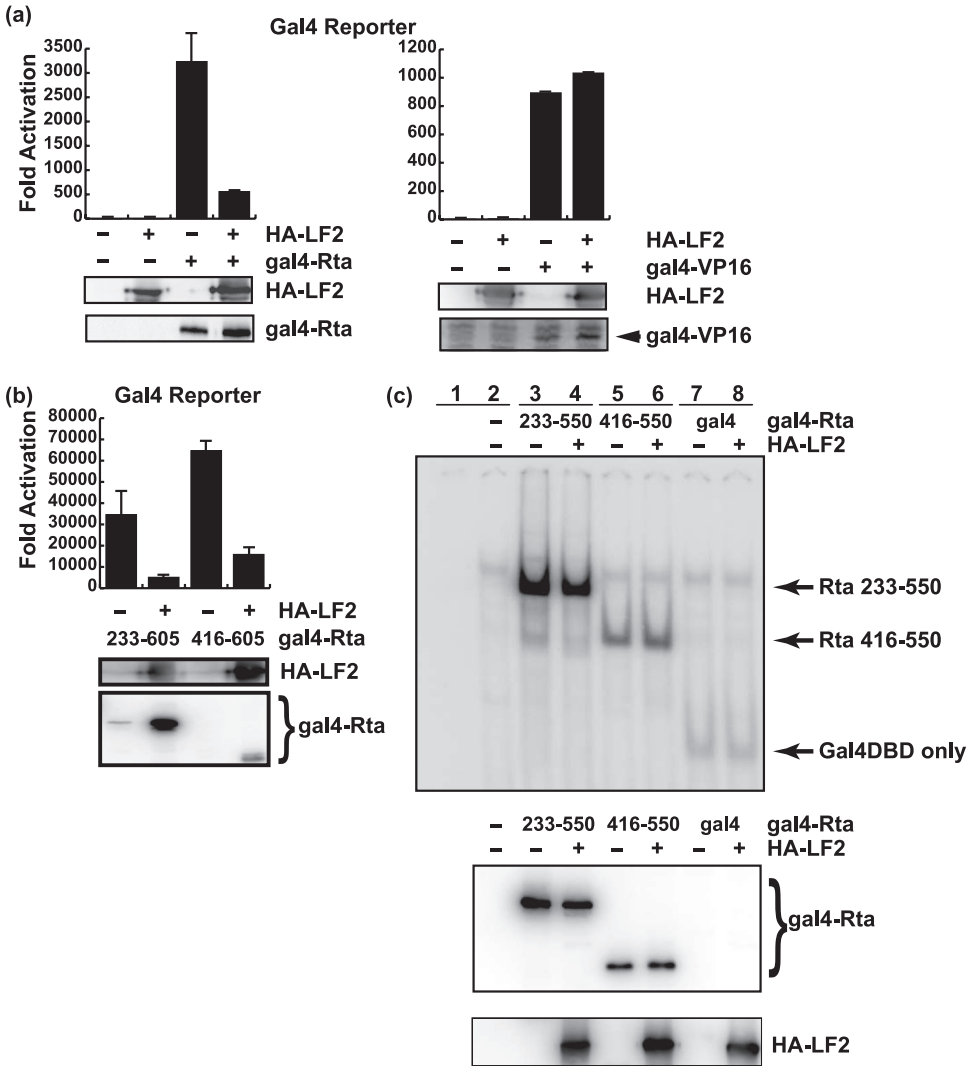

FIG. 6. LF2 represses Gal4-Rta activation but does not affect Gal4-Rta DNA binding. (a) Reporter assay results from 293T cells transfected with a multimerized Gal4 luciferase reporter construct and plasmids expressing Gal4-Rta (aa 11 to 605) or Gal4-VP16 in the presence or absence of HA-LF2. Luciferase activities are shown as *n*-fold activation over that with the reporter alone and were normalized for transfection efficiency as determined by β-galactosidase activity. Western blots of the cell lysates with anti-HA and anti-Rta or anti-Gal4 DBD antibodies are shown below each graph to demonstrate protein expression levels. (b) Reporter assay results demonstrating the effects of cotransfected HA-LF2 on Gal4-Rta_233-605 and Gal4-Rta_416-605 activation. (c) EMSA demonstrating the effects of cotransfected LF2 on DNA binding by Gal4-Rta fusions. The DNA binding activities of Gal4-Rta_233-550, Gal4-Rta_416-550, and the Gal4 DBD are indicated (arrows). Nuclear extracts for EMSA were prepared from 293T cells transiently transfected with plasmids expressing Gal4-Rta fusions or the Gal4 DBD vector alone in the presence or absence of HA-LF2 proteins, as indicated. For each lane, Western blots of nuclear extracts for Rta and LF2 protein levels are shown.

sion. Coexpression of LF2 reduced sCP induction by either Rta or Zta to undetectable levels. Strikingly, LF2 also reduced sCP induction by Rta and Zta coexpression to nearly undetectable levels (Fig. 7a, compare lanes 6 and 7).

Western blotting of 293T/B95-8 cell lysates was also used to confirm the expression of transfected LF2, Rta, and Zta (Fig. 7b). This also demonstrated that Rta induced endogenous Zta expression (Fig. 7b, Zta blot, lane 2) and Zta induced endogenous Rta expression (Fig. 7B, Rta blot, lane 3). Cotransfection of LF2 did not significantly affect Zta protein levels but resulted in the appearance of higher-molecular-weight forms of Rta, presumably due to SUMO2/3 modification. In summary, LF2, when expressed in B95-8-infected 293T cells, blocks EBV replication induced by Zta or Rta. These effects are partially surmountable by strong Zta and Rta coexpression. Overall, the effects of LF2 expression on DNA replication and EBV protein expression are strikingly similar to the phenotype of the Rta knockout virus (23).

Mutation of Rta lysines 19, 213, and 517 does not affect LF2 repression. Previous studies have reported that SUMO1 modification of Rta occurs at lysines 19, 213, and 517 (10). We speculated that LF2 might promote SUMO2/3 modification of these residues. Three lysine-to-arginine mutations were introduced into Rta to give Rta K19R K213R K517R (referred to below as Rta_3K-R). Surprisingly, activation by Rta_3K-R of the BALF2, BMRF1, and BMLF1 promoters was not impaired

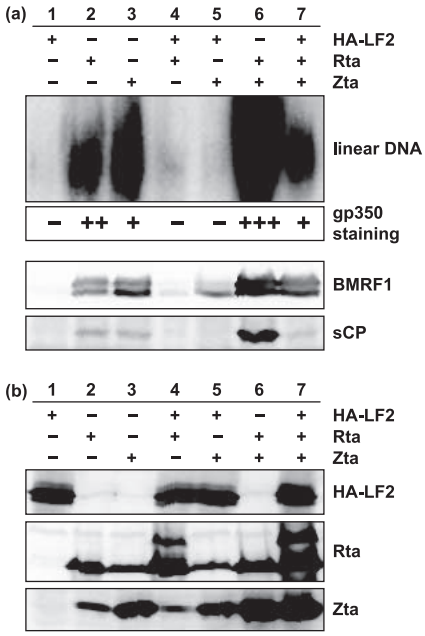

FIG. 7. LF2 inhibits the induction of EBV replication by Rta or Zta. (a) Viral DNA replication and protein expression in 293T/B95-8 cells 48 h after transfection with Rta, Zta, and/or LF2 as indicated. (Top panel) Gardella gel assay showing replicated EBV DNA detected by Southern blotting with the BamHI W fragment of the EBV genome. (Second panel) Immunofluorescence staining for gp350 using the primary antibody 72A1 was measured by FACS analysis, and the percentages of positive cells are indicated as follows: −, <0.5%; +, 0.5 to 1%; ++, 1.1 to 4.9%; +++, ≥5%. (Third and bottom panels) Western blots showing BMRF1 and sCP protein levels, respectively, from the same lysates prepared 48 h after transfection. (b) Western blot for LF2, Rta, and Zta protein levels in transfected 293T/B95-8 cells.

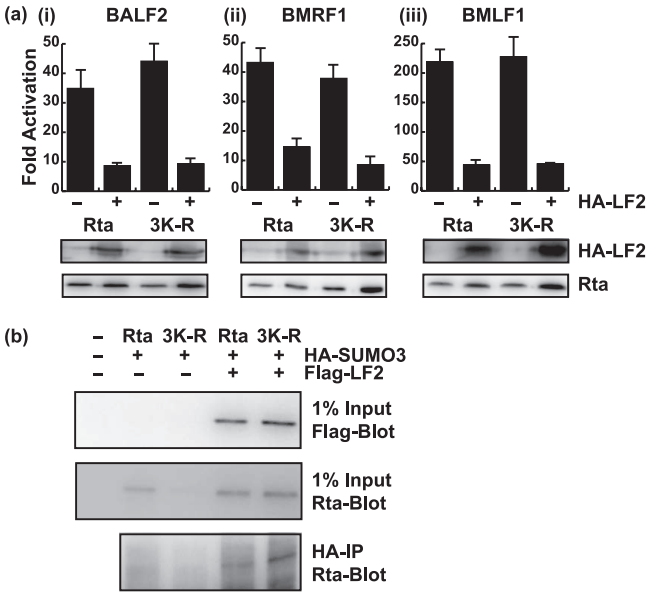

FIG. 8. Rta_3K-R is equivalent to wild-type Rta in lytic promoter activation, LF2 repression, and SUMO3 modification. (a) Reporter assay results from 293T cells transfected with luciferase reporter constructs from the indicated promoters with Rta or the Rta K19R K213R K517R triple point mutant (Rta_3K-R) in the presence or absence of HA-LF2. Luciferase activities are shown as *n*-fold activation compared to that with the reporter alone and were normalized for transfection efficiency as determined by β-galactosidase activity. Data are averages for six transfections from three independent experiments. Western blots of the cell lysates with anti-HA and anti-Rta antibodies are shown below each graph to demonstrate LF2 and Rta protein expression levels. (b) HA immunoprecipitation from 239T cells transfected with wild-type Rta or Rta_3K-R and HA-SUMO3 in the presence or absence of Flag-LF2 was conducted under partially denaturing conditions. Input lysates (1%) Western blotted for Flag (top) and Rta (center) are shown. A Western blot with anti Rta antibodies (bottom) demonstrated the presence of HA-SUMO3-modified wild-type Rta and Rta_3K-R in the presence (right lanes), but not in the absence (left lanes), of LF2.

(Fig. 8a); impairment had been reported for the triple-alanine mutant Rta K19A K213A K517A. Moreover, LF2 repression of Rta_3K-R activation of each promoter was similar to that seen for Rta. As with Rta, Western blotting demonstrated that Rta_3K-R levels were increased by LF2 cotransfection. HA-SUMO immunoprecipitation experiments revealed that Rta_3K-R was SUMO3 modified in the presence of LF2 at levels equivalent to or greater than that of wild-type Rta (Fig. 8b). Thus, lysines 19, 213, and 517 do not appear to be required for LF2-mediated repression or SUMO3 modification. Furthermore, our results are in apparent conflict with previous reports that SUMO modification at these lysines is essential for Rta-mediated activation.

DISCUSSION

The data presented here support a central role for LF2 in EBV replication as a modulator of Rta activity. In epithelial and B cells, LF2 associates with and potently inhibits Rta activation of multiple lytic promoters without decreasing Rta protein levels. This association can be demonstrated in EBV-positive B cells induced for lytic replication expressing endogenous LF2. Furthermore, in EBV-positive epithelial cells, LF2 expression can block the induction of EBV replication following transfection of either Rta or Zta. Thus, LF2, a protein deleted from the B95-8 reference genome, can interact with Rta at physiologic levels of expression and has the ability to dramatically alter EBV replication. Rta transactivates viral promoters both directly and indirectly, and RREs have been identified in numerous Rta-responsive promoters. Our results suggest that a major mechanism by which LF2 exerts its repressive effects is inhibition of the direct pathway by blocking Rta binding to RREs. Consistent with this mechanism, LF2 was unable to inhibit Rta activation of two promoters lacking RREs: Rp in reporter assays and Zp in EBV-infected epithelial cells. Interestingly, when Rta was targeted to DNA through a heterologous DBD fusion, it was still inhibited by LF2. This suggests that LF2 also inhibits the Rta activation domain. Our mapping experiments demonstrated that LF2 requires the Rta C terminus for efficient binding; thus, LF2 is positioned to regulate the Rta activation domain. Because LF2 did not supershift Gal4-Rta complexes, a model where LF2 masks the Rta activation domain seems less likely than a model where LF2 modifies (e.g., by SUMOylation [see below]) the Rta activation domain. Taken together, these data suggest that LF2 can inhibit Rta when it is directly or indirectly targeted to a promoter. The ability of Rta to activate Rp and Zp in the

presence of LF2 may be due to activation of cytoplasmic signaling pathways that may be unaffected by these inhibitory mechanisms (2, 17).

Another significant finding presented here is that LF2 induces covalent modification of Rta by SUMO2 and SUMO3. SUMO modification of target lysines on proteins parallels the ubiquitin pathway and consists of the sequential action of E1, E2, and E3 enzymes. However, unlike ubiquitylation, SUMO modification does not target proteins for degradation but can affect protein localization, stability, activity, and binding partners (28). In general, SUMO modification of a transcription factor is repressive, due in part to the presence of SUMO-interacting motifs on transcriptional repressor proteins such as Daxx (44). Thus, SUMO2/3 modification of Rta could be a mechanism by which LF2 exerts its repressive effects. This model is in apparent conflict with prior reports that SUMOylation of Rta is required for activation (10). Those studies characterized SUMO1 modification, and one possibility is that SUMO2 and SUMO3, which are ~95% homologous with each other but share only ~50% homology with SUMO1, exert opposite effects on Rta activity. Indeed, this has been reported for the MBD1 transcription factor, which is repressed by SUMO2/3 modification and derepressed by SUMO1 modification (52). Alternatively, different lysine residues in Rta could be involved in LF2-mediated SUMO2/3 modification. For SUMO1, three putative target lysines in Rta (lysines 19, 213, and 517) were identified. Mutation of all three residues was required to eliminate SUMO1 modification completely. Interestingly, of the single-lysine mutants, only the K213A mutant was negative for transcriptional activation, whereas Rta K19A and Rta K517A approximated wild-type transcriptional-activation levels. Thus, the degree of SUMO1 modification did not correlate with transcriptional activation. Our results demonstrate that SUMO modification of these residues is not required for Rta activation or LF2-mediated repression. Moreover, the K213A mutation has subsequently been reported to disrupt Rta DNA binding (9). We have found that another lysine mutation (K156A) within the DBD renders Rta defective for activation and DNA binding (unpublished data). A similar point mutant has been described for KSHV Rta (11). Thus, loss of the positive charge within the Rta DBD, rather than loss of SUMO1 modification, may explain the K213A phenotype.

An important outstanding question is which Rta lysines are modified by SUMO2/3. Identification of these lysines is an important step in addressing the broader question of whether Rta SUMOylation is required for LF2-mediated repression. This is certainly plausible, since SUMOylation of Zta inhibits its activation of lytic promoters (1, 3). If SUMO2/3 modification is found to play a central role in LF2-mediated repression, this raises the possibility that LF2 may be functioning as an E3 SUMO ligase. LF2 has no sequence homology to known E3 ligases and could represent a novel E3 ligase family. Alternatively, LF2 may recruit an E3 ligase, such as PIAS proteins or HDAC4, which is reported to be a ligase specific for SUMO2/3 modification (32, 69).

Another important question is why gammaherpesviruses would encode a protein that inhibits their replication. One possibility is that LF2 permits the expression of a restricted subset of genes without committing the cell to viral replication. Many replication genes could confer a survival advantage (e.g., the v-bcl2 protein encoded by BHRF1) on the cell in contexts other than replication. Alternatively, LF2 accumulation during replication may allow the cell to exit "lytic" infection prior to cell death. These possibilities are not mutually exclusive. Studies using Akata cells have shown that LMP1 is upregulated during replication in response to Rta (12, 51, 67). LF2 could selectively repress genes such as BALF2, blocking further DNA replication, while permitting Rta-mediated LMP1 upregulation. Although the Rta-responsive elements of the LMP1 promoter have not been fully delineated, the multiple Sp1 binding sites within suggest that it, like Rp, may be resistant to LF2-mediated repression (63).

Implicit in these examples is the suggestion that LF2 expression or activity must be regulated in order to allow EBV replication to occur. Whether the effects of LF2 are controlled primarily by the timing or the level of its expression are important questions for future work. Alternatively, the role of LF2 may differ between B cells and epithelial cells. LF2 kinetics in B cells are consistent with its being an early gene, but no information is available regarding protein expression. Whether and when LF2 is expressed in epithelial cells is also unknown. Our results demonstrate that LF2 is a competent repressor in both B cells and epithelial cells, but the timing and level of its expression may differ greatly between the two cell types, resulting in cell type-specific LF2 effects. Consistent with this possibility, we found that an LF2-positive EBV BAC constructed from the P3HR1 genome could be induced to replicate in a lymphoblastoid cell line but not in 293T cells (unpublished data). By contrast, EBV B95-8-based BACs replicate readily in both cell types (13, 19, 20).

LF2 homologues in KSHV and MHV68 are incorporated into virions. It is not currently known if this is the case for EBV, because experiments to determine its virion composition used the LF2-negative B95-8 cell line (42). If LF2 is a virion protein in wild-type EBV, one role for LF2 may be to promote the establishment of latent infection by suppressing replication upon primary infection. It is possible that its sequestration in nascent virions makes it available to inhibit Rta only once the tegument is delivered to the newly infected cell. Because neither Rta nor Zta is present in significant quantities in virions, the delivery of LF2 to the infected cell would significantly reduce the chance of a lytic infection being established (if, for example, initial conditions in the cell were favorable for Rp or Zp activation). Thus, LF2 may account for the propensity of gammaherpesviruses to establish latent infections, just as the incorporation of VP16 into herpes simplex virus virions serves to activate lytic replication.

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