JOURNAL OF VIROLOGY, June 2001, p. 5059–5068 Vol. 75, No. 11
0022-538X/01/$04.00+0 DOI: 10.1128/JVI.75.11.5059–5068.2001
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Activation of TRAF5 and TRAF6 Signal Cascades Negatively Regulates the Latent Replication Origin of Epstein-Barr Virus through p38 Mitogen-Activated Protein Kinase

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Received 30 November 2000/Accepted 9 March 2001

Latent Epstein-Barr virus (EBV) is maintained by the virus replication origin *oriP* that initiates DNA replication with the viral *oriP*-binding factor EBNA1. However, it is not known whether *oriP*'s replicator activity is regulated by virus proteins or extracellular signals. By using a transient replication assay, we found that a low level of expression of viral signal transduction activator latent membrane protein 1 (LMP1) suppressed *oriP* activity. The binding site of the tumor necrosis factor receptor-associated factor (TRAF) of LMP1 was essential for this suppressive effect. Activation of the TRAF signal cascade by overexpression of TRAF5 and/or TRAF6 also suppressed *oriP* activity. Conversely, blocking of TRAF signaling with dominant negative mutants of TRAF5 and TRAF6, as well as inhibition of a downstream signal mediator p38 MAPK, released the LMP1-induced *oriP* suppression. Furthermore, activation of TRAF6 signal cascade by lipopolysaccharides (LPS) resulted in loss of EBV from Burkitt's lymphoma cell line Akata, and inhibition of p38 MAPK abolished the suppressive effect of LPS. These results suggested that the level of *oriP* activity is regulated by LMP1 and extracellular signals through TRAF5- and TRAF6-mediated signal cascades.

Epstein-Barr virus (EBV) is related to Burkitt's lymphoma, T-cell lymphoma, gastric carcinoma, infectious mononucleosis, and opportunistic lymphoma in immunosuppressed patients (31), but resting memory B lymphocytes are normal cells infected latently with EBV in vivo (43, 44). During latent infection, the 170-kb EBV genome forms a circular plasmid DNA and is maintained by the 2.2-kb region *oriP* containing an origin of bidirectional DNA replication (62, 64). *oriP* is comprised of two EBNA1-binding elements, dyad symmetry (DS) and family of repeats (FR), separated by 960 bp. The DS element functions as a replication origin (16, 20, 43, 55, 65), and the FR element plays a major role in nuclear retention of the genome (26, 41). DNA replication from *oriP* (DS-dependent replication) requires only the viral *oriP*-binding protein EBNA1 and occurs once in a single S phase through a mechanism of replication licensing (21, 56, 62, 63). However, it is not known whether *oriP*'s replicator activity is regulated by virus proteins or extracellular signals.

In contrast to these studies, in some EBV-positive lymphoma cell lines, replication of the EBV genome is initiated mostly in a broad initiation zone distant from *oriP* (DS-independent replication) (25). The occurrence of DS-independent replication was initially found in Raji and Daudi by 2D gel analysis (38) and then was demonstrated using the *oriP*-containing plasmid in several cell lines, including C33, HEK293, and P3HR1 (2, 35; unpublished data). Recently, Norio et al. (49) showed more direct evidence, using recombinant EBV virus, that the DS element is dispensable for EBV replication in BL30 and a P3HR1 clone. When DS-independent replication occurs, the DS-dependent replication from *oriP* is rare (38). The initiation region used for DS-independent replication may be preferentially used over *oriP* in lymphomas. Alternatively, the *oriP* activity may be negatively regulated by latent virus proteins expressing in these cell lines. To explore this possibility, we examined the effect of latent membrane protein 1 (LMP1) on *oriP* activity. LMP1 is an EBV integrated membrane protein that plays an essential role in immortalization of human B lymphocytes by EBV (29, 34, 45) and transforms rodent fibroblasts (3, 61). LMP1 induces activation of several signal mediators: NF-κB (22, 42), c-Jun amino-terminal kinase (JNK) (12, 32), extracellular signal-regulated kinases (ERKs) (52), p38 mitogen-activated protein kinase (MAPK) (13), and Janus kinase 3 (17). LMP1 has two C-terminal terminal activating regions, CTAR1/TES1 (amino acids [aa] 187 to 232) and CTAR2/TES2 (aa 351 to 386), which are responsible for activation of these signal mediators. CTAR1/TES1 contains the PxQxT motif that is a binding site for the tumor necrosis factor receptor-associated factors (TRAFs) (9, 22, 42, 46). TRAFs are the signal mediators of the cellular membrane receptors of TNFR and Toll/IR-1R superfamilies and initiate distinct but overlapping signal cascades (7, 23, 46, 47). Among the six TRAFs identified to date, TRAF1, TRAF2, TRAF3, and TRAF5 but not TRAF6 associate with the PxQxT motif of CTAR1/TES1 (5, 9, 10, 46, 52). TRAF2 also associates indirectly with CTAR2/TES2 via TRADD and RIP and mediates signal cascades leading to activation of NF-κB and JNK (12, 14, 24, 25, 32, 33, 58).

In this study, we showed that *oriP* activity is negatively regulated by the TRAF5-mediated signal initiated from LMP1 and the TRAF5- and TRAF6-mediated signals from cellular

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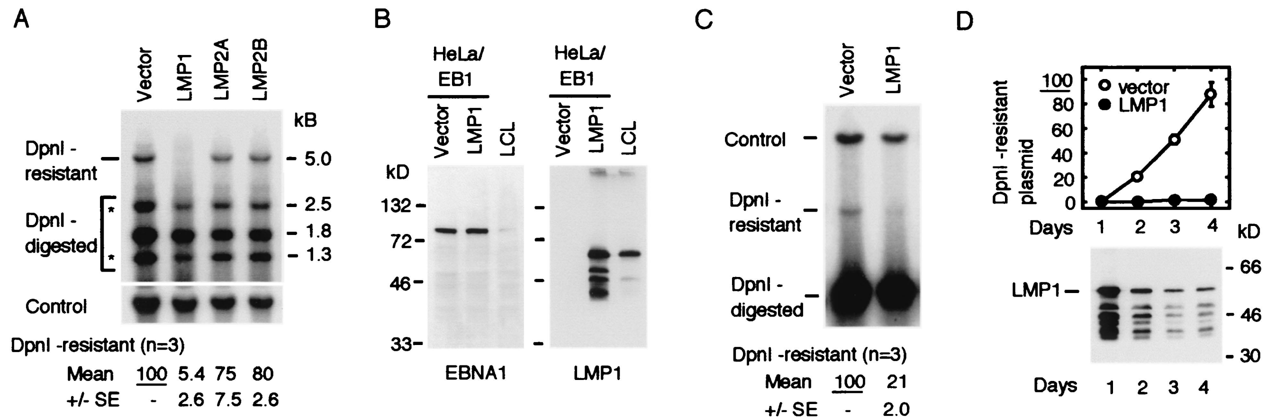

FIG. 1. Expression of LMP1 suppressed replication of the *oriP* plasmid in HeLa/EB1 cells. (A) Transient replication assay of the *oriP* plasmid. The *oriP* plasmid (2 μg), the control plasmid (1 μg), and the expression plasmid of LMP1, LMP2A, or LMP2B (0.5 μg) were transfected. Total amounts of plasmids were adjusted to 3.5 μg with the vector plasmid. Hirt's extracts were prepared 2 days after transfection, and plasmids were analyzed by *Dpn*I digestion and Southern blot hybridization. The linearized *Dpn*I-resistant plasmid (5.0 kb) and three *Dpn*I-digested fragments (2.5, 1.8, and 1.3 kb) are shown. Two fragments indicated by asterisks (2.5 and 1.3 kb) are products of replication intermediates that were accumulated by the replication fork barrier at the FR element of *oriP*. Amounts of the *Dpn*I-resistant plasmid (replicated plasmid) are normalized with that of the control plasmid and shown below. Data represent averages of three experiments with the standard errors (SE). (B) Expression of LMP1 and EBNA1 in transfected cells. Short polypeptides reacted with LMP1 antibody were digested products of LMP1. As a control, a similar number of LCL cells were analyzed in a parallel lane. (C) Replication assay of the DS plasmid. Experimental conditions are described above for panel A. Data represent averages of three experiments with the standard errors (SE). (D) Time course of the *oriP* plasmid replication. Transfected plasmids were the same as in panel A, and cells were collected for *Dpn*I assay at the days indicated. A summary of two experiments is shown. Expression of LMP1 in one experiment is shown at the bottom. The same amount of total proteins was loaded on each lane.

receptors. We also identified the p38 MAPK, a common downstream kinase in these signal cascades, as playing an important role in this negative regulation of EBV replication.

MATERIALS AND METHODS

Plasmids. The *oriP* plasmid (KORI) containing the *oriP* region, the DS plasmid (KD11), the SV40 plasmid, and the internal control plasmid were described previously (55, 56). The expression plasmids of the LMP1 deletion derivatives were constructed from the LMP1 expression plasmid pNH-LMP1 (59). The plasmids expressing the LMP1 point mutants were described elsewhere (25). The expression plasmids of TRAF and their dominant negative mutants were also described elsewhere (27). A cDNA clone of mouse p38 MAPK was obtained by PCR from 15-day embryos using primers according to the p38 sequence (19) and was inserted into the expression vector pactEF (50). The dominant negative p38 mutant, $p38^{AGF}$, was prepared by replacing the wild-type Thr^{180} (ACA) and Tyr^{182} (TAC) with an Ala (GCC) and a Phe (TTC), respectively, by oligonucleotide-directed mutagenesis.

Transient replication assay. The *oriP* plasmid (2 μg) was transfected into HeLa/EB1 cells (2×10^6) (55) with the unmethylated control plasmid (1 μg) and the effector plasmid(s) by the calcium phosphate method. Transfection efficiency was estimated as 50% on average. After transfection, cells were cultured for 2 days in the experiments described in Fig. 1, 2, and 3, and for 3 days in the experiments described in Fig. 4, 5, and 6. *Dpn*I digestion and Southern hybridization analysis were performed as described previously (56) using the *oriP* region (*Eco*RI-*Sac*II) as a hybridization probe. The salt concentration in the *Dpn*I reaction buffer was lowered to 50 mM in this study. Aliquots of about 1/10 of the extracts were used for a single *Dpn*I assay. Radioisotope signals on Southern blots were analyzed quantitatively with a BAS2000 image analyzer (Fuji). The same membranes were rehybridized to detect the control plasmid. The hybridization signal of the *Dpn*I-resistant *oriP* plasmid was normalized with the signal of the internal control plasmid in the same sample and was represented relatively to that of the vector-transfected sample. Expression of LMP1 and of EBNA1 was analyzed using a monoclonal antibody against LMP1 (S12) and a rabbit polyclonal antibody against EBNA1.

[^{3}H]thymidine incorporation of the LMP1/GFP expressing cells. The pNH-LMP1 (0 to 4 μg) was transfected with the green fluorescent protein (GFP) expression plasmid pEGFP-C1 (4 μg) into HeLa/EB1 (2×10^6) in 100-mm dishes. Transfection efficiency was determined by counting GFP-expressing cells at 24 h after transfection. Then, cells were replated into 96-well plates (2,000 cells in 200 μl) and were cultured for 4 h in the presence of [^{3}H]thymidine (1 μCi). Incorporation by the GFP/LMP1-expressing cells (2,000 cells) was calculated using the following equation: $cpm^G = [cpm^S - (1 - f) \times cpm^O]/f$, where cpm^G is uptake by GFP-expressing cells; cpm^S is total uptake by cells transfected with the GFP plasmid and the LMP1 plasmid; cpm^O is total uptake by cells transfected with the GFP plasmid alone; and f is the ratio of GFP-expressing cells.

NF-κB activity. The NF-κB reporter plasmid pκB-tkLuc (27) (2 μg) was transfected with pSV-β-gal (1 μg) and pNH-LMP1 (1 μg) into HeLa/EB1 (10^6) in 60-mm dishes. Luciferase activity was determined 2 days after transfection, and β-galactosidase activity was used as internal control.

Stimulation of Burkitt's lymphoma B cell lines. Bacterial lipopolysaccharide (LPS) (Difco) (5 mg/ml) was added at 10 μg/ml to growing B cells (15 ml; 10^5 cells/ml). Two days after stimulation, cells (10 ml) were collected. Fresh culture medium containing LPS (10 ml) was added to the rest of the cells (5 ml), which were then cultured again for 2 days. Total DNA was prepared from these LPS-stimulated cells and unstimulated cells. The total DNA (4 μg) was digested with *Bam*HI and analyzed by Southern hybridization methods using an EBV (B95-8) *Bam*HI-C fragment for a probe. Hybridized signals were analyzed quantitatively with a BAS2000 image analyzer.

RESULTS

Expression of LMP1 induced suppression of *oriP* plasmid replication in HeLa/EB1 cells. We have previously demonstrated that when the *dam*-methylated *oriP* plasmid is transfected into HeLa/EB1 cells, the *Dpn*I-resistant replicated *oriP* plasmid is accumulated during 2 days after transfection (55, 56; Fig. 1A). When we analyzed the recovered *oriP* plasmid by *Dpn*I digestion and Southern hybridization using the *oriP* region for a probe, we detected one linearized *Dpn*I-resistant plasmid (5.0 kb) and five *Dpn*I-digested fragments (2.5, 1.8, 1.3, 0.8, and 0.6 kb). The 0.8-kb and 0.6-kb fragments are not shown in the figures. Among these *Dpn*I-digested fragments, the 1.8-kb, 0.8-kb, and 0.6-kb fragments were predicted from the restriction sites. The 2.5-kb and 1.3-kb fragments were the products of replication intermediates accumulated by a replication fork barrier at an FR element (16). Therefore, the

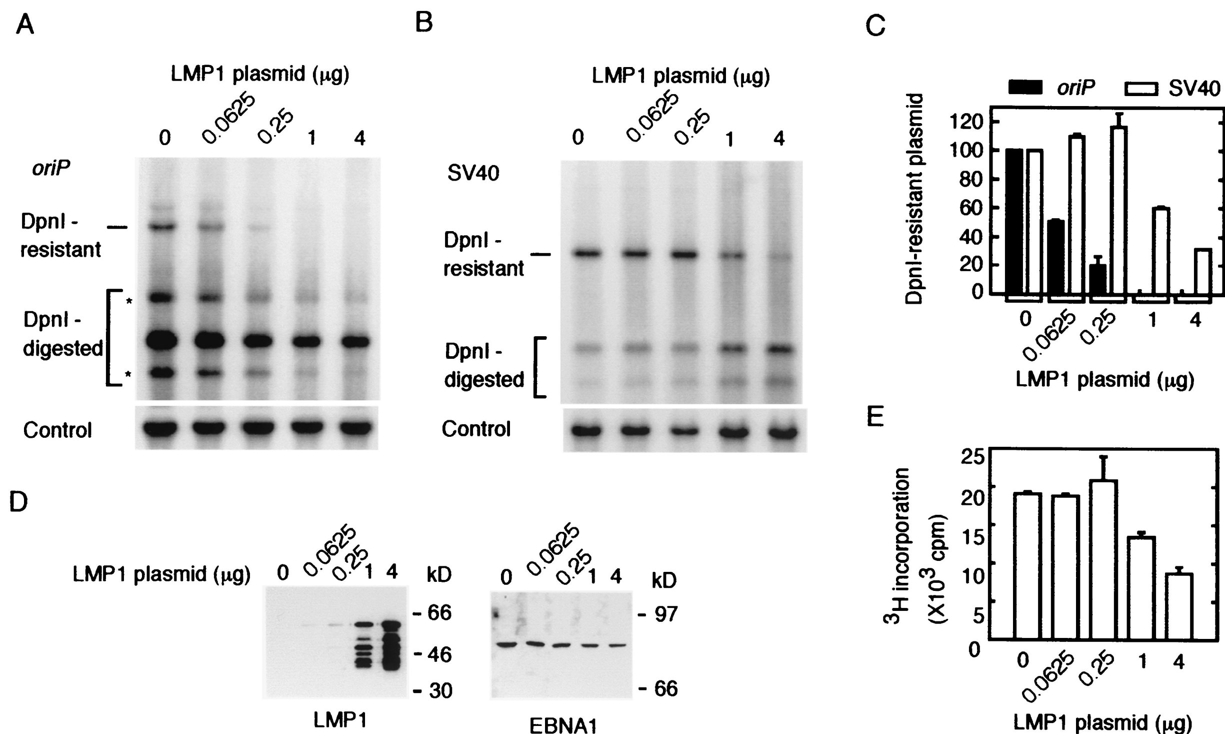

FIG. 2. The LMP1 plasmid dose dependency of *oriP* suppression. (A) Transient replication assay of the *oriP* plasmid. The *oriP* plasmid (2 μg), the control plasmid (1 μg), and the LMP1 plasmid (0, 0.0625, 0.25, 1, and 4 μg) were transfected. Total amounts of plasmids were 7 μg. Two fragments indicated by asterisks are products of replication intermediates. (B) Transient replication assay of the SV40 plasmid. The SV40 plasmid (2 μg) was transfected with other plasmids as described above for panel A. (C) Summary of experiments described in panels A and B. Data represent averages of two experiments with standard errors (SE). (D) Expression of LMP1 and EBNA1 in the transfected cells. (E) [^{3}H]thymidine incorporation of the LMP1/GFP expressing cells. The LMP1 plasmid was transfected with pEGFP-C1 (4 μg). Data represent averages of three experiments with standard errors (SE).

amount of these replication intermediates was less than that of the 1.8-kb *Dpn*I-digested fragment and related to the amount of replicated *Dpn*I-resistant plasmid. To examine the effect of LMP1 expression on *oriP* activity, we cotransfected the LMP1 expression plasmid in this transient replication assay and found that expression of LMP1 significantly suppressed replication of the *oriP* plasmid (Fig. 1A). The amount of replicated plasmid was normalized to the amount of the internal control plasmid in the same sample and then was compared. The replicated plasmid in the LMP1-transfected cells was about 5% of that of the vector-transfected cells. We also examined the other latent membrane proteins LMP2A and LMP2B but observed only a weak suppression of *oriP* activity. Western analysis confirmed that expression of LMP1 did not affect expression of EBNA1, suggesting that insufficient expression of EBNA1 was not a cause of *oriP* suppression (Fig. 1B). Like the *oriP* plasmid, the DS plasmid containing only the replication origin was also sensitive to LMP1 expression, indicating that the origin element was responsible for LMP1-induced suppression (Fig. 1C). Suppressive effects of LMP1 continued for at least 4 days, while expression of LMP1 was highest 1 day after transfection and then decreased significantly at later time points (Fig. 1D). We then examined the dose dependency of the LMP1 plasmid for *oriP* suppression and found that the lowest dose of LMP1 plasmid (0.0625 μg) was significantly effective for *oriP* suppression (Fig. 2A). The amount of LMP1 plasmid required for 50% inhibition was 0.0625 μg for 2×10^6 cells (Fig. 2C). Fig. 2E shows the amount of LMP1 expressed in these transfected cells. When 0.5 μg of LMP1 plasmid was transfected into 2×10^6 cells, the amount of LMP1 was about equal to that expressed in an EBV-positive lymphoma cell line, Raji (results not shown).

A low level of LMP1 expression that induced *oriP* suppression did not inhibit cell growth. Several studies have demonstrated that high levels of LMP1 expression inhibit cell growth (11, 18, 30). To monitor the growth inhibitory effect of LMP1 in our transient replication assay, we used the SV40 plasmid containing SV40 origin and the T-antigen gene (56). The SV40 plasmid was transfected with the LMP1 plasmid and its replication was analyzed at 2 days after transfection (Fig. 2B). In contrast to the *oriP* plasmid, a lower dose of the LMP1 plasmid (0.0625 and 0.25 μg) did not suppress the SV40 plasmid replication. With a higher dose of LMP1 plasmid (1 and 4 μg), replication of the SV40 plasmid was suppressed by 60 and 40%, respectively, suggesting that the growth inhibitory effect of LMP1 appeared at these doses. To confirm this result by another assay, [^{3}H]thymidine incorporation by LMP1-expressing cells was examined. We transfected the same amount of the GFP plasmid (4 μg) with several different amounts of the LMP1 plasmid (0 to 4 μg) into HeLa/EB1. ^{3}H incorporation by the GFP-expressing cells was estimated from the ratio of GFP-positive cells and ^{3}H incorporation by total cells. ^{3}H incorporation by GFP-positive cells was not affected with lower doses of LMP1 plasmid (0.0625 and 0.25 μg) but was reduced by 71

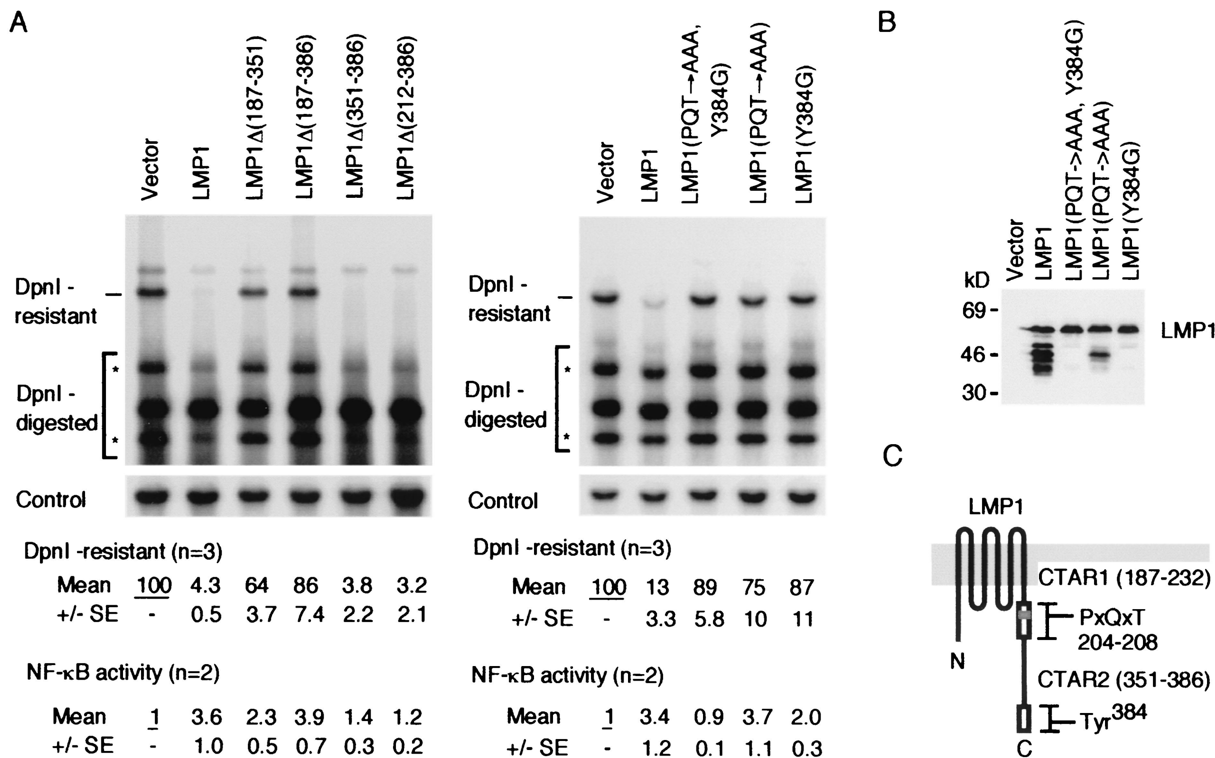

FIG. 3. Mutational analysis of LMP1 domains for *oriP* suppression. (A) Transient replication assay of the *oriP* plasmid. The *oriP* plasmid (2 μg), the control plasmid (1 μg), and the LMP1 plasmid (0.5 μg) were transfected. Total amounts of plasmids were 3.5 μg. The amino acid numbers in parentheses indicate the region deleted from LMP1. Amino acid substitutions in the LMP1 point mutants are also shown in parentheses. Normalized amounts of *Dpn*I-resistant plasmid are shown below. Data represent averages of three experiments with standard errors (SE). Activation of NF-κB activity by these LMP1 mutants was examined using the pκB-Luc luciferase reporter plasmid. Data represent averages of two experiments with standard errors (SE). (B) Expression of LMP1 and its point mutants. The same amount of total protein was loaded in each lane. (C) Structure of LMP1.

and 46% with higher doses (1 and 4 μg) (Fig. 2E). Thus, a lower level of LMP1 expression that induced *oriP* suppression did not inhibit cell growth.

The TRAF-binding motif of LMP1 was mostly responsible for induction of *oriP* suppression. To determine the signal cascade leading to *oriP* suppression, we examined the LMP1 domain responsible for induction of *oriP* suppression. An LMP1 mutant, LMP1Δ(351-386), had a deletion of CTAR2 (aa 351 to 386) but retained CTAR1 (aa 187 to 232). Expression of LMP1Δ(351-386) suppressed *oriP* replication to the same extent as that of the wild-type molecule (Fig. 3A). Similarly, LMP1Δ(212-386), in which CTAR2 and most of CTAR1 were deleted but the PxQxT motif (aa 204 to 208) was retained, also showed the wild-type function. However, complete deletion of CTAR1 and CTAR2, including the PxQxT motif in LMP1Δ(187-386), eliminated most of the suppressive effect, and the internal deletion of CTAR1 in LMP1Δ(187-351) showed only a weak suppressive effect. This indicated the importance of the PxQxT motif for *oriP* suppression, which was further confirmed using LMP1 point mutants. The LMP1 mutant LMP1(PQT→AAA) had amino acid substitutions Pro^{204} to Ala, Gln^{206} to Ala, and Thr^{208} to Ala in the PxQxT motif and did not bind TRAFs (32, 33). As shown in Fig. 3A, LMP1 (PQT→AAA) lost most of the suppressive effect.

We also examined Tyr^{384} in CTAR2, the amino acid residue important for binding of the TRADD-TRAF2 complex (14, 24, 33). Unexpectedly, the point mutant LMP1(Y384G) showed complete loss of the suppressive effect, although CTAR2 was not required for most of the LMP1's suppressive effect (Fig. 3A). Western analysis confirmed expression of a similar level of LMP1 protein (Fig. 3B). As the Y384G mutation abolishes the binding of the TRADD-TRAF2 complex to the CTAR2 domain, this result suggested that the absence of the TRADD-TRAF2 complex on the CTAR2 domain may have induced a large conformational change in LMP1 and interfered with the function of CTAR1.

Overexpression of TRAF5 and TRAF6 suppressed *oriP* activity. TRAF1, TRAF2, TRAF3, and TRAF5 associate with the PxQxT motif of CTAR1 (5, 9, 10, 46, 53). To identify the TRAF-mediated signal cascade leading to *oriP* suppression, we overexpressed each TRAF in the absence of LMP1 and examined its effect on *oriP* activity. TRAF expression vectors used in this experiment were constructed with the same mammalian expression plasmid, and cells were collected 3 days after transfection because transfection of a large amount of plasmids reduced the efficiency of *oriP* replication. By this transient replication assay, we found that overexpression of TRAF5, but not of TRAF2 or TRAF3, reduced *oriP* activity by 60% (Fig. 4A). We also examined TRAF6, although it did not bind to the PxQxT motif, and found that overexpression of TRAF6 suppressed *oriP* activity to a similar extent as TRAF5.

Because TRAF2 or TRAF3 might work synergistically with

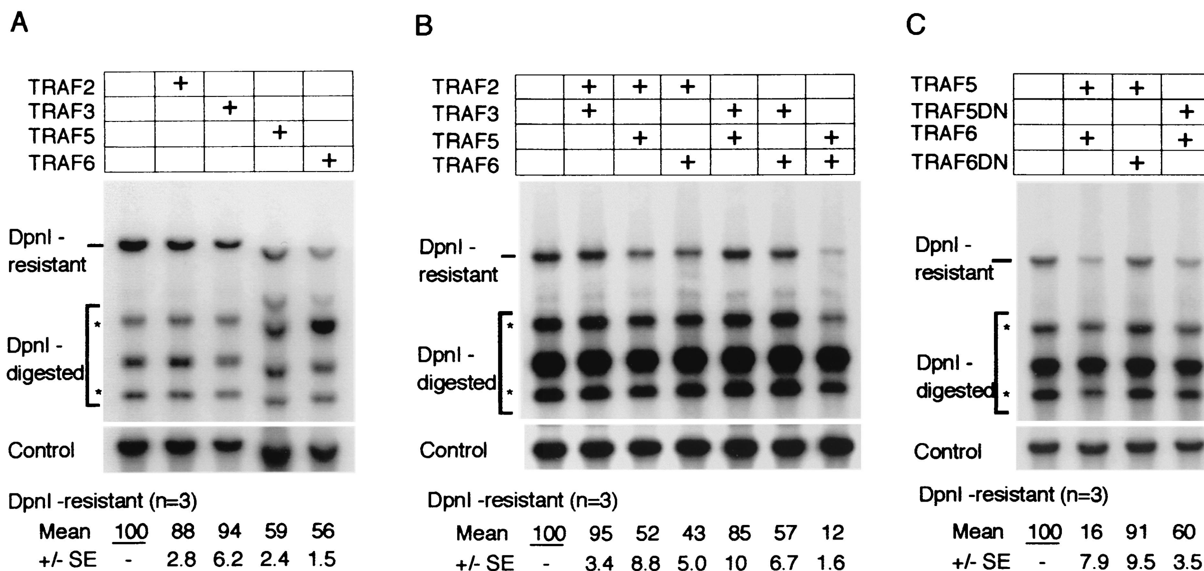

FIG. 4. Effects of TRAF expression on *oriP* activity. Shown are the results of a transient replication assay of the *oriP* plasmid. (A) Effects of single expression of TRAF. The *oriP* plasmid (2 μg), the control plasmid (0.5 μg), and the TRAF expression plasmid (4 μg) were transfected. Total amounts of plasmids were 6.5 μg. (B) Effects of coexpression of TRAFs. The *oriP* plasmid (2 μg), the control plasmid (0.5 μg), and the TRAF expression plasmids (4 μg each) were transfected. Total amounts of plasmids were 10.5 μg. (C) Effects of coexpression of TRAF and the dominant negative TRAF mutants, TRAF5DN and TRAF6DN. Normalized amounts of *Dpn*I-resistant plasmid are shown below. Data represent averages of three experiments with standard errors (SE). Two fragments indicated by asterisks are products of replication intermediates.

TRAF5 and TRAF6, we examined combinations of TRAFs in a similar assay. However, coexpression of TRAF2 with TRAF5 or TRAF6 showed suppressive effects similar to those of TRAF5 and TRAF6 alone, indicating that TRAF2 did not interfere with the functions of TRAF5 or TRAF6 (Fig. 4B). Coexpression of TRAF3 reduced TRAF5-induced *oriP* suppression but it did not affect TRAF6-induced suppression. In contrast, when TRAF5 and TRAF6 were coexpressed, their suppressive effects were added and the *oriP* activity was reduced by 10%. This additive effect was not observed when one of TRAF5 and TRAF6 had a deletion in the effector domain (Fig. 4C). We also examined expression of TRAF1. Like TRAF2, expression of TRAF1 neither suppressed *oriP* activity nor interfered with the suppressive effects of TRAF5 and TRAF6 (results not shown).

***oriP* was activated by inhibition of the TRAF-mediated signaling in HeLa/EB1.** Because overexpression of TRAF5 and TRAF6 induced *oriP* suppression, we next examined the effects of inhibition of the TRAF-mediated signaling. Under normal culture conditions, the TRAF-mediated signal cascade was activated at a low level. We inhibited this basal activity of TRAF signaling by expressing the dominant negative mutant of TRAF, TRAFDN, which had deletions in the amino-terminal effector domain. When TRAF5DN or TRAF6DN was expressed, *oriP* replication was moderately activated, by 126 or 150% (Fig. 5A). Coexpression of TRAF5DN and TRAF6DN showed further activation, by 242%, indicating that the effects of TRAF5DN and TRAF6DN were added like those of TRAF5 and TRAF6. This result confirmed that TRAF5- and TRAF6-mediated signal cascades negatively regulated *oriP* activity and also indicated that *oriP* was sensitive to even a basal level of signaling in HeLa/EB1 cells.

Interestingly, expression of TRAF3DN also activated *oriP* replication by 150%, although overexpression of TRAF3 did not suppress *oriP*. Coexpression of TRAF3DN with TRAF5DN or TRAF6DN showed further activation, by 215 and 325%, respectively. An in vitro study indicated that TRAF5 does not bind to the PxQxT motif directly, but that TRAF3 forms TRAF3-TRAF5 hetero-oligomers through the amino-terminal effector domain and mediates binding of TRAF5 to the motif (51). Because TRAF3DN lacked the effector domain, TRAF3DN competitively inhibited the binding of TRAF3-TRAF5 hetero-oligomers to the PxQxT motif and inhibited TRAF5-mediated signaling. Therefore, TRAF3DN was functionally similar to TRAF5DN.

We also examined the effects of TRAFDN on LMP1-induced suppression. Single expression of TRAF3DN or TRAF5DN did not affect the LMP1Δ(212-386)-induced suppression of *oriP* activity, but coexpression of both mutants partially released the suppression, confirming that TRAF5 mediated the LMP1-induced signal for *oriP* suppression (Fig. 5B). Although LMP1 does not bind TRAF6, TRAF6DN also partially released *oriP* suppression when it was coexpressed with TRAF3DN or TRAF5DN. This suggested that the LMP1-induced (TRAF5-mediated) signal and the TRAF6-mediated signal had a common downstream mediator for *oriP* suppression.

The p38 MAPK regulated *oriP* activity. Because expression of LMP1, TRAF5, or TRAF6 induced activation of p38 MAPK in HeLa cells (4, 13; unpublished data), the kinase was a candidate common signal mediator for *oriP* regulation. We examined whether p38 MAPK was involved in the signal cascade leading to *oriP* suppression. Under the condition that *oriP* replication was suppressed by about 35% with LMP1, expression of the dominant negative mutant of p38 MAPK, p38^{AGF}, released the LMP1-induced *oriP* suppression by about 90% (Fig. 6A). Similarly, treatment of cells with the specific inhib-

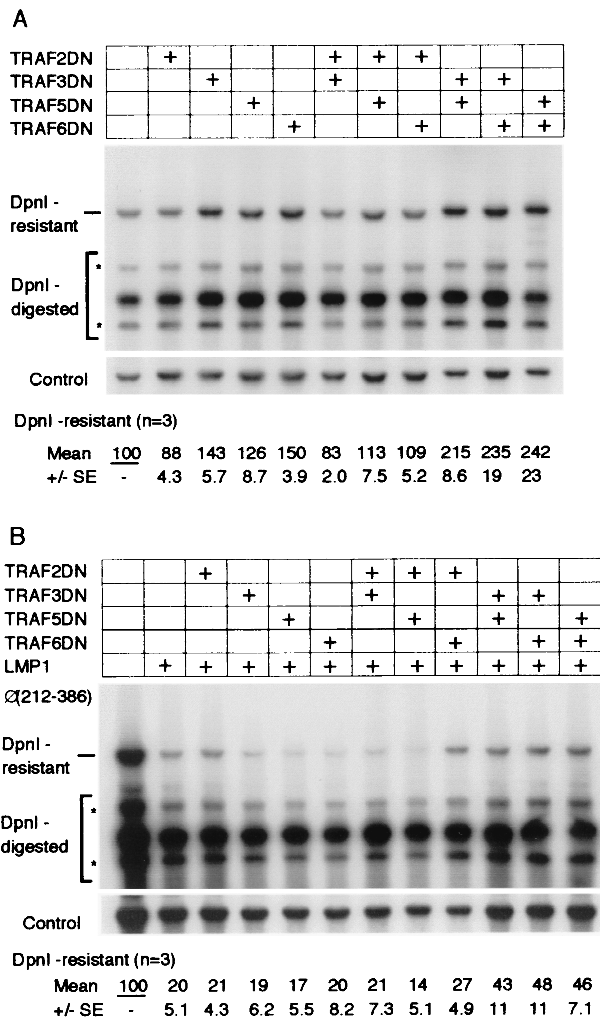

FIG. 5. Effects of dominant negative TRAF expression on *oriP* activity. Shown are the results of a transient replication assay of the *oriP* plasmid. (A) Effects of dominant negative TRAF (TRAFDN) in the absence of LMP1 expression. The *oriP* plasmid (2 μg), the control plasmid (0.5 μg), and the expression plasmids of TRAFDN (4 μg each) were transfected. Total amounts of plasmids were 10.5 μg. (B) Effects of dominant negative TRAFDN on LMP1-induced suppression. The *oriP* plasmid (2 μg), the control plasmid (0.5 μg), the TRAF expression plasmids (4 μg each), and LMP1Δ(212-386) (0.25 μg) were transfected. Total amounts of plasmids were 10.75 μg. Normalized amounts of *Dpn*I-resistant plasmid are shown at the bottom. Data represent averages of three experiments with standard errors (SE). Two fragments indicated by asterisks are products of replication intermediates.

itor of p38 MAPK, SB203580 (20 μM), also released *oriP* suppression. In contrast, the wild-type p38 MAPK did not affect *oriP* replication. Because $p38^{AGF}$ and SB203580 did not stimulate [^{3}H]thymidine incorporation, these results suggested that p38 MAPK was a downstream mediator of LMP1 for *oriP* suppression. Furthermore, in the absence of LMP1, expression of $p38^{AGF}$ also activated *oriP* activity by 195%, and treatment with SB208530 showed further activation, by 315%. This indicated that *oriP* activity was negatively regulated by p38 MAPK and that its basal level activity in HeLa/EB1 cells reduced *oriP* replication by threefold.

We next examined the involvement of the *ras*-raf1-MEK-ERK signal pathway in *oriP* regulation (52). Expression of the dominant positive mutant of H-*ras*, 12V*ras*, and the constitutively active mutant of MEK1, $MEK1^{EE}$, were reported to induce activation of ERKs. However, expression of 12V*ras* and $MEK1^{EE}$ did not suppress *oriP* activity (Fig. 6B). Thus, activation of the ERK signal pathway did not induce *oriP* suppression. We also analyzed NF-κB activation by LMP1 mutants and compared it with their ability to induce *oriP* suppression. LMP1Δ(351-386) and LMP1Δ(212-386) suppressed *oriP* replication as effectively as the wild type but these mutants did not activate NF-κB (Fig. 3). In contrast, LMP1Δ(187-351) and LMP1(PQT→AAA) suppressed *oriP* only weakly but they ac-

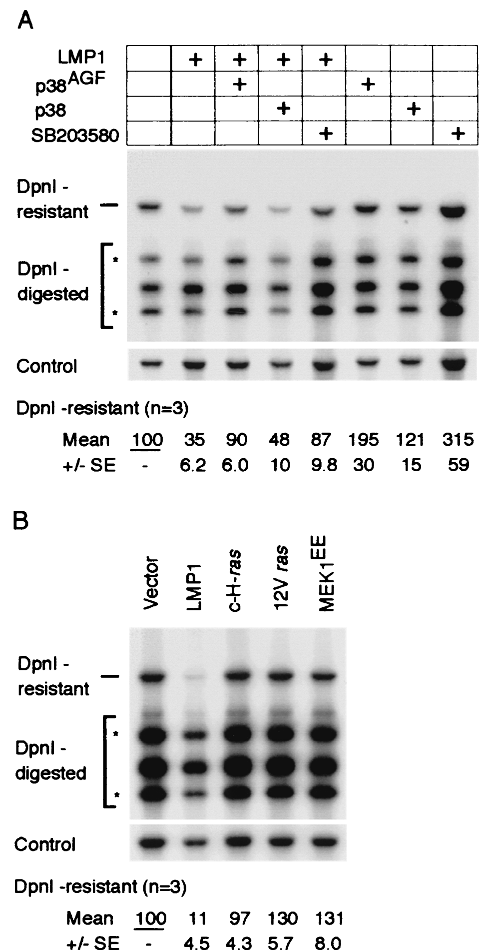

FIG. 6. Examination of the signal mediators that affect *oriP* activity. Shown are the results of a transient replication assay of the *oriP* plasmid. (A) The p38 MAPK. The *oriP* plasmid (2 μg), the control plasmid (0.5 μg), LMP1Δ(212-386) (0.25 μg), and the expression plasmid of p38 MAPK or the dominant negative mutant of p38 MAPK, $p38^{AGF}$ (8 μg), were transfected. Total amounts of plasmids were 10.75 μg. The specific inhibitor for SB208350 was added into the culture medium at a concentration of 20 μM. (B) *Ras* and MEK. The *oriP* plasmid (2 μg), the control plasmid (0.5 μg), one of the expression plasmids of LMP1 (0.5 μg), c-H-*ras* (4 μg), the dominant active *ras* 12V*ras* (4 μg), or the constitutively active mutant of MEK1, $MEK1^{EE}$ (4 μg), were transfected. Total amounts of plasmids were 6.5 μg. Normalized amounts of *Dpn*I-resistant plasmid are shown at the bottom. Data represent averages of three experiments with standard errors (SE). Two fragments indicated by asterisks are products of replication intermediates.

tivated NF-κB similarly to the wild-type LMP1. These results indicated that distinct domains of LMP1 induced activation of NF-κB and *oriP* suppression. Similarly, CTAR2 and the region between CTAR1 and CTAR2 were essential for activation of JNK and Janus kinase 3, respectively (12, 17, 32), but both regions were dispensable for *oriP* suppression (Fig. 3).

Activation of the TRAF6-mediating signal cascade by LPS resulted in loss of the EBV genome from Akata. Given the results suggesting that activation of TRAF5- and TRAF6-mediating signal cascades suppresses replication of the *oriP* plasmid in HeLa/EB1 cells, we next examined whether the same signalings negatively affected EBV replication in the infected cells. To see the suppression of *oriP* activity, it was essential that the EBV genome was maintained predominantly by the DS-dependent replication from *oriP* in the infected cells. A Burkitt's lymphoma cell line, Akata, showed the latency type I phenotype and did not express LMP1. In addition, the spontaneous loss of Akata EBV was also reported (54). Therefore it was very likely that Akata EBV was maintained by the DS-dependent replication from *oriP*. To activate TRAF-mediated signal cascades, we used LPS. It was shown that LPS activated cells through Toll-like receptors, and TRAF6 was the signaling mediator from Toll-like receptors to NF-κB and MAPKs (1, 28, 39, 40). We cultured growing Akata cells (10^5 cells/ml) in the presence of 10 μg of LPS/ml for 2 or 4 days. To examine the copy number of the Akata EBV genome, total DNA was prepared from these cells and was analyzed by Southern blot hybridization using the same amount of DNA (4 μg) and a *Bam*HI-C fragment for a probe. As shown in Fig. 7A, Akata EBV decreased significantly after LPS stimulation. Quantitative analysis indicated that EBV DNA was decreased by 28% during 4 days of LPS stimulation. In a control experiment, we examined another Burkitt's lymphoma cell line, Raji. Raji EBV DNA was maintained by the replication initiated in a region out of *oriP* (DS-independent replication) (38), and *oriP* was not used for replication origin, presumably because the cell expressed LMP1. As we expected, activation of the TRAF6 signal cascade by LPS did not reduce the copy number of Raji EBV, suggesting that LPS stimulation suppressed the *oriP* activity in Akata. We also examined Daudi EBV replication for another control. Like Akata, Daudi did not express LMP1 but Daudi EBV was replicated by both DS-dependent and DS-independent mechanisms (38). LPS stimulation resulted in only a little loss of Daudi EBV. These results suggested that activation of the TRAF6 signal cascade suppressed EBV replication in the infected cells when EBV was maintained by the DS-dependent replication from *oriP*. To confirm that p38 MAPK mediated the signal cascade leading to the suppression of Akata EBV replication, Akata cells were stimulated with LPS in the presence of SB208350. As shown in Fig. 7B, when p38 MAPK was inhibited, Akata EBV was not lost by LPS stimulation. We also examined the effects of SB208350 on replication of the EBV genome in infected cells. In contrast to HeLa/EB1, in which initial accumulation of the replicated *oriP* plasmid was increased by inhibiting the basal activity of p38 MAPK, similar treatment of the EBV-infected cells for 4 days did not increase the copy number of Akata and Raji EBV. Similar results were also obtained with LCLs and AG876 cell lines (data not shown).

DISCUSSION

We demonstrated that the replicator activity of *oriP* was negatively regulated by the TRAF5-mediated signal cascade from LMP1 and the TRAF5 and TRAF6 signal cascades from cellular receptors. This negative regulation was shown in the transient replication assay of the *oriP* plasmid and was also demonstrated in the analysis of Akata EBV replication.

While the DS element of *oriP* initiates DNA replication, the FR element of *oriP* functions as a replication terminator where two replication forks proceeding to opposite directions meet and a round of DNA replication is completed (16). After bidirectional replication is initiated from the DS element, one replication fork proceeds through most of the EBV plasmid, and the other fork proceeds only a short distance, directly to the FR element in the opposite direction. Two-dimensional gel analysis showed that this replication fork, after a short distance, was stopped by a replication fork barrier at the FR element, and the replication intermediates were accumulated (16). In the transient replication assay of the *oriP* plasmid replication, we found that two *Dpn*I-digested *oriP* fragments (2.5 and 1.3 kb) were not predicted from the *Dpn*I sites in *oriP*. Amounts of these fragments are roughly related to that of the *Dpn*I-resistant *oriP* plasmid. Longer enzyme digestion and use of excess enzymes did not reduce these products, indicating that the fragments were not products of incomplete digestion of *Dpn*I. Furthermore, these fragments were not detected in the samples when the *oriP* plasmid was transfected into replication-incompetent HeLa cells (55) and the DS plasmid lacking the FR element was transfected into HeLa/EB1 cells (Fig. 1B). From these results, we considered that these *Dpn*I-sensitive fragments are products of replication intermediates that were accumulated by the replication fork barrier at the FR element.

The LMP1 expression required to suppress 90% of *oriP* activity was almost equal to that in Raji cells. This indicated that *oriP* activity was sensitive enough to be suppressed by LMP1 expressed in EBV-infected cells. TRAF1, TRAF2, TRAF3, and TRAF5 bind to the PxQxT motif of LMP1 (5, 9, 10, 46, 53). TRAF1 participates in the antiapoptotic activity of LMP1 (6, 60) and does not mediate regulation of *oriP*. TRAF2 and TRAF5 initiate signal cascades which are overlapped in the activation of NF-κB and JNK. However, only TRAF5 regulates *oriP* activity through p38 MAPK. Thus, activation of the signal cascade leading to *oriP* suppression is a TRAF5-specific function. TRAF3 facilitates the function of TRAF5 by binding the TRAF3-TRAF5 hetero-oligomer to LMP1 (51). In addition, TRAF3 may mediate its own signal cascade, because overexpression of TRAF3 reverses the TRAF5-induced *oriP* suppression. This suggests the importance of balance between TRAF3 and TRAF5 in this signal transduction. TRAF6 binds to CD40, RANK, and p75 NGFR and also associates with IL-1R indirectly. As with TRAF5, overexpression of TRAF6 activates NF-κB, JNK, and p38 MAPK. Our results suggest that p38 MAPK is a common downstream mediator for *oriP* suppression in the signal cascades activated by LMP1, TRAF5, and TRAF6.

Eliopoulos et al. (13) showed that both CTAR1 and CTAR2 of LMP1 contribute to activation of p38 MAPK. In contrast, our results showed that CTAR1 contributed mostly for *oriP*

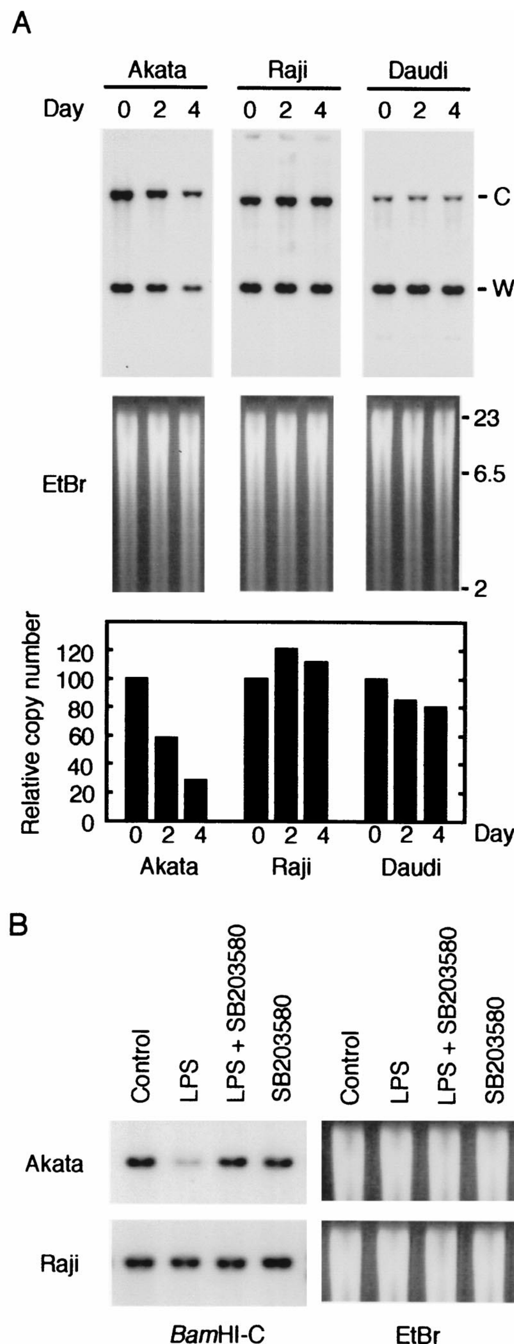

FIG. 7. LPS simulation resulted in loss of Akata EBV. (A) LPS stimulation of EBV-infected B cell lines. Cells (10^5 cells/ml) were stimulated with 10 μg of LPS/ml for 2 or 4 days. Total DNA of these stimulated and unstimulated cells (4 μg) was digested with *Bam*HI and was analyzed by Southern blot hybridization using an EBV(B95-8) *Bam*HI-C fragment for a probe, which cross-hybridized with *Bam*HI-W (3.1 kb). For the loading controls, EtBr-staining images of agarose gels are shown in the middle panel. Hybridization signals were measured and shown as a relative copy number in the lower panel. Raji and Daudi EBVs were replicated by the DS-independent mechanism (16). (B) The effects of SB208350 on EBV replication. EBV-infected B cell lines (10^5/ml) were cultured in the presence of the specific inhibitor for p38 MAPK SB208350 (20 μM) with or without LPS stimulation (5 μg/ml) for 4 days. Total DNA (4 μg) was prepared and analyzed as described above for panel A. Hybridization signals of the *Bam*HI-C fragment are shown in the left panels. EtBr-staining images of agarose gels are shown in the right panels.

suppression and the contribution of CTAR2 was only a part. There are four isozymes of p38 MAPK, p38α, p38β, p38γ, and p38δ. Among these isozymes, p38α and p38β are sensitive to SB203580 and appear to mediate distinct functions (15). A difference in the contribution of CTAR1 and CTAR2 to p38 activation and *oriP* suppression may be explained by identifying the p38 isozyme that is activated by LMP1 and suppresses *oriP* activity. The p38 MAPK is activated by phosphorylation by MAPK kinases (MAPKK) MAKK3 and MAKK6. These MAPKKs are activated by a group of MAPKK kinases (MAP KKK). Since MAPKKKs also activate the signal pathways leading to JNK, there may be cross-talk between the signal pathways leading to p38 MAPK and JNK (8). This suggests that the signal pathway initiated from CTAR2 and leading to JNK activation may also contribute to *oriP* suppression. Our results showed that LMP1 mutants lacking CTAR1 but retaining CTAR2, LMP1Δ(187-351), induced *oriP* suppression weakly. A mechanism by which p38 MAPK suppresses *oriP* activity is not yet known. The p38 MAPK may modify EBNA1 directly or indirectly in nuclei.

We confirmed that TRAF-mediated signaling suppressed *oriP* activity in EBV-infected B cells (Fig. 7). Using EBV-positive Burkitt's lymphoma cell line Akata, we showed that activation of the TRAF6-mediated signal cascade with LPS decreased the copy number of EBV by 28% after stimulation for 4 days (Fig. 7). Loss of 72% of the genome copy during three cell cycles corresponded to 74% suppression of EBV replication in each cell cycle, indicating that suppression with LPS stimulation was significant. The specificity of this suppression of EBV replication was shown by the result that LPS stimulation did not suppress replication of Raji EBV that was maintained by the DS-independent replication. Furthermore, we showed that p38 MAPK was involved in suppression of both EBV and the *oriP* plasmid replication, suggesting that the same mechanism regulated negatively the *oriP* activity of Akata EBV and the *oriP* plasmid in HeLa/EB1 cells. Consistent with these results, spontaneous loss of the EBV genome was observed in the EBV-positive Burkitt's lymphoma cell lines Akata and Mutu (54, 57).

In HeLa/EB1 cells, the p38 MAPK was activated at a low level under normal culture conditions, which was enough to suppress the initial accumulation of the replicated *oriP* plasmid by 30% (Fig. 5 and 6). This negative pressure imposed on *oriP* activity may cause constant loss of the *oriP* plasmid from transfected cells for longer cultures, which was reported in several studies (41, 56, 62). In contrast to HeLa/EB1, inhibition of p38 MAPK by SB208350 under unstimulated conditions did not increase Akata EBV in a short time (4 days). We speculated that the basal activity of p38 MAPK was lower in Akata than in HeLa/EB1 and may suppress Akata EBV replication only slightly in normal culture conditions. This is also consistent with the observation that spontaneous loss of Akata EBV (54) was a relatively slow process compared to LPS-induced loss (Fig. 7A).

Latently infected EBV appears to replicate by two distinct mechanisms, DS-dependent replication and DS-independent replication. The DS-dependent replication is initiated from the DS element of *oriP* and requires EBNA1 for initiation of DNA replication (62, 64). In contrast, DS-independent replication is initiated in a broad region out of *oriP* and EBNA1 functions

only for maintenance of the EBV chromosome (38). DS-independent replication appears to be performed by cellular replication factors without EBNA1 and is activated only in certain cell lines (unpublished data). DS-dependent and DS-independent mechanisms are not mutually exclusive and occur simultaneously, as is observed in Daudi. Another significant difference in these replication mechanisms is in their sensitivity to LMP1- and TRAF-induced signaling. As we showed in this study, activation of these signal cascades suppressed DS-dependent replication but not DS-independent replication. Based upon this knowledge of EBV replication, it is possible to make several speculations about latent infection of EBV. When latent EBV is replicated mainly by the DS-dependent mechanism, activation of TRAF5 and TRAF6 signal cascades or induction of LMP1 expression suppresses DS-dependent replication and the copy number of the EBV genome in infected cells may decrease. Normal cells infected latently with EBV in vivo are resting memory B cells (43, 44), which are eventually activated by $CD4^+$ T cells. Upon activation, TRAF5- and TRAF6-mediated signaling are initiated from CD40, TNFRII, and IL-1R. Therefore, when the EBV-infected B cell is latency phenotype I (EBNA1-only cells), it is likely that *oriP* activity is suppressed in activated B cells and EBV may be reduced or eventually lost from activated B cells. In contrast, when latent EBV is maintained predominantly by DS-independent replication, expression of LMP1 does not suppress latent EBV replication. Therefore, this type of infected cell can express LMP1 continuously. In vitro experiments have shown that continuous expression of LMP1 induced immortalization and the transformation phenotype in cultured cells (3, 34, 45, 61). Therefore, activation of DS-independent replication may facilitate lymphoproliferative disorders. It is unknown why some cell lines activate DS-independent replication of EBV but others do not. Because most B cell lines that were infected with EBV in vitro are latency phenotype III and expressing LMP1, activation of DS-independent replication may be related to immortalization of cells.

The DS-independent mechanism of EBV replication is apparently important in establishing the latent infection status in vitro, because LMP1 is expressed in both EBV-infected peripheral blood mononuclear cells and immortalized LCL clones established later. However, EBV can promote cell growth without expression of LMP1 by expressing virus-encoded poly$(A)^-$ RNA EBER (36, 37). Interestingly, when EBV-infected cell lines are prepared using normal gastric epithelial cells, the EBV-infected epithelial cells do not express LMP1 (48). Therefore, the DS-dependent replication from *oriP* may also play an important role during infection and establishing of the latent state in nonlymphoid cells.

ACKNOWLEDGMENTS

We thank E. Kieff for the S12 antibody and cDNA clones of TRAF1, TRAF2, and TRAF3, W. Hammerschmidt and A. Kieser for the LMP1 mutant plasmids, G. Mosialos for the TRAF1 clone, H. Nakano for TRAF5 clones, J. Inoue for TRAF6 clone, and H. Kitayama for *ras* mutant clones.

This work is supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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