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Inhibition of Epstein-Barr Virus OriP Function by Tankyrase, a Telomere-Associated Poly-ADP Ribose Polymerase That Binds and Modifies EBNA1

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Tankyrase (TNKS) is a telomere-associated poly-ADP ribose polymerase (PARP) that has been implicated along with several telomere repeat binding factors in the regulation of Epstein-Barr virus origin of plasmid replication (OriP). We now show that TNKS1 can bind to the family of repeats (FR) and dyad symmetry regions of OriP by using a chromatin immunoprecipitation assay and DNA affinity purification. TNKS1 and TNKS2 bound to EBNA1 in coimmunoprecipitation experiments with transfected cell lysates and with purified recombinant proteins in vitro. Two RXXPDG-like TNKS-interacting motifs in the EBNA1 amino-terminal domain mediated binding with the ankyrin repeat domain of TNKS. Mutations of both motifs at EBNA1 G81 and G425 abrogated TNKS binding and enhanced EBNA1-dependent replication of OriP. Small hairpin RNA targeted knockdown of TNKS1 enhanced OriP-dependent DNA replication. Overexpression of TNKS1 or TNKS2 inhibited OriP-dependent DNA replication, while a PARP-inactive form of TNKS2 (M1045V) was compromised for this inhibition. We show that EBNA1 is subject to PAR modification in vivo and to TNKS1-mediated PAR modification in vitro. These results indicate that TNKS proteins can interact directly with the EBNA1 protein, associate with the FR region of OriP in vivo, and inhibit OriP replication in a PARP-dependent manner.

Epstein-Barr virus (EBV) is a human herpesvirus that establishes lifelong latent infections causally linked to several cancers, including Burkitt's lymphoma and nasopharyngeal carcinoma (23, 32). The latent form of EBV exists as a multicopy extrachromosomal plasmid that replicates semiconservatively during the S phase of the cell cycle (1, 50). Epstein-Barr nuclear antigen 1 (EBNA1) is the only viral protein required for stable episomal maintenance of the viral genome (21, 26, 51) (reviewed in references 27 and 43). EBNA1 binds to two regions of the viral origin of plasmid replication (OriP), referred to as the family of repeats (FR) and the dyad symmetry (DS) element (31). FR is essential for plasmid maintenance, while DS is required for initiation of OriP-dependent DNA replication (18, 52). Cellular factors, like the origin recognition complex (ORC) and minichromosome maintenance (MCM) proteins, which regulate chromosomal DNA replication and cell cycle licensing, have been shown to associate with the DS region of OriP (9, 16, 35). In addition to these replication factors, several telomere-associated proteins, including telomere repeat factors (TRF1 and TRF2) and hRap1, interact with three nonamer repeats (TTAGGGTTA) that are interspersed with EBNA1 binding sites in the DS (13, 14).

In a previous study, we had shown that TRF2 and hRap1 facilitate DS-dependent DNA replication and OriP plasmid maintenance (13). In addition to TRF1, TRF2, and hRap1, we had also found that the telomere-associated poly-ADP ribose polymerase tankyrase 1 (TNKS1) was associated with the DS, using DNA affinity chromatography and chromatin immunoprecipitation assays (14). TNKS1 was originally identified as a TRF1-interacting protein that could modulate telomere length by disrupting binding of TRF1 to telomeric repeats (41, 42). TNKS2, a highly related paralogue, was also found to interact with TRF1 (22), as well as with several additional cellular proteins including the aminopeptidase IRAP4, a vesicular protein that undergoes regulated translocation to the cell surface (10). TNKS1 and -2 share similar structural and biochemical properties and can interact with each other through their SAM domains (15, 34). Subcellular localization studies indicated that only a fraction of TNKS protein colocalized to telomeres (40), but more recent short interfering hairpin RNA (shRNA) knock-down studies demonstrated that TNKS1 is required for sister telomere separation during anaphase (17). TNKS1 was found to be highly enriched in DS-specific DNA affinity-purified protein preparations (14), but its molecular associations and potential functions at OriP have not been explored in detail.

Posttranslational modification by poly-ADP ribosylation (PAR) has been implicated in several physiological and pathophysiological processes, including chromatin regulation, recognition of DNA damage, and response to oxidative stress (reviewed in references 6, 7, 11, 25, 37, 39, and 46). To date, 12 poly-ADP ribose polymerase (PARP) activities have been found in metazoan organisms (reviewed in references 4, 11, 37, and 39). PARP1, the most abundant and prototypical member of the enzyme family, has well-characterized functions in DNA damage response pathways (11, 37) and transcription regulation (25, 44, 45). PARP2 was recently shown to bind and poly-ADP ribosylate TRF2 (12). TNKS1 and -2 have dual roles in telomere homeostasis and in vesicular trafficking (reviewed in reference 39). PARP enzymes utilize NAD^+ as a substrate for the transfer of linear and branched chains of poly-ADP ribose onto glutamic acid residues of various protein sub-

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strates. PARP1 is one of the most abundant substrates of PARP1; other substrates for PARP1 modification include histones, p53, various transcription factors, and proteins implicated in DNA damage response. PAR modifications are transient and rapidly degraded by poly-ADP ribose glycohydrolase. The precise role of PAR modification remains unclear, but it is likely to alter electrostatic charge, protein interactions, and signaling potential.

Identification of in vivo substrates of various PARPs has been difficult due to the highly unstable nature of poly-ADP ribose-modified forms which are thought to have a half-life of less than 1 min in vivo (11). However, several proteins that interact with the ankyrin repeat domain of TNKS1 and -2 have been considered likely candidates for in vivo substrates of poly-ADP ribosylation. Telomere lengthening induced by TNKS1 or -2 overexpression correlates with TRF1 binding and TNKS-associated PARP activity, suggesting that their functions in vivo are mediated through PAR modification of target interacting proteins like TRF1 (41, 42). The ankyrin repeat domains of TNKS1 and -2 have been shown to bind to several additional proteins, including IRAP, NuMA, and TAB182, through a small RXXPDG consensus motif (33, 36). The recruitment of TNKS1 to the OriP DS region was originally thought to result from its interaction with TRF1, which can bind directly to nonamer sequences in DS. However, in several experiments we were able to identify TNKS1 without detecting significant amounts of TRF1. This raised the question of whether TNKS1 may be recruited to OriP through more-direct interactions with EBNA1 itself. In this study, we present evidence that TNKS1 and -2 can bind directly to EBNA1 through two RXXPDG-like motifs in the amino-terminal domains of EBNA1. We also show that TNKS binding to EBNA1 downregulates OriP replication and plasmid maintenance and that this inhibition correlates with poly-ADP ribosylation of EBNA1.

MATERIALS AND METHODS

Cells. HeLa N2 and 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics in a 5%-CO_2 incubator at 37°C. EBV-positive Raji and ZKO-293 cells (kindly provided by H. Delecluse) were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics in a 5%-CO_2 incubator at 37°C.

Plasmids and proteins. The OriP plasmid (N503) was described previously (14). shRNA expression vectors were generated as described previously (30). Briefly, short hairpins of 27 to 29 nucleotides for TNKS1 were expressed by the U6 promoter in the pENTR/D-Topo vector (Invitrogen). The pGEM1 plasmid, containing the U6 promoter, was used as the template for PCR with the Sp6 primer (CACCGATTTAGGTGACACTATAG) and the TNKS1 primer (AAAAAAAATAATTCTCCTCAGACACCTCCTTCTGCCCAAGCTTCGACAGAAGGAAGTGTCTGAGGAGAATCACGGTGTTTCGTCCTTTCCACAA). Single-amino-acid substitution and deletion mutants of EBNA1 were generated by using the plasmid N523 as the DNA template with the QuickChange II site-directed mutagenesis Kit (Stratagene). The N523 plasmid was derived from pREP10 (Invitrogen) and expresses hemagglutinin-tagged EBNA1 under control of the cytomegalovirus promoter. pCMV-FLAG-TNKS1, pCMV-FLAG-TNKS2, and pCMV-FLAG-TNKS2-M1054V were described previously (33, 34). For pFLAG-TNKS2-ANK, which expresses the ANK domain of TNKS2 with an N-terminal FLAG tag, the 2.2-kb NheI-XbaI fragment of pFLAG-TNKS-2 was replaced with a 150-bp PCR product to remove sequences beyond codon 812.

Recombinant hexahistidine-tagged EBNA1, TNKS1, and TRF1 were overexpressed in baculovirus-infected Sf9 cells and purified over nickel-nitrilotriacetic acid (Ni-NTA) agarose as described previously (5, 42). Recombinant bacterial hexahistidine-tagged EBNA1 was sequentially purified by using Ni-NTA agarose and DNA affinity purification with magnetic beads coupled to DS as described previously (14). Glutathione *S*-transferase (GST)-EBNA1 and truncation mutants were generated by PCR-based site-directed mutagenesis in the pGEX-2T vector (Amersham). TNKS1(641-831) was expressed as a hexahistidine fusion protein in the NheI-XhoI sites of pRSET (Invitrogen) and purified to homogeneity by Ni-NTA-agarose chromatography. The fusion proteins were purified and used in pull-down assays as described previously (34). In this work, recombinant EBNA1 refers to the truncation mutant lacking the glycine-alanine copolymer (ΔGA) from amino acids (aa) 104 to 329.

Transfections. All transfections were performed by the use of the Lipofectamine 2000 reagent, using 2 to 5 μg of plasmid DNA for 1.5×10^6 cells, which were seeded in 6-cm plates 12 to 16 h prior to transfection (14).

Western blotting. Primary antibodies to EBNA1 (Advanced Biotechnologies, Inc.), FLAG (Sigma M2 and polyclonal), PCNA (Santa Cruz Biotechnology), TNKS1 (IMGENEX), and PAR (monoclonal; Trevigen) were used according to the manufacturer's specifications. Rabbit antibodies to EBNA1 and TNKS1 were generated against recombinant protein and affinity purified. T1S and T12 antibodies to TNKS1 and -2, respectively, have been described (34).

Immunoprecipitation. For PAR immunoprecipitation, 3×10^7 Raji cells were cultured in the absence or in the presence of 3-aminobenzamide (3 mM) for 4 h and then resuspended in 1.5 ml of cold lysis buffer (0.1% sodium dodecyl sulfate [SDS], 50 mm Tris-HCl [pH 7.4], 1% Triton X-100, 1 mM EDTA, 400 mM NaCl) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and protease inhibitor cocktail at 4°C. After 10 min on ice, an equal volume of cold water was added and lysates were centrifuged for 10 min at $16,000 \times g$ in an Eppendorf microfuge. Supernatants were collected and precleared with protein G-Sepharose beads for 1 h and then incubated overnight with indicated antibodies with rotation at 4°C. The immune complexes were collected with protein G-Sepharose beads for 2 h at 4°C; beads were washed three times with cold 1:1-diluted lysis buffer and resuspended in 50 μl of 2× Laemmli buffer at 65°C. In some cases, samples were eluted at 95°C to reduce the diffuse electrophoretic properties of the PAR-modified species (Fig. 8B). For transfected 293 cells, 3×10^7 cells were harvested 48 h posttransfection and processed as described above. For coimmunoprecipitation with purified recombinant EBNA1 and TNKS1, combinations of each protein or bovine serum albumin were resuspended in 1 ml of cold NET-N buffer (100 mM NaCl, 2 mm EDTA, 20 mM Tris-HCl [pH 8.0], 0.2% Igepal) supplemented with 1 mM PMSF and protease inhibitor cocktail (Sigma) and incubated overnight with appropriate antibodies with rotation at 4°C. The immune complexes were collected with protein G-Sepharose beads for 2 h at 4°C; beads were washed four times with cold NET-N buffer and resuspended in 50 μl of 2× Laemmli buffer. The sample (25 μl) was subject to a 4 to 20% Tris-glycine polyacrylamide gel, followed by Western blotting analysis.

ChIP assay. Chromatin immunoprecipitation (ChIP) assays were performed as described previously (14). The primers for amplification were as follows: DS (CCCGTGACAGCTCATGGGGTGGGAGAT and CAATCAGAGGGGCCTGTGTAGCTACCG), FR (GACTCTGCTTTTCTGCCGTCT and TTGGCAAAAGGATGGTTAGG), and BZLF1p (CAGCTGAGGTGCTGCATAAGCTTG and ACCTTGCCGGCACCTTTGCTATC). Rabbit polyclonal antibodies to ORC2 and MCM3 were purchased from BD Pharmingen and Abcam, respectively. Rabbit antibodies to EBNA1, TRF2, TNKS1, and TRF1 were generated against recombinant proteins and affinity purified.

DNA replication and plasmid maintenance assays. DNA replication and plasmid maintenance assays were performed as described previously (14). For a replication assay with shRNAs, the OriP plasmid (3 μg) and a plasmid expressing siRNA (3 μg) were cotransfected into 1.5×10^6 293-ZKO cells and harvested 72 h posttransfection. For a replication assay with overexpression of TNKS, 293-ZKO cells were cotransfected with the OriP plasmid (3 μg) and expression vectors of FLAG-TNKS1, FLAG-TNKS2, or FLAG-TNKS2 (M1054V) (3 μg). EBNA1 mutants were analyzed for DNA replication and plasmid maintenance in EBNA1-negative HeLa N2 cells. EBNA1 mutants were expressed on plasmids that also contained OriP. For plasmid maintenance of EBNA1 mutants, 1.5×10^6 HeLaN2 cells were transfected with either the EBNA1 wild type (wt) or mutants. Twenty-four hours posttransfection, cells were washed with phosphate-buffered saline, replated onto 10-cm plates, and then cultured for 14 to 21 days under hygromycin selection (250 μg/ml). Selected cells were subjected to replating every fourth day. Plasmid DNA was isolated by Hirt extraction, digested with BamHI, and then detected by Southern blotting. Southern blots were quantified by PhosphorImager analysis, and DNA replication efficiency was calculated by comparing the ratio of DpnI-resistant DNA to BamHI-linearized DNA; values were presented as a percentage of EBNA1 wt activity included in the same experiment.

DNA pull-down assay. Nuclear extract binding with biotinylated DNA bound to streptavidin was described previously (14). DNA affinity purification with recombinant proteins involved generating in vitro-translated TNKS1 or lucif-

erase with [^{35}S]methionine using T7/T3 coupled reticulocyte lysate systems (Promega). Recombinant EBNA1 was prebound to DS, FR, or BKS DNA coupled to magnetic beads for 45 min at room temperature (RT). The bound material was washed three times with 1 ml of D150 buffer (20 mM HEPES [pH 7.9], 0.2 mM EDTA, 20% glycerol, 150 mM KCl, 1 mM PMSF, 1 mM DTT) containing 0.05% Ipegal and then further incubated with ^{35}S-labeled TNKS1 or luciferase for 45 min at RT. The bound material was washed three times with 1 ml of D150 containing 0.05% Ipegal and then boiled for 15 min in 2× SDS-loading buffer. The eluted sample was run on a 10% polyacrylamide gel, the gel was dried, and the radioactive species were detected by PhosphoImager.

GST-pull down assay. GST-EBNA1 proteins were expressed and purified from *Escherichia coli* by glutathione-Sepharose affinity purification and washing with sonication buffer (20 mM Tris-Cl [pH 7.5], 1 mM EDTA, 150 mM NaCl, 0.05% Ipegal, 1 mM PMSF, 1 mM DTT). Glutathione-Sepharose (20 μl)-bound GST-EBNA1 proteins (~50 μg) were incubated with TNKS proteins (either purified or from cell extracts) for 30 min and then subjected to three washings with 1 ml of sonication buffer at 4°C for 15 min each. Glutathione-Sepharose-bound complexes were disrupted with Laemli buffer and subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Complexes were visualized by Western blotting or Coomassie staining.

In vitro poly-ADP ribosylation assays. Recombinant proteins (10 to 500 ng) were incubated in 20 μl of reaction buffer containing 10 mM Tris (pH 7.0), 10% glycerol, 6 mM $MgCl_2$, 50 mM KCl, 1 mM PMSF, and 5 mM 2-mercaptoethanol with or without 1 mM NAD^+. Reactions were incubated for 30 min at RT, inactivated by the addition of 2× SDS-PAGE loading buffer and subjected to Western blot analysis. Binding reactions used baculovirus-derived EBNA1 (~100 nM) and TNKS1 (~100 nM).

RESULTS

TNKS1 interacts with the FR region of OriP in vivo. The ChIP assay was used to determine if several proteins, including TNKS1 and TRF1, could be detected at either the FR region or the DS region of OriP in vivo (Fig. 1A). EBV-positive Raji cells were formaldehyde cross-linked, and total cell lysates were sonicated to produce DNA fragments of an average length of 300 to 600 bp. Amplicons for EBV DNA were selected at the left side of FR (relative to standard EBV coordinates) or adjacent to DS and were thus spaced more than 1.7 kb from each other. A third amplicon was the BZLF1 promoter region (Zp) located more than 40 kb from OriP. Thus, there should be limited overlap in these amplicons from sheared DNA fragments used in the ChIP assays. Antibodies to EBNA1, TRF2, TNKS1, TRF1, ORC2, MCM3, or control rabbit immunoglobulin G (IgG) were compared for their ability to ChIP DS, FR, or Zp DNA. EBNA1 antibodies precipitated DNA from DS and FR but not from Zp (Fig. 1A, lanes 7 to 9). The preferential precipitation of DS over FR is likely caused by the difference in amplicon proximity to EBNA1 binding sites and not a reflection of the in vivo binding affinity, which is likely to be much higher at FR than at DS. TRF2 bound DS but did not precipitate significant amounts of FR or Zp, consistent with the known TRF2 binding sites in DS (Fig. 1A, lanes 10 to 12). Similarly, TRF1, ORC2, and MCM3 precipitated more DS than FR or Zp DNA (Fig. 1A, lanes 16 to 24). In contrast, TNKS1 antibody precipitated FR DNA to a greater extent than DS DNA, and levels of both regions were significantly above Zp levels (Fig. 1A, lanes 13 to 15). This suggests that the TNKS1 protein can bind both FR and DS but preferentially binds to the FR region of OriP.

The ability of TNKS1 to bind FR was reexamined in vitro using DNA affinity purification with recombinant TNKS1 and EBNA1 (Fig. 1B). ^{35}S-labeled in vitro-translated TNKS1 or control luciferase protein were compared for their binding to FR, DS, or control BKS DNA in the presence or absence of recombinant EBNA1 expressed and purified from baculovirus infected sf9 cells. We found that ^{35}S-TNKS1 bound to FR and DS in the presence of EBNA1 but not when EBNA1 was absent. ^{35}S-luciferase did not bind DS or FR significantly, and ^{35}S-TNKS1 did not bind to control BKS DNA, indicating that the binding of TNKS1 was specific for EBNA1-bound DS and FR. ^{35}S-TNKS1 also bound to DS when recombinant TRF1 was substituted for EBNA1, indicating that TNKS1 can be recruited to DS through direct interactions with TRF1, as well as with EBNA1.

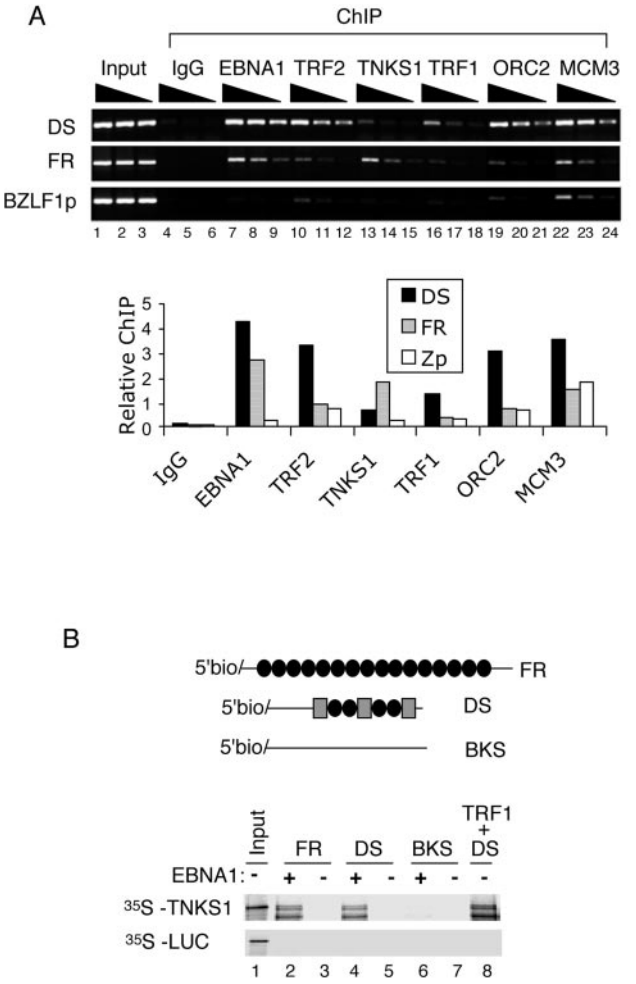

FIG. 1. TNKS binds the FR region of OriP. (A) Raji cells were subjected to ChIP assay with control IgG (lanes 4 to 6) or specific antibodies against EBNA1 (lanes 7 to 9), TRF2 (lanes 10 to 12), TNKS1 (lanes 13 to 15), TRF1 (lanes 16 to 18), ORC2 (lanes 19 to 21), or MCM3 (lane 22 to 24). ChIP DNA was amplified for EBV regions DS, FR, and BZLF1p as indicated. Input (lanes 1 to 3) and chromatin immunoprecipitated DNA were serially diluted threefold (lanes 1 to 3). Quantification of the average intensity of ethidium bromide-stained DNA products was done relative to total input DNA and shown in the bar graph below. (B) FR, DS, or BKS DNA affinity binding was assayed by using in vitro-translated ^{35}S-TNKS1 or ^{35}S-luciferase (^{35}S-LUC) control protein. Binding was measured with (+) or without (−) recombinant baculovirus-expressed and purified EBNA1. For the positive control, recombinant TRF1 was used instead of EBNA1 (lane 8). The schematic above depicts biotinylated (Bio) templates with EBNA1 sites (circles) and TRF binding sites (squares).

Association of TNKS with EBNA1. The above-described experiments suggest that EBNA1 can bind TNKS1 in the absence of TRF1. To further explore this possibility, we assayed whether EBNA1 could coimmunoprecipitate with FLAG-tagged TNKS1 in transiently cotransfected 293 cells (Fig. 2A). Immunoprecipitation with antibodies specific for EBNA1 revealed that FLAG-TNKS1 coimmunoprecipitated with ~1% of the input EBNA1 (Fig. 2A). No FLAG-TNKS1 was detected in IgG control immunoprecipitates, indicating that the TNKS1 coimmunoprecipitation was specific for EBNA1. Similarly, immunoprecipitation with FLAG antibodies revealed that EBNA1 coimmunoprecipitated with anti-FLAG-TNKS1, while no EBNA1 was detected with rabbit IgG control (lower panel). Nearly identical results were found when FLAG-TNKS2 was substituted for FLAG-TNKS1 (Fig. 2B, lower panel). Since many TNKS-interacting proteins bind the ankyrin repeat domain, we assayed the ability of the TNKS2-ankyrin domain alone to interact with EBNA1 in coimmunoprecipitation assays. FLAG-TNKS2 -ANK was also found to bind EBNA1, similar to full-length TNKS proteins. These results indicate that TNKS1 and TNKS2 can interact with EBNA1 in transfected cells in the absence of OriP DNA.

To further test the possibility of a direct interaction between the EBNA1 and TNKS proteins, we assayed the ability of purified *Escherichia coli*-derived GST-EBNA1 to bind to FLAG-TNKS1 or FLAG-TNKS2 protein that was expressed in transfected 293 cells (Fig. 2B). Purified GST-EBNA1(1–440ΔGA) or GST protein control was incubated with nuclear extracts from transfected cells and assayed with antibodies specific for TNKS1 (top panel) or TNKS2 (lower panel). In both cases, we found that GST-EBNA1 bound TNKS proteins expressed in 293 cells, further supporting our findings that these proteins can interact independently of OriP DNA.

To eliminate the possibility that other cellular factors mediate the interaction between TNKS and EBNA1, we tested the ability of recombinant TNKS1 and EBNA1 proteins expressed and purified from baculovirus-infected cells to interact in vitro (Fig. 2C). TNKS1 was incubated with EBNA1 and subjected to immunoprecipitation with antibodies specific for EBNA1, TNKS1, or control IgG (Fig. 2C). We observed that anti-EBNA1 antibodies efficiently precipitated TNKS1 protein in an EBNA1-dependent fashion (Fig. 2C, lane 8) and that anti-TNKS1 precipitated EBNA1 in a TNKS1-dependent fashion (Fig. 2C, lane 11). These results indicate that full-length EBNA1 and TNKS1 can interact with each other in the absence of other cellular and viral proteins.

TNKS binds to two related motifs in EBNA1. The highly conserved ankyrin domain of TNKS1 and -2 interacts with an RXXPDG motif in several binding partners, including TRF1, IRAP, and TAB182 (33). We observed that the TNKS2 ankyrin domain also conferred binding to transfected full-length and bacterially expressed GST-EBNA1 (Fig. 2A). To determine if EBNA1 has any similar TNKS binding motifs, we assayed various GST-EBNA1 fusion proteins for binding to the TNKS2 ankyrin domain (Fig. 3A). EBNA1 can be divided into at least three functional regions: the C-terminal DNA binding domain (aa 450 to 607), an N-terminal region that confers chromosome tethering and replication activity (aa 1 to 103 and 330 to 440), and a glycine-alanine copolymer (aa 103 to 330) which is dispensable for DNA replication and plasmid main-

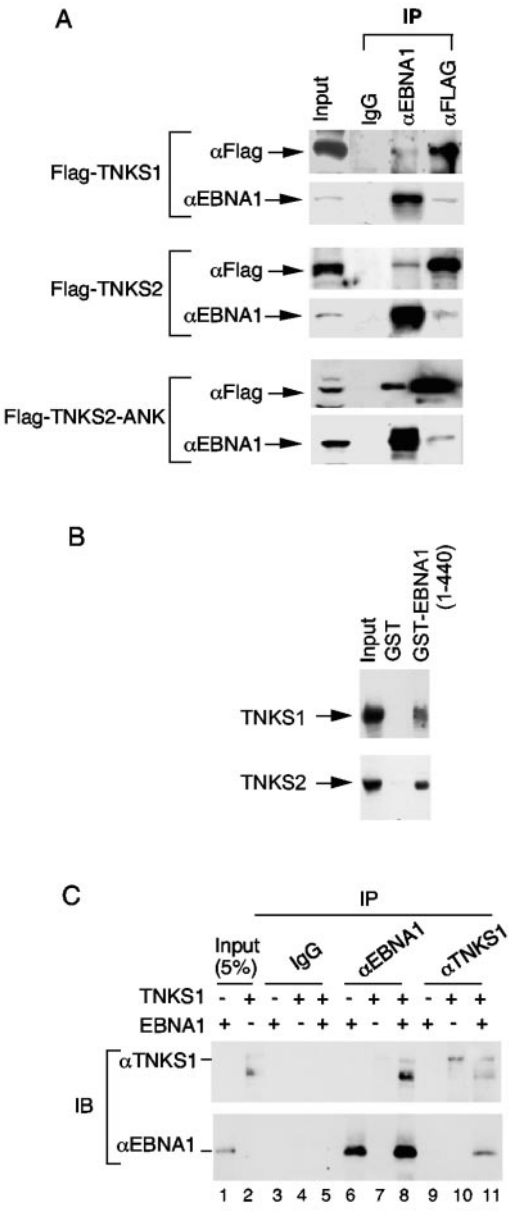

FIG. 2. TNKS binds EBNA1 directly. (A) 293 cells were cotransfected with plasmids expressing EBNA1 and either FLAG-TNKS1 (top panel), FLAG-TNKS2 (middle panel), or FLAG-TNKS2-ANK (lower panel). Immunoprecipitates using rabbit anti-EBNA1, anti-FLAG, or control rabbit IgG were assayed by Western blotting with monoclonal antibodies specific for FLAG or EBNA1. (B) Cell extracts containing full-length FLAG-TNKS1 (top panel) or FLAG-TNKS2 (lower panel) were assayed for binding GST or GST-EBNA1(1-440ΔGA). Bound proteins were assayed by Western blotting with the anti-TNKS antibodies T1S (upper panel) and T12 (lower panel). (C) Purified baculovirus-expressed TNKS1 and EBNA1 form a complex in vitro. EBNA1 alone (lanes 3, 6, and 9), TNKS alone (lanes 4, 7, and 10), or the two proteins mixed in vitro (lanes 5, 8, and 11) were subjected to immunoprecipitation (IP) with IgG (lanes 3 to 5) or antibodies against EBNA1 (lanes 6 to 8) or TNKS1 (lanes 9 to 11). Immunoprecipitates were washed extensively and assayed by Western immunoblotting (IB) with monoclonal antibodies to TNKS1 (top panel) or EBNA1 (bottom panel). Inputs (lanes 1 and 2) represent 5% of the total material used for immunoprecipitates.

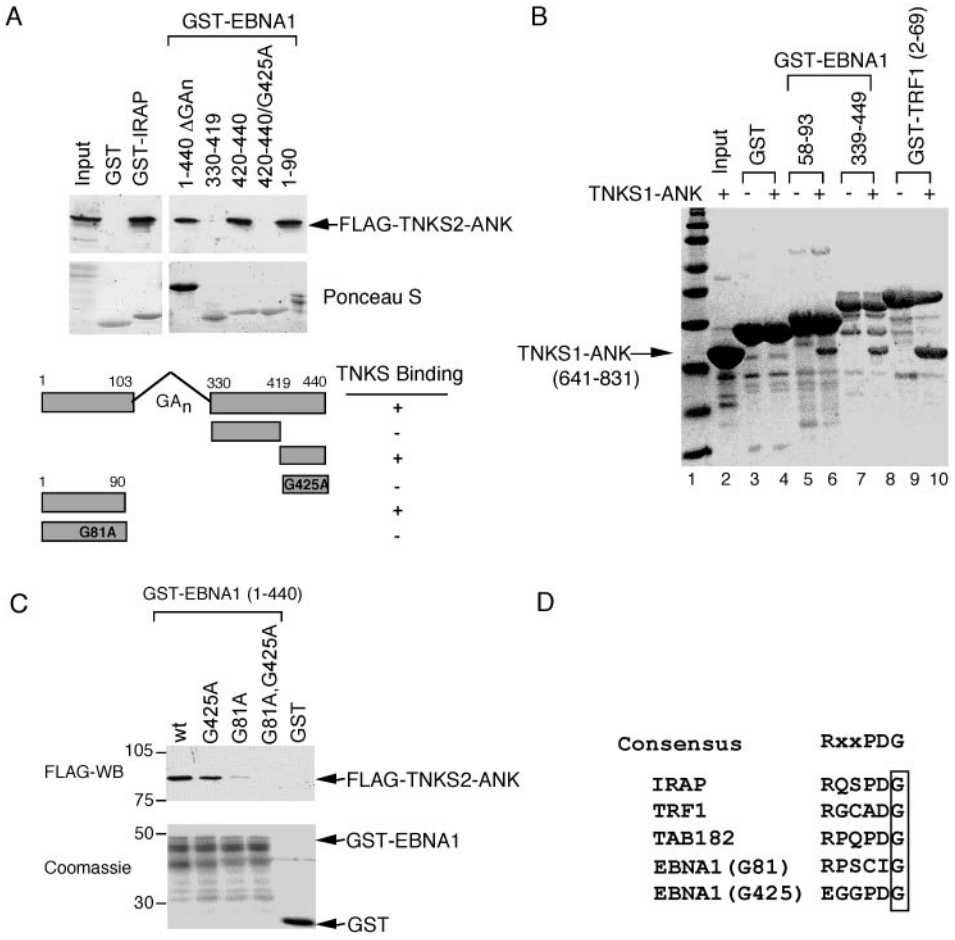

FIG. 3. TNKS binds to two RXXPDG-like motifs in EBNA1. (A) Extracts from 293 cells transfected with the FLAG-tagged ankyrin domain of TNKS2 (FLAG-TNKS2-ANK) were used to assay binding to GST fusions of various EBNA1 subdomains, as indicated above. Bound proteins were detected by anti-FLAG immunoblotting (top panel). GST proteins were visualized by Ponceau S staining of nitrocellulose blots (lower panel). GST-IRAP is a positive control for TNKS2 binding. A summary of several independent binding experiments is given in the schematic below. (B) Purified GST (lanes 3 and 4), GST-EBNA1(58–93) (lanes 5 and 6), or GST-EBNA1(339–449) (lanes 7 and 8) were incubated with (+) or without (−) the purified TNKS1 ankyrin repeat domain (aa 641 to 831). Glutathione agarose-bound proteins were visualized by Coomassie blue staining in SDS-PAGE. (C) GST-EBNA1(1–440ΔGA) containing substitution mutations at G425, G81, or both was assayed for binding to TNKS2 as described for panel A. Coomassie staining of GST-EBNA1 proteins is shown below. (D) Alignment of the RXXPDG-like motifs from EBNA1 and other TNKS-interacting proteins.

tenance activity (3, 28, 29, 48, 49). We found that the N-terminal region (aa 1 to 103 plus aa 330 to 440), lacking any DNA binding activity, bound the TNKS2 ankyrin repeat domain (Fig. 3A). Further deletion analysis of the EBNA1 N-terminal region uncovered a TNKS-binding domain within aa 1 to 90 and a second binding domain within aa 419 to 440 (Fig. 3A).

The TNKS1 ankyrin domain (aa 641 to 831) containing five ankyrin repeats was purified to near-homogeneity and assayed for its ability to bind purified GST-EBNA1 fragments in the absence of any additional factors (Fig. 3B). TNKS1(641–831) did not bind to GST protein (Fig. 3B, lane 4) but bound efficiently to GST-EBNA1(58–93) (Fig. 3B, lane 6) and to GST-EBNA1(339–449) (Fig. 3B, lane 8). TNKS1(641–831) bound efficiently to GST-TRF1(2–69), as expected (Fig. 3B, lane 10). These findings demonstrate that at least two regions of EBNA1 can interact directly with the ankyrin domains of TNKS1 and TNKS2.

Inspection of these EBNA1 regions revealed two hexapeptide sequences, the RPSCIG (aa 76 to 81) and EGGPDG (aa 420 to 425) sequences, that partially match the consensus TNKS binding motif RXXPDG (Fig. 3D). Substitution mutations in EBNA1 G425A eliminated the binding of the EBNA1 420–440 fragment to TNKS2 (Fig. 3A), and a similar G81A substitution abolished the binding of EBNA1 aa 1 to 90 to TNKS2 (data not shown). In the context of the N-terminal domain (aa 1 to 103 plus aa 330 to 440), this single-substitution mutation did not completely eliminate TNKS binding (Fig. 3C). However, the combination of G81A and G425A led to a significant reduction in TNKS binding (Fig. 3C). These results demonstrate that two RXXPDG-like motifs in the EBNA1 amino-terminal domain confer binding to the TNKS ankyrin repeat domain.

To verify that the EBNA1 G81A/G425A mutation diminished TNKS binding in the context of full-length proteins in vivo, we assayed the ability of wt and mutant EBNA1 proteins to coimmunoprecipitate with full-length FLAG-TNKS2 in transfected HeLa cells (Fig. 4A). FLAG-TNKS2 was found to

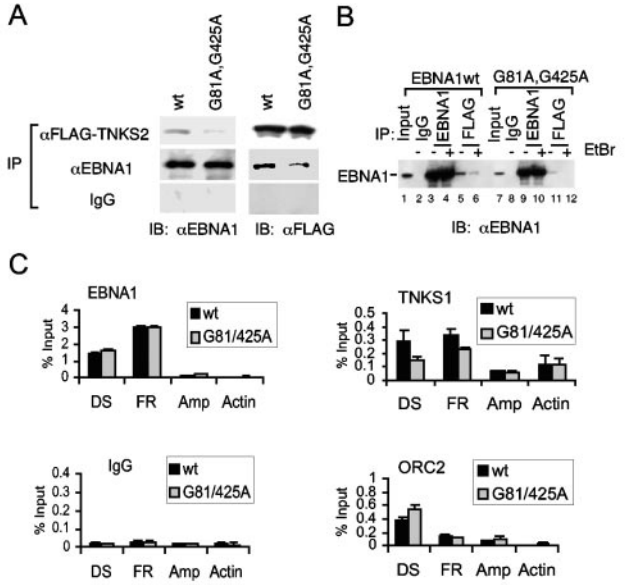

FIG. 4. EBNA1 (G81A/G425A) diminishes TNKS binding in vivo. (A) wt or G81A/G425A EBNA1 was coexpressed with FLAG-TNKS2 in HeLa cells and assayed for coimmunoprecipitation. Immunoprecipitation with anti-FLAG, anti-EBNA1, or IgG control was analyzed by immunoblotting with anti-EBNA1 (left panel) or anti-FLAG (right panel). (B) wt or G81A/G425A EBNA1 was coexpressed with FLAG-TNKS2 in HeLa cells and assayed for coimmunoprecipitation in the absence (−) or presence (+) of 100 μg of EtBr/ml. Immunoprecipitates were analyzed by immunoblotting with anti-EBNA1. (C) wt (black fill) or G81A/G425A (grey fill) EBNA1 was coexpressed in HeLa cells with OriP-containing plasmid and assayed by ChIP for amplification of DS, FR, ampicillin gene (Amp), or cellular actin DNA, using antibody to EBNA1, TNKS1, ORC2, or IgG.

coimmunoprecipitate with wt EBNA1, as expected. In contrast, significantly less FLAG-TNKS2 coimmunoprecipitated with EBNA1 (G81A/G425A). Similar levels of EBNA1 were expressed and immunoprecipitated by EBNA1 antibody (middle panel), and no EBNA1 proteins were detected with IgG control immunoprecipitates (lower panel). The same experiment was repeated and assayed by immunoblotting with anti-FLAG (right panels). Again, we found that EBNA1 (G81A/G425A) had diminished capacity to immunoprecipitate FLAG-TNKS2 (right middle panel). Nearly identical amounts of FLAG-TNKS2 were expressed and immunoprecipitated by FLAG antibody (right top panel), and no proteins were detected with IgG control immunoprecipitations. These results indicate that EBNA1 residues G81 and G425 are important for TNKS interaction in the context of full-length proteins in vivo. To determine if DNA might be mediating some of the interactions between full-length EBNA1 and TNKS proteins, we performed immunoprecipitation of wt EBNA1 and G81A/G425A EBNA1 in the presence or absence of 100 μg of ethidium bromide (EtBr)/ml, which disrupts most DNA-protein complexes (Fig. 4B). We found that addition of EtBr reduced but did not eliminate EBNA1 coimmunoprecipitation with FLAG-TNKS2 (Fig. 4B, lanes 5 and 6). In contrast, addition of EtBr completely eliminated any detectable coimmunoprecipitation between G81A/G425A EBNA1 and FLAG-TNKS2 (Fig. 4B, 12). These findings suggest that EBNA1 can bind TNKS in the absence of DNA and that this interaction is dependent on the EBNA1 amino acid residues G81 and G425. However, these results also suggest that DNA binding stabilizes the interaction of EBNA1 with TNKS, since EtBr reduced coprecipitation of EBNA1 with TNKS.

To further evaluate the effect of the EBNA1 G81A/G425A mutation in vivo, we analyzed the binding of EBNA1, TNKS, and ORC2 to regions of OriP using a ChIP assay (Fig. 4C). We found that wt and G81A/G425A EBNA1 bound to DS and FR specifically and with nearly equal efficiencies (Fig. 4C, top left). In contrast, we found that TNKS1 binding at DS was reduced to ~40% in cells expressing G81A/G425A EBNA1 relative to EBNA1 wt-expressing cells (Fig. 4C, top right). A similar reduction of TNKS1 binding (~60%) was found at FR. In contrast, ORC2 binding was slightly elevated at DS in cells expressing G81A/G425A EBNA1 compared to results with EBNA1 wt (Fig. 4C, lower right). No specific binding was detected at a plasmid control region (Amp) or at a cellular gene (Actin). Similarly, no significant binding was found with control IgG ChIP assays (Fig. 4C, lower left). These data indicate that the EBNA1 residues G81A and G425 reduce the association of TNKS protein at OriP regions DS and FR in vivo. These results also suggest that EBNA1 (G81A/G425A) augments ORC association with the DS region of OriP.

Disruption of TNKS binding enhances EBNA1 replication activity. To determine if the RXXPDG-like TNKS interaction motifs in EBNA1 were functionally important, we measured the DNA replication and plasmid maintenance activities of an EBNA1 protein containing a substitution mutation in both of these motifs (Fig. 5). EBNA1-dependent DNA replication was assayed by transient transfection and quantitated by determining the amount of DpnI-resistant plasmid (upper panel) relative to that of total BamHI linearized plasmid (lower panel) at 72 h posttransfection. As expected, no replication was detected in the presence of an EBNA1 mutant lacking the DNA binding domain (ΔDB) (Fig. 5A, lanes 1 to 2). By contrast, the substitution mutations disabling both TNKS-interacting motifs of EBNA1 (at G81A and G425A) led to a ~3-fold increase in DNA replication (lanes 5 to 6) relative to that of wt EBNA (lanes 3 and 4). These EBNA1 mutants were also assayed for plasmid maintenance activity after 14 days in culture (Fig. 5B). We found that substitution mutants produced a similar three- to fourfold increase in OriP plasmid maintenance (Fig. 5B). These results indicate that the disruption of both TNKS interaction motifs in EBNA1 leads to an increase in DNA replication and plasmid maintenance activities. EBNA1 protein levels 72 h after transfection revealed that these mutations may also have an effect on EBNA1 stability, since the G81/425A mutant was expressed at slightly elevated levels relative to wt EBNA1 (Fig. 5C).

TNKS1 negatively regulates OriP replication. To investigate the functional role of TNKS1 in OriP-dependent DNA replication, we used shRNA-targeted depletion of TNKS1 (Fig. 6). EBV-positive 293-ZKO cells were transfected with shRNA control plasmid or TNKS1-shRNA target plasmid and assayed by Western blotting for depletion of the TNKS1 protein (Fig. 6A). The TNKS1-shRNA target plasmid reduced the TNKS1 protein to less than ~20% of control plasmid levels (Fig. 6A, upper panel). Control PCNA protein levels were not affected by shRNA plasmid transfection (Fig. 6A, lower panel). These same cells were cotransfected with OriP-containing plasmids

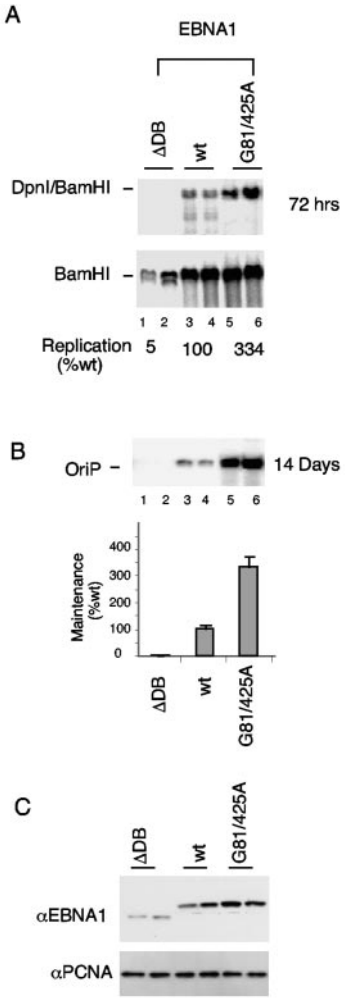

FIG. 5. Mutation of the EBNA1 TNKS interaction motifs enhances replication and plasmid maintenance. (A) EBNA1-dependent DNA replication was assayed in HeLa cells transfected with OriP plasmids expressing EBNA1 lacking its DNA binding domain (ΔDB), EBNA1 (wt), or EBNA1 (G81A/G425A), as indicated above each lane. Dpn1 and BamHI double cuts (top panel) indicate replicated DNA relative to the total recovered DNA linearized with BamHI alone (bottom panel). Replication activity was quantified for at least three independent experiments, and standard deviations were less than 15%. (B) Plasmid maintenance was assayed for OriP plasmids encoding ΔDB (lanes 1 and 2), wt (lanes 3 and 4), or G81/425A (lanes 5 and 6) EBNA1. Recovered plasmid was linearized with BamHI and detected by Southern hybridization. Quantification of three independent experiments is presented in the bar graph below. (C) Western blot analysis of hemagglutinin-tagged EBNA1 derivatives from transfected cell extracts described for panel A above.

and assayed for DNA replication (Fig. 6B). Plasmid replication was determined by the ratio of Dpn1-resistant to total BamHI-linearized plasmid recovered 72 h after transfection. shRNA-directed depletion of TNKS1 led to a 2.6-fold increase in OriP-dependent DNA replication (Fig. 6B). These findings are consistent with a role of TNKS1 as a negative regulator of OriP replication.

We next examined the effect of TNKS1 and TNKS2 overexpression on OriP DNA replication (Fig. 7). Ectopic expression of FLAG-tagged TNKS1 and -2 inhibited OriP replication by three- and sixfold, respectively (Fig. 7A and B). The increased efficiency of TNKS2 inhibition relative to that of TNKS1 most likely reflects the higher expression levels of TNKS2 in transient-transfection experiments (Fig. 7C, αFlag panel). These findings are consistent with those of the shRNA studies and further suggest that the TNKS proteins negatively regulate OriP replication.

To determine if the PAR enzymatic activity of TNKS was important for this inhibition, we assayed TNKS2 (M1054V), which contains a point mutation in the PARP domain that destroys its enzymatic activity (34). TNKS2 (M1054V) was expressed to levels identical to those of wt TNKS2, as determined by anti-FLAG immunoblotting (Fig. 7C). We found that TNKS2 (M1054V) (57% of wt activity) was compromised in its ability to inhibit OriP replication relative to wt TNKS2 (14% of wt activity) (Fig. 7A and B). These findings indicate that overexpression of TNKS1 and -2 inhibit OriP replication and that the PARP activity of TNKS2 contributes to this inhibition. Ectopic expression of TNKS1 and TNKS2 caused a slight reduction in EBNA1 protein levels as detected by Western blotting (Fig. 7C, middle panel), suggesting that TNKS proteins may inhibit OriP replication in part by reducing EBNA1 protein stability.

Poly-ADP ribosylation of EBNA1 by TNKS. We have previously shown that EBNA1 can be ADP ribosylated in vitro by using DS DNA affinity-purified proteins (14). We next set out to determine whether PAR modification could be detected on endogenous EBNA1 in latently infected Raji Burkitt lymphoma cells (Fig. 8A). Long-branched PAR modifications are thought to have extremely short half-lives in vivo and therefore may not be recovered fully in immunoprecipitation assays. Nevertheless, we sought to determine if EBNA1 protein in

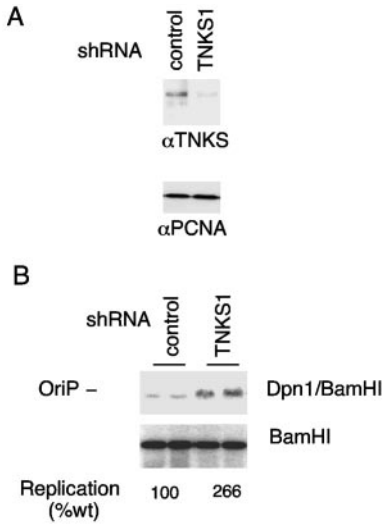

FIG. 6. shRNA-mediated depletion of TNKS1 enhances OriP replication. EBNA1-positive ZKO-293 cells were cotransfected with OriP plasmid and either shRNA control plasmid or shRNA target plasmid for TNKS1. (A) Seventy-two hours posttransfection, cell lysates were assayed by Western blotting for expression of TNKS1 (top panel) or control PCNA protein (bottom panel). (B) The same transfected cells were assayed for OriP-dependent replication of transfected plasmids. Quantification of replication was the average from three independent experiments.

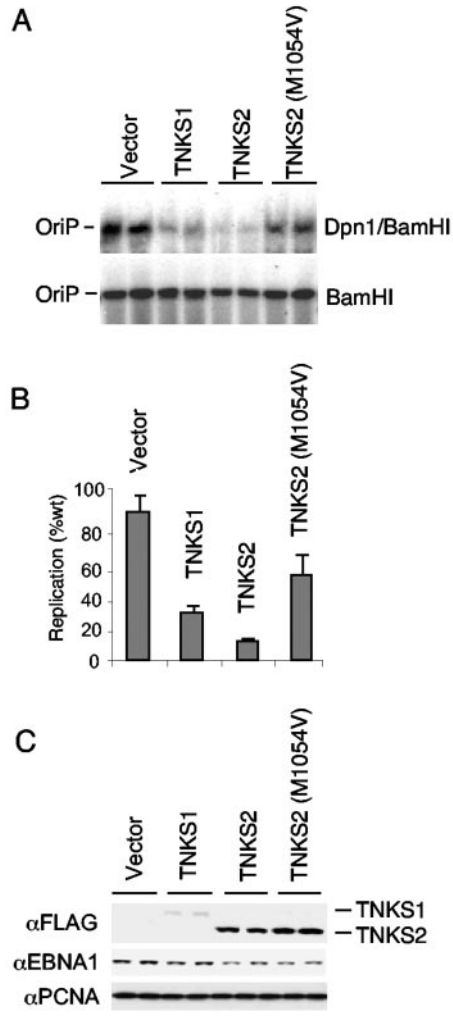

FIG. 7. Ectopic expression of TNKS proteins inhibits OriP replication. (A) EBNA1-positive ZKO-293 cells were cotransfected with OriP plasmid and expression vector for TNKS1, TNKS2, or a PARP-defective mutant of TNKS2 (M1054V), as indicated above each lane. The transfected cells were assayed for OriP-dependent DNA replication. (B) Quantification of OriP DNA replication represents the average for three independent experiments. (C) Western blot analysis of transfected cell extracts shown above. Antibodies to FLAG-tagged TNKS1 or TNKS2 (top panel), EBNA1 (middle panel), and control protein PCNA (bottom panel) are indicated.

Raji cells had reactivity to the PAR-specific monoclonal antibody, using immunoblotting of immunoprecipitates with antibodies to EBNA1. To enhance the isolation of PAR-modified forms of EBNA1, immunoprecipitates were eluted with Laemmli buffer at 65°C to avoid excessive heat treatment. EBNA1 protein was readily detected in EBNA1 immunoprecipitates and could be weakly detected in immunoprecipitates with PAR antibody (Fig. 8A, left panel). Immunoblotting of the same immunoprecipitate with PAR-specific antibody revealed abundant and diffuse species of PAR-modified proteins in the PAR immunoprecipitates (Fig. 8A, middle panel). Similar but lower-abundance diffuse species could be detected in EBNA1 immunoprecipitates. In contrast, no PAR-reactive species was detected in IgG control immunoprecipitates. The same immunoblot was probed with TNKS1-specific antibody and revealed a ~150-kDa protein specific to the EBNA1 immunoprecipitation, indicating that TNKS1 coimmunoprecipitates with EBNA1 in Raji cell extracts under these conditions (Fig. 8A, right panel). These results suggest that EBNA1 can be isolated as PAR-modified species in Raji cell extracts.

To further verify that the EBNA1 was subject to PAR mod-

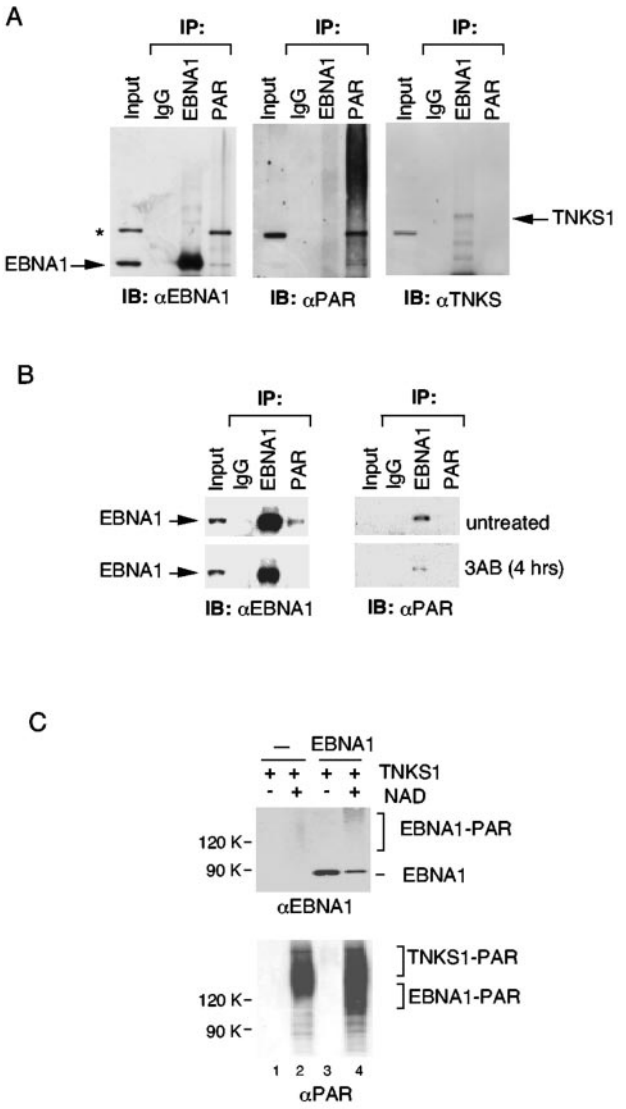

FIG. 8. PAR modification of EBNA1. (A) EBV-positive Raji cells were immunoprecipitated (IP) with anti-EBNA1, anti-PAR, or IgG control and then analyzed by immunoblotting (IB) with anti-EBNA1 (left panel), anti-PAR (middle panel), or anti-TNKS1 (right panel). Immunoprecipitated proteins were eluted at 65°C in Laemmli buffer. The positions of EBNA1 and TNKS1 proteins are indicated by the arrows. The asterisk indicates a cross-reacting band of unknown identity. (B) EBV-positive Raji cells left untreated (top panels) or treated with 3-AB for 4 h (lower panels) were subjected to immunoprecipitation with antibodies against EBNA1, PAR, or control mouse IgG. Immunoprecipitates were immunoblotted with anti-EBNA1 antibodies (left panels) or anti-PAR antibodies (right panels). Immunoprecipitated proteins were eluted with Laemmli buffer at 95°C for 5 min. (C) Purified recombinant TNKS1 protein was incubated with purified recombinant EBNA1 with (+) or without (−) 1 mM NAD^+ as indicated and assayed by Western blotting with anti-EBNA1 (top) or anti-PAR (lower panel) antibodies.

ification in vivo, we compared PAR immunoprecipitates in Raji cells that were pretreated with the PARP inhibitor 3-aminobenzamide (3-AB). Raji cells were treated with or without 3 mM 3-AB for 4 h and then subjected to immunoprecipitation with antibodies to EBNA1, PAR, or control IgG (Fig. 8B). To reduce the heterogeneous migration of PAR-modified EBNA1 proteins, immunoprecipitates were eluted in Laemmli buffer at 95°C for 5 min. EBNA1 was readily detected in PAR immunoprecipitates from untreated cells but was no longer detectable in PAR immunoprecipitates from 3-AB-treated Raji cells (Fig. 8A, left panels). Similarly, when immunoblots were probed with PAR-specific antibody, EBNA1 was more readily detected in untreated cells than in 3-AB-treated cells (Fig. 8A, right panels). These results indicate that PAR-modified EBNA1 can be isolated from Raji cell extracts and that 3-AB can partially inhibit this modification.

We next determined whether EBNA1 could be a direct substrate of purified recombinant TNKS1 in cell-free reactions (Fig. 8C). PAR modifications in vitro can be detected by the diffuse electrophoretic mobility of the heterogeneously PAR-modified protein or by direct detection with anti-PAR antibodies in Western blots. TNKS1 by itself was subjected to significant levels of automodification in these reactions (Fig. 8C, lower panel, lane 2). Incubation of EBNA1 with TNKS1 and NAD^+ resulted in a diffuse trailing of the EBNA1 protein as detected by anti-EBNA1 immunoblotting (Fig. 8C, top panel, lane 4). PAR-modified EBNA1 was also revealed by the reactivity to anti-PAR antibodies and the change in electrophoretic distribution of PAR modified proteins relative to auto-modified TNKS1 (Fig. 8C, lower panel, compare lanes 4 and 2). These results demonstrate that EBNA1 can be PAR modified by TNKS1 in vitro.

DISCUSSION

In this work, we provide evidence that the telomere-associated PARPs, TNKS1 and TNKS2, can bind and modify EBNA1 and negatively regulate OriP replication and plasmid maintenance. The association of TNKS proteins with EBNA1 was shown by several complementary approaches. We presented evidence that TNKS1 associates with the FR and DS regions of OriP in vivo by ChIP assay and in vitro by DNA affinity purification with partially purified components (Fig. 1). TNKS1 and TNKS2 proteins could be coimmunoprecipitated with EBNA1 from transfected cells and isolated from transfected cell extracts by GST-EBNA1 affinity purification (Fig. 2). Purified recombinant EBNA1 and TNKS1 coprecipitated in vitro, indicating that no other cellular proteins were required for binding. This interaction between EBNA1 and TNKS1 was mapped by deletion and substitution mutagenesis to the TNKS ankyrin repeats and to two RXXPDG-like motifs in the EBNA1 protein (Fig. 3). These data strongly indicate that EBNA1 contains at least two TNKS ankyrin-domain interaction motifs, that full-length TNKS1 and -2 can bind to EBNA1, and that TNKS1 binds the FR region of OriP in B cells latently infected with EBV.

The functional consequence of TNKS interaction with EBNA1 was also investigated. Our data indicate that TNKS proteins inhibit OriP replication and plasmid maintenance. First, a substitution mutant of EBNA1 (G81/425A) that disrupts TNKS binding to EBNA1 and to OriP DNA (Fig. 4) enhanced EBNA1 replication and plasmid maintenance activity in vivo (Fig. 5). Secondly, shRNA directed against TNKS1 enhanced OriP DNA replication (Fig. 6). Third, overexpression of TNKS1 and TNKS2 inhibited OriP-dependent DNA replication (Fig. 7). These data strongly indicate that TNKS proteins act as negative regulators of OriP replication and plasmid maintenance.

Inhibitory domains in EBNA1 have been reported previously. Deletion of EBNA1 amino acids 61 to 83 or 395 to 450 enhance DNA replication and plasmid maintenance activity (19, 48). These deletions overlap the two domains that we found to interact with TNKS proteins in this study and support our finding that TNKS interaction with EBNA1 inhibits DNA replication and plasmid maintenance. A protein profiling study of EBNA1 complexes found that the interaction with the ubiquitin protease HAUSP was dependent upon EBNA1 amino acid residues 395 to 440, a region which overlaps with one of the TNKS interaction motifs (19, 20). TNKS proteins were not found among the many EBNA1-associated proteins in the protein-profiling study or in two-hybrid analysis (2, 24, 38, 47). Our data indicate that the TNKS-EBNA1 complex is not highly abundant under typical cell culture conditions and may bind to only a small percentage of EBNA1 molecules in asynchronous cell cultures. Thus, it is not surprising that TNKS polypeptides were not identified in these previous screens for EBNA1-associated proteins. Also, the potential modification of EBNA1 by TNKS would also limit the recovery of a stable EBNA1-TNKS complex from cell lysates. In addition, it is possible that TNKS1 and HAUSP may compete for overlapping binding sites on EBNA1 and direct EBNA1 to different pathways, leading to either degradation or stabilization, respectively.

The mechanism of TNKS inhibition of OriP function is not completely understood. Our data suggest that poly-ADP ribosylation of EBNA1 is an important component of this inhibitory activity, but other possibilities still exist. We found that a PARP-defective mutant of TNKS2 was reduced in its ability to inhibit EBNA1 replication (Fig. 7). However, this mutant was still capable of inhibiting replication, albeit to a lesser extent than wild-type TNKS2. TNKS1 was shown to PAR modify EBNA1 in vitro in the absence of any other proteins, indicating that EBNA1 is a candidate substrate for TNKS proteins (Fig. 8C). We also found that PAR-modified EBNA1 could be isolated from EBV-positive cell extracts, suggesting that EBNA1 is subject to PAR modification in vivo (Fig. 8A and B). Previous work indicated that EBNA1 was efficiently PAR modified in cell-free reactions with DS DNA affinity-purified proteins, which included TNKS1 as well as PARP1 (14). It was also shown that small-molecule inhibitors of PARP enzymes, such as 3-AB and niacinamide, enhance OriP-dependent plasmid maintenance (14). Thus, it is likely that PAR modifications inhibit EBNA1 function at OriP and that TNKS proteins contribute to these inhibitory PAR modifications.

TNKS1 is known to increase telomere length through PAR-dependent inhibition of TRF1 binding to telomere repeats. PAR modification of TRF1 by TNKS1 leads to the loss of DNA binding and the consequent polyubiquitination and proteasome-dependent degradation of TRF1 (8). TNKS may also regulate TRF1 binding to the DS region of OriP. Previously,

we observed that TRF1 binding increases in G_2/M phase of the cell cycle, and its ectopic expression inhibited OriP replication (13). TNKS may coordinate the cell cycle-dependent binding and release of TRF1 at DS. Alternatively, TNKS may regulate the dissociation and degradation of EBNA1 from OriP, a process that may be essential for completion of DNA replication. While we have observed that ectopic expression of TNKS1 leads to a slight decrease in the steady-state levels of EBNA1 as detected by Western blotting of transfected cells, we have not been able to demonstrate that purified TNKS leads to a dissociation of EBNA1 from OriP or targeting of EBNA1 for degradation similar to that seen with TRF1 (data not shown). Such a mechanism seems plausible but awaits further investigation.

We have previously found that telomere repeat binding factors bind the nonamer repeats of the OriP DS region and regulate replication activity (13, 14). In this work, we demonstrate that another telomere-associated protein, TNKS, regulates OriP activity through a direct interaction with the EBNA1 protein and the OriP FR region. The FR region of OriP is structurally reminiscent of telomeric repeats, and this may suggest that TNKS has additional complex functions at OriP. TNKS has been proposed to bind and organize tandem arrays of TRF1 at telomeres (36), and a similar structural function of TNKS at the EBNA1 tandem arrays in FR may also be an aspect of its function at OriP. A recent study demonstrated that TNKS is required for separation of sister chromatid telomeres in late stages of metaphase (17). It is tempting to speculate that TNKS binding and PAR modification of EBNA1 regulate aspects of OriP-dependent plasmid segregation at the end of metaphase, similar to what is observed at cellular telomeres. TNKS2 overexpression has been reported to induce apoptosis in some cell types, and it is possible that some of the inhibitory activity of TNKS2 may be attributed to an indirect effect on cell proliferation (22). Although the precise mechanism of TNKS function at OriP remains unclear, our findings that TNKS binds and modifies EBNA1 provide further evidence that OriP has borrowed features of cellular telomere maintenance to regulate viral genome stability.

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REFERENCES

1. **Adams, A.** 1987. Replication of latent Epstein-Barr virus genomes in Raji cells. J. Virol. **61:**1743–1746.
2. **Aiyar, A., C. Tyree, and B. Sugden.** 1998. The plasmid replicon of EBV consists of multiple cis-acting elements that facilitate DNA synthesis by the cell and a viral maintenance element. EMBO J. **17:**6394–6403.
3. **Ambinder, R. F., M. A. Mullen, Y. N. Chang, G. S. Hayward, and S. D. Hayward.** 1991. Functional domains of Epstein-Barr virus nuclear antigen EBNA-1. J. Virol. **65:**1466–1478.
4. **Ame, J. C., C. Spenlehauer, and G. De Murcia.** 2004. The PARP superfamily. Bioessays **26:**882–893.
5. **Bianchi, A., S. Smith, L. Chong, P. Elias, and T. de Lange.** 1997. TRF1 is a dimer and bends telomeric DNA. EMBO J. **16:**1785–1794.
6. **Burkle, A.** 2001. Physiology and pathophysiology of poly(ADP-ribosyl)ation. Bioessays **23:**795–806.
7. **Burkle, A.** 2001. Poly(APD-ribosyl)ation, a DNA damage-driven protein modification and regulator of genomic instability. Cancer Lett. **163:**1–5.
8. **Chang, W., J. N. Dynek, and S. Smith.** 2003. TRF1 is degraded by ubiquitin-mediated proteolysis after release from telomeres. Genes Dev. **17:**1328–1333.
9. **Chaudhuri, B., H. Xu, I. Todorov, A. Dutta, and J. L. Yates.** 2001. Human DNA replication initiation factors, ORC and MCM, associate with oriP of Epstein-Barr virus. Proc. Natl. Acad. Sci. USA **98:**10085–10089.
10. **Chi, N. W., and H. F. Lodish.** 2000. Tankyrase is a Golgi-associated mitogen-activated protein kinase substrate that interacts with IRAP in GLUT4 vesicles. J. Biol. Chem. **275:**38437–38444.
11. **D'Amours, D., S. Desnoyers, I. D'Silva, and G. G. Poirier.** 1999. Poly(ADP-ribosyl)ation reactions in the regulation of nuclear functions. Biochem. J. **342:**249–268.
12. **Dantzer, F., M. J. Giraud-Panis, I. Jaco, J. C. Ame, I. Schultz, M. Blasco, C. E. Koering, E. Gilson, J. Menissier-de Murcia, G. de Murcia, and V. Schreiber.** 2004. Functional interaction between poly(ADP-ribose) polymerase 2 (PARP-2) and TRF2: PARP activity negatively regulates TRF2. Mol. Cell. Biol. **24:**1595–1607.
13. **Deng, Z., C. Atanasiu, J. S. Burg, D. Broccoli, and P. M. Lieberman.** 2003. Telomere repeat binding factors TRF1, TRF2, and hRAP1 modulate replication of Epstein-Barr virus OriP. J. Virol. **77:**11992–12001.
14. **Deng, Z., L. Lezina, C. J. Chen, S. Shtivelband, W. So, and P. M. Lieberman.** 2002. Telomeric proteins regulate episomal maintenance of Epstein-Barr virus origin of plasmid replication. Mol. Cell **9:**493–503.
15. **De Rycker, M., R. N. Venkatesan, C. Wei, and C. M. Price.** 2003. Vertebrate tankyrase domain structure and sterile alpha motif (SAM)-mediated multimerization. Biochem. J. **372:**87–96.
16. **Dhar, S. K., K. Yoshida, Y. Machida, P. Khaira, B. Chaudhuri, J. A. Wohlschlegel, M. Leffak, J. Yates, and A. Dutta.** 2001. Replication from oriP of Epstein-Barr virus requires human ORC and is inhibited by geminin. Cell **106:**287–296.
17. **Dynek, J. N., and S. Smith.** 2004. Resolution of sister telomere association is required for progression through mitosis. Science **304:**97–100.
18. **Gahn, T. A., and C. L. Schildkraut.** 1989. The Epstein-Barr virus origin of plasmid replication, oriP, contains both the initiation and termination sites of DNA replication. Cell **58:**527–535.
19. **Holowaty, M. N., Y. Sheng, T. Nguyen, C. Arrowsmith, and L. Frappier.** 2003. Protein interaction domains of the ubiquitin-specific protease, USP7/HAUSP. J. Biol. Chem. **278:**47753–47761.
20. **Holowaty, M. N., M. Zeghouf, H. Wu, J. Tellam, V. Athanasopoulos, J. Greenblatt, and L. Frappier.** 2003. Protein profiling with Epstein-Barr nuclear antigen-1 reveals an interaction with the herpesvirus-associated ubiquitin-specific protease HAUSP/USP7. J. Biol. Chem. **278:**29987–29994.
21. **Humme, S., G. Reisbach, R. Feederle, H. J. Delecluse, K. Bousset, W. Hammerschmidt, and A. Schepers.** 2003. The EBV nuclear antigen 1 (EBNA1) enhances B cell immortalization several thousandfold. Proc. Natl. Acad. Sci. USA **100:**10989–10994.
22. **Kaminker, P. G., S. H. Kim, R. D. Taylor, Y. Zebarjadian, W. D. Funk, G. B. Morin, P. Yaswen, and J. Campisi.** 2001. TANK2, a new TRF1-associated poly(ADP-ribose) polymerase, causes rapid induction of cell death upon overexpression. J. Biol. Chem. **276:**35891–35899.
23. **Kieff, E.** 1996. Epstein-Barr virus and its replication, p. 2343–2396. *In* D. Knipe and P. M. Howley (ed.), Field's virology, 3rd ed., vol. 2. Lippincott-Raven Publishers, Philadelphia, Pa.
24. **Kim, A. L., M. Maher, J. B. Hayman, J. Ozer, D. Zerby, J. L. Yates, and P. M. Lieberman.** 1997. An imperfect correlation between the DNA replication activity of Epstein-Barr virus nuclear antigen 1 (EBNA1) and the binding to the nuclear import receptor, Rch1/importin alpha. Virology **239:**340–351.
25. **Kraus, W. L., and J. T. Lis.** 2003. PARP goes transcription. Cell **113:**677–683.
26. **Lee, M. A., M. E. Diamond, and J. L. Yates.** 1999. Genetic evidence that EBNA-1 is needed for efficient, stable latent infection by Epstein-Barr virus. J. Virol. **73:**2974–2982.
27. **Leight, E. R., and B. Sugden.** 2000. EBNA-1: a protein pivotal to latent infection by Epstein-Barr virus. Rev. Med. Virol. **10:**83–100.
28. **Mackey, D., and B. Sugden.** 1999. The linking regions of EBNA1 are essential for its support of replication and transcription. Mol. Cell. Biol. **19:**3349–3359.
29. **Marechal, V., A. Dehee, R. Chikhi-Brachet, T. Piolot, M. Coppey-Moisan, and, N. J. C.** 1999. Mapping EBNA-1 domains involved in binding to metaphase chromosomes. J. Virol. **73:**4385–4392.
30. **Paddison, P. J., A. A. Caudy, E. Bernstein, G. J. Hannon, and D. S. Conklin.** 2002. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev. **16:**948–958.
31. **Rawlins, D. R., G. Milman, S. D. Hayward, and G. S. Hayward.** 1985. Sequence-specific DNA binding of the Epstein-Barr virus nuclear antigen

(EBNA-1) to clustered sites in the plasmid maintenance region. Cell **42:** 859–868.
32. **Rickinson, A. B., and E. Kieff.** 1996. Epstein-Barr Virus, p. 2397–2446. Fields virology, 3rd ed. Lippincott-Raven Publishers, Philadelphia, Pa.
33. **Sbodio, J. I., and N. W. Chi.** 2002. Identification of a tankyrase-binding motif shared by IRAP, TAB182, and human TRF1 but not mouse TRF1. NuMA contains this RXXPDG motif and is a novel tankyrase partner. J. Biol. Chem. **277:**31887–31892.
34. **Sbodio, J. I., H. F. Lodish, and N. W. Chi.** 2002. Tankyrase-2 oligomerizes with tankyrase-1 and binds to both TRF1 (telomere-repeat-binding factor 1) and IRAP (insulin-responsive aminopeptidase). Biochem. J. **361:**451–459.
35. **Schepers, A., M. Ritzi, K. Bousset, E. Kremmer, J. L. Yates, J. Harwood, J. F. Diffley, and W. Hammerschmidt.** 2001. Human origin recognition complex binds to the region of the latent origin of DNA replication of Epstein-Barr virus. EMBO J. **20:**4588–4602.
36. **Seimiya, H., and S. Smith.** 2002. The telomeric poly(ADP-ribose) polymerase, tankyrase 1, contains multiple binding sites for telomeric repeat binding factor 1 (TRF1) and a novel acceptor, 182-kDa tankyrase-binding protein (TAB182). J. Biol. Chem. **277:**14116–14126.
37. **Shall, S., and G. de Murcia.** 2000. Poly (ADP-ribose) polymerase 1: what have we learned from the deficient mouse model? Mutat. Res. **460:**1–15.
38. **Shire, K., D. F. Ceccarelli, T. M. Avolio-Hunter, and L. Frappier.** 1999. EBP2, a human protein that interacts with sequences of the Epstein-Barr virus nuclear antigen 1 important for plasmid maintenance. J. Virol. **73:** 2587–2595.
39. **Smith, S.** 2001. The world according to PARP. Trends Biochem. Sci. **26:** 174–179.
40. **Smith, S., and T. de Lange.** 1999. Cell cycle dependent localization of the telomeric PARP, tankyrase, to nuclear pore complexes and centrosomes. J. Cell Sci. **112:**3649–3656.
41. **Smith, S., and T. de Lange.** 2000. Tankyrase promotes telomere elongation in human cells. Curr. Biol. **10:**1299–1302.
42. **Smith, S., I. Giriat, A. Schmitt, and T. de Lange.** 1998. Tankyrase, a poly-(ADP-ribose) polymerase at human telomeres. Science **282:**1484–1487.
43. **Sugden, B., and E. R. Leight.** 2001. EBV's plasmid replicon: an enigma in cis and trans. Curr. Top. Microbiol. Immunol. **258:**3–11.
44. **Tulin, A., and A. Spradling.** 2003. Chromatin loosening by poly(ADP)-ribose polymerase (PARP) at Drosophila puff loci. Science **299:**560–562.
45. **Tulin, A., D. Stewart, and A. C. Spradling.** 2002. The Drosophila heterochromatic gene encoding poly(ADP-ribose) polymerase (PARP) is required to modulate chromatin structure during development. Genes Dev. **16:**2108–2119.
46. **Virag, L., and C. Szabo.** 2002. The therapeutic potential of poly(ADP-ribose) polymerase inhibitors. Pharmacol. Rev. **54:**375–429.
47. **Wang, Y., J. E. Finan, J. M. Middledorp, and S. D. Hayward.** 1997. P32/TAP, a cellular protein that interacts with EBNA-1 of Epstein-Barr virus. Virology **236:**18–29.
48. **Wu, H., P. Kapoor, and L. Frappier.** 2002. Separation of the DNA replication, segregation, and transcriptional activation functions of Epstein-Barr nuclear antigen 1. J. Virol. **76:**2480–2490.
49. **Yates, J., and S. M. Camiolo.** 1988. Dissection of DNA replication and enhancer activation functions of Epstein-Barr virus nuclear antigen 1. Cancer Cells **6:**197–205.
50. **Yates, J. L., and N. Guan.** 1991. Epstein-Barr virus-derived plasmids replicate only once per cell cycle and are not amplified after entry into cells. J. Virol. **65:**483–488.
51. **Yates, J. L., N. Warren, P. Reisman, and B. Sugden.** 1984. A *cis*-acting element from Epstein-Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. Proc. Natl. Acad. Sci. USA **81:**3806–3810.
52. **Yates, J. L., N. Warren, and B. Sugden.** 1985. Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. Nature **313:** 812–815.