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STAP-2 Negatively Regulates both Canonical and Noncanonical NF-κB Activation Induced by Epstein-Barr Virus-Derived Latent Membrane Protein 1

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Epstein-Barr virus (EBV) belongs to the herpesvirus group and can infect most human individuals. Although the majority of infected carriers remain asymptomatic, the virus may sometimes play a role in the pathogenesis of lymphoid and epithelial malignancies, such as Burkitt’s lymphoma, Hodgkin’s lymphoma, and nasopharyngeal carcinoma (42).

EBV-infected cells express several latent antigens, including EBV nuclear antigens and latent membrane proteins (LMPs). These EBV-derived antigens activate resting B cells to produce EBV nuclear antigens and latent membrane proteins (LMPs)

The signal-transducing adaptor protein 2 (STAP-2) is a recently identified adaptor protein that contains a pleckstrin homology (PH) and Src homology 2 (SH2)-like domains, as well as a proline-rich domain in its C-terminal region. In previous studies, we demonstrated that STAP-2 binds to MyD88 and IKK-α or IKK-β and modulates NF-κB signaling in macrophages. In the present study, we found that ectopic expression of STAP-2 inhibited Epstein-Barr virus (EBV) LMP1-mediated NF-κB signaling and interleukin-6 expression. Indeed, STAP-2 associated with LMP1 through its PH and SH2-like domains, and these proteins interacted with each other in EBV-positive human B cells. We found, furthermore, that STAP-2 regulated LMP1-mediated NF-κB signaling through direct or indirect interactions with the tumor necrosis factor receptor (TNFR)-associated factor 3 (TRAF3) and TNFR-associated death domain (TRADD) proteins. STAP-2 mRNA was induced by the expression of LMP1 in human B cells. Furthermore, transient expression of STAP-2 in EBV-positive human B cells decreased cell growth. Finally, STAP-2 knockout mouse embryonic fibroblasts showed enhanced LMP1-induced cell growth. These results suggest that STAP-2 acts as an endogenous negative regulator of EBV LMP1-mediated signaling through TRAF3 and TRADD.
Recently, we cloned two novel adaptor molecules, signal-transducing adaptor protein 1 (STAP-1) and STAP-2 (26, 27). STAP-1 was identified as a c-kit-interacting protein, while STAP-2 is a c-fms-interacting protein. The human STAP-2 protein is identical to a recently cloned adaptor molecule, BKS, which is a substrate of breast tumor kinase tyrosine kinase (28). Both STAP-1 and STAP-2 contain an N-terminal pleckstrin homology (PH) domain and a region related to the Src homology 2 (SH2) domain (26, 27). We previously reported that STAP-2 interacts with STAP3 through its YXXQ motif and with STAP3 through its PH and SH2-like domains (38). Notably, STAP-2 also plays important roles in LPS- and NF-κB-induced activation via complex formation with MyD88 and IKKs (39). Here, we investigated the influence of STAP-2 on LMP1-induced NF-κB activation.

Our most important findings were that STAP-2 could enhance the formation of LMP1-TRAF3 complexes and downregulate LMP1-induced NF-κB activation. We found, furthermore, that STAP-2 acts as a negative modulator of LMP1-induced NF-κB activation by displacing TRADD from LMP1. These negative regulatory activities resulted in the suppression of LMP1-induced cell growth. Taken together with the finding that STAP-2 mRNA was induced by the expression of LMP1 in human B cells, STAP-2 is likely to act as an endogenous negative regulator of EBV infection.

MATERIALS AND METHODS

Reagents and antibodies. Dorsomycin (Don) was purchased from MP Biomedicals (Irvine, CA). Expression vectors for TRAF1, TRAF2, TRAF3, TRAF5, TRAF6, TRADD, RIP1, B629, NF-κB-LUC, and PI-6-LUC were kindly provided by J. Inoue (University of Tokyo, Tokyo, Japan), H. Kobayashi (Kyushu University, Fukuoka, Japan), N. Inohara (University of Michigan Medical School, Ann Arbor, MI), H. Shibuya (Tokyo Medical and Dental University, Tokyo, Japan), T. Fujita (Kyoto University, Kyoto, Japan), and S. Akira (Osaka University, Osaka, Japan). The epitope-tagged STAP-2, glutathione S-transferase (GST) fusion STAP-2 mutants (GST-PH, GST-SH2, and GST-C) and the epitope-tagged LMP1 constructs were described previously (20, 29). The FLAG-tagged LMP1 mutants (DM, ID, and 3A) were described previously (20). The FLAG-tagged LMP1 deletion mutants [LMP1(1-92), LMP1(1-231), and LMP1(Δ232-352)], the GST-fused LMP1 deletion mutants [CTAR1(193-231) and CTAR2(353-386)], and the FLAG-tagged TRAF3 mutants [TRAF3(Δ2-92), TRAF3(Δ2-190), TRAF3(Δ2-367), TRAF3(Δ22-568), and TRAF3(Δ177-568)] were generated by PCR methods and then sequenced (primer sequences are available upon request). Primary antibodies were obtained commercially, as follows: anti-TRAF2, anti-TRADD, anti-Myc, anti-GST, anti-p52, anti-TRAF3, anti-TRAF6, and anti-GFP antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA); the human STAP-2 and human TRAF2 antibodies were from Sigma-Aldrich (St. Louis, MO); anti-IκBα antibodies were from Cell Signaling Technology (Beverly, MA); and antiactin MAb was from Chemicon International (Temecula, CA). An anti-LMP1 MAb (product no. S12) was prepared as described previously (25). The anti-human STAP-2 antibody was described previously (38).

Cell culture and transfection. Human EBV-positive B lymphoma cell lines (B4 and Raji) and a human EBV-negative Burkitt’s lymphoma B cell line (Ramos) were maintained in RPMI 1640 medium containing 10% fetal calf serum (FCS). Ramos tetracycline-controlled transactivator (TCA) control and Ramos tTA LMP1 transfectants carrying a tetracycline-regulated control or an LMP1 expression plasmid were prepared as described previously (20). The number of Raji cells that were viable after the indicated treatments and the number of MEFs were measured using a 2(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) assay (Cell Counting Kit-8; Wako Pure Chemicals). Briefly, 10 μl of WST-8 solution was added to the cells in each well, and cells were incubated for 2 h. The absorbance levels were measured at a test wavelength of 450 nm and a reference wavelength of 595 nm, using an Infinite F200 pro microplate reader (Bio-Rad, Hercules, CA).

RESULTS

STAP-2 negatively regulates LMP1-induced NF-κB activation. We previously demonstrated that STAP-2 plays important roles in LPS/TLR4-mediated NF-κB activation (39). The observations that the EBV-derived protein LMP1 functions like constitutively activated TNFR and transmits signals to activate NF-κB led us to investigate the effects of STAP-2 on...
LMP1-induced NF-kB activation by using transient reporter assays with NF-kB-LUC. NF-kB-LUC activities were markedly induced by the overexpression of LMP1 in both 293T and HeLa cells (Fig. 1a). It is important to note that the LMP1-induced NF-kB-LUC activities were significantly reduced in parallel with the expression of STAP-2.

To assess the effects of STAP-2 on LMP1-induced canonical and noncanonical NF-kB activation, we established stable transformants of HeLa/STAP-2 or HeLa/pcDNA3. First, we examined the effects of STAP-2 on LMP1-induced IkBα degradation, which leads to canonical NF-kB activation. Ectopic expression of LMP1 markedly enhanced IkBα degradation in HeLa/pcDNA3 but failed to induce IkBα degradation in HeLa/STAP-2 (Fig. 1b). Next, we examined the effects of STAP-2 on the processing of LMP1-induced p100/NF-kB2 to p52, which leads to noncanonical NF-kB activation. Ectopic expression of LMP1 in HeLa/pcDNA3, but not HeLa/STAP-2, markedly enhanced p100/NF-kB2 processing (Fig. 1b). These results indicated that STAP-2 suppressed both the canonical and the noncanonical NF-kB activation induced by LMP1.

NF-kB activation induces the production of inflammatory cytokines, such as IL-6. To confirm the inhibition of LMP1-induced NF-kB activation by STAP-2, the LMP1-induced activation of IL-6 promoter activity and the IL-6 mRNA expression level were analyzed with 293T and HeLa cells with or without STAP-2 expression. LMP1-induced activation of pIL-6-LUC was suppressed by STAP-2 in a dose-dependent manner, and the inhibition of luciferase activity was approximately 50% for both 293T and HeLa cells when 300 ng of a STAP-2 expression plasmid was transfected (Fig. 1c). IL-6 mRNA expression was also significantly reduced in parallel with the expression of STAP-2.
pression was detected in correspondence with the expression levels of LMP1 in RNA samples obtained from LMP1-transfected HeLa/pcDNA3 (Fig. 1d). However, IL-6 mRNA expression was not influenced by ectopic LMP1 expression in HeLa/STAP-2.

STAP-2 physically associates with LMP1. One mechanism, consistent with the above data, may involve direct interactions between STAP-2 and LMP1. To examine this possibility, co-immunoprecipitation experiments were performed using 293T cells transfected with the expression vectors for HA-tagged LMP1 together with Myc-tagged STAP-2. The immunoprecipitates with an anti-HA antibody contained the STAP-2 protein (Fig. 2a), indicating that STAP-2 was associated with LMP1 in 293T cells. To further determine the physical interactions between STAP-2 and LMP1, the human EBV-positive B cell lines IB4 and Raji, which express endogenous STAP-2 and large amounts of LMP1, were employed. The immunoprecipitates with an anti-LMP1 antibody contained the STAP-2 protein (Fig. 2b), indicating that endogenous STAP-2 protein interacted with the EBV-derived LMP1 protein in human B cells.

We also examined the cellular colocalization of STAP-2 and LMP1 in vivo, using confocal microscopy. HeLa cells were transfected with HA-tagged LMP1 and Myc-tagged STAP-2. The STAP-2 protein was concentrated in punctate vesicle-like and perinuclear structures, where LMP1 was also mainly localized (Fig. 2c), demonstrating that LMP1 colocalized with STAP-2 in vivo.

The PH and SH2-like domains of STAP-2 interact with the CTAR1 and CTAR2 domains of LMP1. To determine which domains of STAP-2 were involved in the interactions with LMP1, a series of STAP-2 deletion mutants fused with GST (GST-PH, GST-SH2, and GST-C) were employed (Fig. 3a). HA-tagged LMP1 and the respective STAP-2 mutants were transiently expressed in 293T cells, and the binding potentials of these proteins to LMP1 were examined by immunoprecipitation with an anti-HA antibody, followed by Western blotting with an anti-GST antibody. Although the immunoprecipitates for LMP1 contained both GST-PH and GST-SH2, the SH2-like domain of STAP-2 bound to LMP1 more strongly than the PH domain did (Fig. 3b).

To determine which domains of LMP1 were involved in the interactions with STAP-2, 293T cells were transfected with Myc-tagged STAP-2 and/or a series of FLAG-tagged LMP1 deletion mutants (Fig. 3c). The transfectants were lysed, immunoprecipitated with an anti-Myc antibody, and immunoblotted with an anti-FLAG antibody. LMP1(1-192) failed to interact with STAP-2, while both LMP1(1-231) and LMP1(232-352) were able to bind to STAP-2, although the binding potential of LMP1(232-352) was lower than that of LMP1(1-231) (Fig. 3d). These results indicate that STAP-2 may interact with LMP1 through its cytoplasmic CTAR1 and CTAR2 domains, which lie in the long cytoplasmic C-terminal tail of LMP1. Thus, we employed the GST-fused CTAR1 and CTAR2 protein constructs (Fig. 3c). Myc-tagged STAP-2 and the corresponding mutants were transiently expressed in 293T cells. The immunoprecipitates for STAP-2 contained both the GST-CTAR1 and the GST-CTAR2 proteins (Fig. 3e). These results indicate that STAP-2 interacts with the CTAR1 and CTAR2 domains of LMP1.

We further examined the interactions between CTAR1/CTAR2 and STAP-2 PH/SH2. FLAG-tagged STAP-2 PH or STAP-2 SH2 and GST-CTAR1 or GST-CTAR2 were transiently coexpressed in 293T cells. The binding potentials of the CTAR1 and CTAR2 domains of LMP1 with the PH and SH2 domains of STAP-2 were examined by pull-down assays with glutathione-Sepharose, followed by Western blotting with an anti-FLAG antibody. The precipitates for CTAR1 contained both STAP-2 PH and STAP-2 SH2, while the precipitates for CTAR2 contained STAP-2 PH alone (Fig. 3f and g), indicating...
the presence of multidomain interactions between STAP-2 and LMP1.

**Functional role of the PH domain of STAP-2 in LMP1-induced signals.** To assess the functional relevance of STAP-2 and LMP1-induced NF-κB activation, we transfected LMP1 and NK-κB-LUC together with a series of STAP-2 deletion mutants (Fig. 4a) into 293T cells. LMP1-induced NF-κB-LUC activity was significantly reduced by the expression of the STAP-2 full length protein and the STAP-2 SH2 and STAP-2 C mutants in a dose-dependent manner (Fig. 4b). However, STAP-2 PH did not show any influence on the NF-κB activity induced by LMP1. Thus, the PH domain of STAP-2 appears to play an important role in the control of LMP1-induced NF-κB activation.

We also investigated the effects of STAP-2 on CTAR1- or CTAR2-induced NF-κB activation. A series of LMP1 mutants (Fig. 4c; LMP1 DM, LMP1 ID, and LMP1 3A) and NF-κB-LUC together with STAP-2 were transfected into 293T cells, and their effects on LMP1-induced NF-κB-LUC activity were evaluated. Neither of the LMP1 mutants carrying only a single CTAR domain (LMP1 ID and LMP1 3A) could fully reconstitute the NF-κB activation induced by the LMP1 WT (Fig. 4d). Moreover, LMP1 DM showed only basal NF-κB activation, even when a much higher expression of LMP1 DM was
induced (Fig. 4d and data not shown). NF-κB activation induced by the LMP1 WT or the LMP1 ID or LMP1 3A mutant was dose-dependently reduced by the expression of STAP-2. Basal NF-κB activation in 293T cells transfected with vector alone or with LMP1 DM was also reduced by the expression of STAP-2 (data not shown). Thus, STAP-2 negatively regulated both CTAR1- and CTAR2-induced NF-κB activation.

**Molecular interactions among STAP-2, LMP1, and TRAF3.**

One of the important molecular mechanisms underlying LMP1-induced NF-κB activation came from the discovery that LMP1 specifically interacts with TRAFs through a conserved TRAF-binding motif in the CTAR1 domain. Although we demonstrated that STAP-2 failed to associate with TRAF6 in our previous report (39), possible associations of STAP-2 with a series of TRAFs were rescreened. 293T cells were transfected with FLAG-tagged TRAF1, TRAF2, TRAF3, TRAF5, or TRAF6 together with GST-fused STAP-2. Western blot analyses of the associated proteins, using an anti-FLAG antibody, revealed that STAP-2 strongly interacted with TRAF1 and TRAF3 and also showed a weak interaction with TRAF5 (Fig. 5a).

Since STAP-2 associated with TRAF3, we tried to determine which domains of STAP-2 mediated the interactions with TRAF3. FLAG-tagged TRAF3 and a series of the STAP-2 deletion mutants (Fig. 3a) were transiently expressed in 293T cells. Western blot analyses of the associated proteins, using an anti-FLAG antibody, revealed that STAP-2 strongly interacted with TRAF1 and TRAF3 and also showed a weak interaction with TRAF5 (Fig. 5a).

Since STAP-2 associated with TRAF3, we tried to determine which domains of STAP-2 mediated the interactions with TRAF3. FLAG-tagged TRAF3 and a series of the STAP-2 deletion mutants (Fig. 3a) were transiently expressed in 293T cells. The immunoprecipitates with an anti-FLAG antibody contained both the PH and the SH2-like domains of STAP-2 (Fig. 5b). These results indicate that both the PH and the SH-like domains of STAP-2 interact with TRAF3.

We also determined which domains of TRAF3 mediated the interactions with STAP-2 by using a series of deletion mutants of TRAF3 (Fig. 5c). Myc-tagged STAP-2 and the respective TRAF3 mutants were transiently expressed in 293T cells. The binding site of STAP-2 on TRAF3 was mapped to a narrow region (aa 317 to 367) between a leucine zipper domain and a TRAF-binding domain (Fig. 5d). To examine the effects of STAP-2 on the interaction between LMP1 and TRAF3, we estimated the binding potentials of LMP1 to TRAF3 in the presence and absence of STAP-2. The expression of STAP-2 significantly enhanced the interaction between TRAF3 and LMP1 (Fig. 5e). In the reverse situation, expression of TRAF3 also enhanced the interaction between STAP-2 and LMP1 (Fig. 5f). We further tested the interaction of LMP1 with TRAF3 by using MEFs derived from WT or STAP-2 knockout (KO) mice. As shown in Fig. 5g, the absence of endogenous STAP-2 caused a significant reduction in the amount of LMP1-bound TRAF3 in STAP-2 KO MEFs. These results suggest that STAP-2 enhances the formation of LMP1-TRAF3 complexes.

We then tested the involvement of TRAF3 in STAP-2-mediated suppression of LMP1-induced NF-κB activation. In parallel with the results for binding, TRAF3 inhibited the LMP1 WT- and the LMP1 ID-induced, but not the LMP1 3A-induced, NF-κB activation in 293T cells (Fig. 5h).

**Reduction of endogenous STAP-2 or TRAF3 enhances LMP1-induced NF-κB activation.** To determine whether STAP-2 represses LMP1-induced NF-κB activation, we used siRNAs to reduce endogenous expression of STAP-2 in HeLa cells (Fig. 6a). HeLa cells were transfected with specific siRNAs for STAP-2 or with a control siRNA. Total RNA isolated from the
FIG. 5. Molecular interactions among STAP-2, LMP1, and TRAF3. (a) 293T cells (1 × 10^7 cells/well) were transfected with GST-fused STAP-2 (10 μg) with or without FLAG-tagged TRAF1, TRAF2, TRAF3, TRAF5, or TRAF6 (15 μg). At 48 h after transfection, the cells were lysed, pulled down with glutathione-Sepharose, and immunoblotted (IB) with an anti-FLAG or anti-GST antibody. An aliquot of each total cell lysate (TCL) was immunoblotted with the anti-FLAG antibody. (b) 293T cells (1 × 10^7 cells) were transfected with FLAG-tagged TRAF3 (8 μg) with or without GST or GST-fused STAP-2 deletion mutants (10 μg). At 48 h after transfection, the cells were lysed, immunoprecipitated (IP) with an anti-FLAG antibody and immunoblotted with an anti-GST or anti-FLAG antibody. An aliquot of each TCL was immunoblotted with the anti-FLAG antibody. (c) Schematic diagrams of the domain structures of the TRAF3 deletion mutant fragments. (d) 293T cells (1 × 10^7 cells/well) were transfected with Myc-tagged STAP-2 (5 μg) with or without FLAG-tagged TRAF3 deletion mutants (10 μg). At 48 h after transfection, the cells were lysed, immunoprecipitated with an anti-Myc antibody, and immunoblotted with an anti-FLAG or anti-Myc antibody. An aliquot of each TCL was immunoblotted with the anti-FLAG antibody. (e) 293T cells (1 × 10^7 cells/well) were transfected with FLAG-tagged TRAF3 mutants (8 μg) with or without GST-fused STAP-2 deletion mutants (10 μg). At 48 h after transfection, the cells were lysed, immunoprecipitated with an anti-FLAG antibody, and immunoblotted with an anti-GST or anti-FLAG antibody. An aliquot of each TCL was immunoblotted with the anti-GST antibody. (f) MEFs in 10-cm dishes were retrovirally transfected with a control vector or the LMP1 expression vector. At 48 h after transfection, the cells were lysed, immunoprecipitated with control mouse IgG or an anti-TRAF3 antibody and immunoblotted with an anti-LMP1 or anti-TRAF3 antibody. An aliquot of each TCL was analyzed by immunoblotting with the anti-LMP1 antibody. (h) 293T cells in 12-well plates were transfected with FLAG-tagged LMP1 mutants (50 ng) and NF-κB-LUC (100 ng) and/or increasing amounts of FLAG-tagged TRAF3 (10 or 100 ng). At 48 h after transfection, the cells were harvested, and the luciferase activities were measured. An aliquot of each TCL was immunoblotted with an anti-FLAG antibody. Asterisks indicate the migration positions of the TRAFs (a), GST-fused STAP-2 deletion mutants (b), and TRAF3 deletion mutants (d).
FIG. 6. Reduction of endogenous STAP-2 or TRAF3 enhances LMP1-induced NF-κB activation. (a) HeLa cells in 24-well plates were transfected with a control siRNA or siRNA targeting human STAP-2. The cells were then transfected with HA-tagged LMP1 (3 ng) and NF-κB-LUC (100 ng), using jetPEI. At 48 h after transfection, the cells were harvested, and the luciferase activities were measured. At least three
transfected cells was subjected to RT-PCR and quantitative real-time PCR analyses, which confirmed a reduction of STAP-2 mRNA expression. We then determined the effects of STAP-2 siRNAs on LMP1-induced NF-κB activation in these cells. As shown in Fig. 6a, the siRNA-mediated reduced expression of STAP-2 resulted in a significant enhancement of LMP1-induced NF-κB activation in these cells. Furthermore, we tested LMP1-induced NF-κB activation with the STAP-2 KO MEFs. Importantly, enhanced LMP1-induced NF-κB activation was observed for the STAP-2 KO MEFs (Fig. 6b). This finding indicates that STAP-2 is an endogenous negative regulator of LMP1-induced NF-κB activation. To further verify the roles of TRAF3 in STAP-2-mediated suppression of LMP1-induced NF-κB activation, we attempted to knock down the TRAF3 protein in HeLa cells. As shown in Fig. 6c, siRNA-mediated reduced expression of TRAF3 resulted in a significant enhancement of LMP1-induced NF-κB activation in HeLa cells. Furthermore, the TRAF3 knockdown enhanced IL-6 mRNA expression and p100/NF-κB processing into p52 but not IκB degradation in HeLa cells (Fig. 6d). We also tested the effect of the TRAF3 knockdown in human EBV-positive Raji B cells. Importantly, a reduction of endogenous TRAF3 in Raji cells enhanced p100/NF-κB processing into p52 via the noncanonical pathway and the Bcl-2 content, although no significant alteration of the IκB content via the canonical pathway was observed (Fig. 6c). These results indicate that TRAF3 may act as a modulator for noncanonical NF-κB activation in HeLa cells and EBV-infected human B cells.

We next examined the effect of reduced TRAF3 expression on STAP-2 suppression of CTAR1-induced NF-κB activation with HeLa cells. As shown in Fig. 6f, reduction of endogenous TRAF3 caused a significant increase in CTAR1-induced NF-κB activation, and STAP-2-mediated suppression of CTAR1-induced NF-κB was restored in HeLa cells, indicating that STAP-2 fails to suppress NF-κB activation in the absence of TRAF3. Taken together, these results indicate that TRAF3 is a modulator of NF-κB activation by LMP1 through CTAR1 and is involved in STAP-2-mediated suppression through CTAR1.

STAP-2 regulates LMP1-induced NF-κB activation by displacing TRADD from LMP1. To further examine the molecular mechanisms of STAP-2-mediated suppression through CTAR2, we determined whether STAP-2-mediated suppression of LMP1-induced NF-κB activation could be restored by the overexpression of other downstream molecules such as IKK-α/β, TRAF1, TRAF2, TRAF6, TRADD, RIP1, and BS69. As shown in Fig. 7a, b, c, and d, overexpression of IKK-α/β, TRAF1, TRAF2, or BS69 had no effect on STAP-2-mediated suppression of LMP-induced NF-κB activation. However, overexpression of TRAF6, TRADD, or RIP1 restored STAP-2-mediated suppression of LMP-induced NF-κB activation (Fig. 7e, f, and g), suggesting that TRAF6-, TRADD-, and RIP1-mediated signals may be targets of LMP1/STAP-2. To examine the involvement of these proteins in LMP-induced NF-κB activation, we attempted to reduce the endogenous expression of TRAF6, TRADD, RIP1, or RIP2 in HeLa cells, using a specific siRNA for each gene (Fig. 8). We then determined the effects of the siRNAs on LMP1-induced NF-κB activation in HeLa cells. siRNA-mediated reduced expression of TRAF6 or TRADD resulted in a significant reduction of LMP1-induced NF-κB activation in HeLa cells (Fig. 8a and b), whereas the RIP1 and RIP2 knockdowns had no effect on LMP1-induced NF-κB activation (Fig. 8c and d), indicating that TRAF6 and TRADD, but not RIP1 and RIP2, act as positive regulators of LMP1-induced NF-κB activation, consistent with data from previous reports (36, 14, 15). TRAF6 was previously shown to participate in active LMP1 signaling complexes by an indirect mechanism involving both CTAR1 and CTAR2 (36), while TRADD and RIP associate directly with LMP1 via CTAR2 (14). Importantly, an LMP1 CTAR2 mutant that fails to interact with TRADD is defective in LMP1-induced NF-κB activation (14), while RIP is not required for LMP1-induced NF-κB activation (15).

To further delineate the molecular interactions between...
STAP-2 and TRADD or RIP, we determined whether STAP-2 interacts directly with TRADD or RIP in vivo. 293T cells were transfected with either HA-tagged TRADD or FLAG-tagged RIP1 together with Myc-tagged STAP-2. Western blot analysis of the associated proteins with an anti-HA or anti-FLAG antibody revealed that STAP-2 did not interact with either TRADD or RIP1 (Fig. 9a and b). Next, we examined the effects of STAP-2 on the interactions between LMP1 and TRADD or RIP1. Interestingly, TRADD, but not RIP1, failed to bind to LMP1 in the presence of STAP-2 (Fig. 9c and d), indicating that STAP-2 inhibits physical interactions between LMP1 and TRADD, but not LMP1 and RIP1. These results suggest that STAP-2 decreases the formation of the LMP1/ TRADD complexes.

**LMP1 induces STAP-2 mRNA expression in human B cells, and STAP-2 acts as an endogenous negative regulator of LMP1-mediated signaling.** Since STAP-2 downregulated LMP1 function, the EBV-negative Ramos B cell line was used to determine whether LMP1 influenced STAP-2 expression. Ramos cells were stably transfected with a vector system that allows LMP1 expression to be inductively regulated by Dox. Addition of Dox to the growth medium resulted in clear increases in the LMP1 protein levels, in whole-cell extracts from LMP1-transfected, but not in empty vector-transfected, Ramos cells. Importantly, ectopic expression of LMP1 resulted in increased STAP-2 mRNA expression, as determined by quantitative real-time PCR (Fig. 10a). Thus, EBV infection upregulated the expression of STAP-2, which negatively controlled LMP1-mediated signaling. We also determined whether STAP-2 expression affected human B cell growth. The transient expression of the STAP-2 WT, but not the vector alone or the STAP-2 ΔPH mutant, suppressed the growth of EBV-positive Raji B cells (Fig. 10b). These data suggest that LMP1-induced STAP-2 participates in the regulation of human B cell growth via its PH domain. Finally, we examined the effect of STAP-2 on LMP1-induced cell growth of MEFs derived from the STAP-2 WT or the STAP-2 KO mice. To this end, MEFs were retrovirally transfected with the control GFP vector or with an LMP1 expression vector, and LMP1-induced cell growth was evaluated. Interestingly, enhanced LMP1-induced cell growth was observed with the STAP-2 KO MEFs (Fig. 10c). Therefore, STAP-2 acts as an endogenous negative regulator of LMP1-mediated signaling.

**DISCUSSION**

Extensive studies using mice with genetic mutations in NF-κB components have revealed essential roles for NF-κB activation in lymphocyte development, activation, proliferation, and survival (22). The target genes of NF-κB that are relevant to lymphocyte biology include positive cell cycle regulators, antiapoptotic factors, inflammatory and immunoregulatory genes, such as cyclin D1, cyclin D2, and c-Myc and Bcl-2 family members, and immunoregulatory cytokines (12, 17, 35, 41, 43). The constitutive activation of lymphocyte proliferation and/or blockage of cell death induced by NF-κB-activating genes may augment the development of lymphomas. Indeed, aberrant NF-κB activation has been detected in various lymphoid malignancies (16). In addition to EBV, at least two other human lymphomagenic viruses are also known to carry NF-κB-activating oncoproteins. For example, Kaposi's sarcoma-associated herpesvirus (KSHV) and human T-lymphotropic virus type 1 (HTLV-1) play important roles in the development of primary effusion lymphoma and adult T-cell lymphoma/leukemia, respectively. KSHV contains a homologue of the cellular FLICE (FADD-like interleukin-1β-converting enzyme)-like inhibitory protein (FLIP), designated vFLIP, which can activate the NF-κB pathway and facilitate B lymphoma growth in mice (4). HTLV-1-mediated transformation of T lymphocytes is dependent on a 40-kDa Tax oncoprotein (34). Tax is sufficient to immortalize primary human T cells and is able to transform rodent fibroblasts, inducing tumors in nude mice (40). Tax transgenic mice also develop leukemia and lymphoma (11). Thus, viral products that activate NF-κB signaling play important roles in the survival of infected cells and in the development of leukemia or lymphoma. It is noteworthy

**FIG. 7.** TRAF6, TRADD, and RIP1, but not IKK-α, IKK-β, TRAF1, TRAF2, and BS69, overcame STAP-2 mediated suppression of LMP-induced NF-κB activation. (a) 293T cells in 24-well plates were transfected with HA-tagged LMP1 (10 ng) and NF-κB-LUC (100 ng) with or without Myc-tagged STAP-2 (100 ng) and/or increasing amounts of Myc-tagged IKK-α or IKK-β (1.0, 10, or 100 ng). At 48 h after transfection, the cells were harvested, and the luciferase activities were measured. An aliquot of each total cell lysate (TCL) was immunoblotted with an anti-HA or an anti-Myc antibody. (b) 293T cells in 24-well plates were transfected with HA-tagged LMP1 (10 ng) and NF-κB-LUC (100 ng) with or without Myc-tagged STAP-2 (100 ng) and/or increasing amounts of Myc-tagged STAP-2 (100 ng) and/or increasing amounts of Myc-tagged IKK-α or IKK-β (1.0, 10, or 100 ng). At 48 h after transfection, the cells were harvested, and the luciferase activities were measured. An aliquot of each TCL was immunoblotted with an anti-HA, an anti-Myc, or an anti-FLAG antibody. (c) 293T cells in 24-well plates were transfected with HA-tagged LMP1 (10 ng) and NF-κB-LUC (100 ng) with or without Myc-tagged STAP-2 (100 ng) and/or increasing amounts of Myc-tagged TRAF1 (1.0, 10, or 100 ng). At 48 h after transfection, the cells were harvested, and the luciferase activities were measured. An aliquot of each TCL was immunoblotted with an anti-HA, an anti-Myc, or an anti-FLAG antibody. (d) 293T cells in 24-well plates were transfected with HA-tagged LMP1 (10 ng) and NF-κB-LUC (100 ng) with or without Myc-tagged STAP-2 (100 ng) and/or increasing amounts of Myc-tagged TRAF2 (1.0, 10, or 100 ng). At 48 h after transfection, the cells were harvested, and the luciferase activities were measured. An aliquot of each TCL was immunoblotted with an anti-HA, an anti-Myc, or an anti-FLAG antibody. (e) 293T cells in 24-well plates were transfected with HA-tagged LMP1 (10 ng) and NF-κB-LUC (100 ng) with or without Myc-tagged STAP-2 (100 ng) and/or increasing amounts of Myc-tagged TRAF2 (1.0, 10, or 100 ng). At 48 h after transfection, the cells were harvested, and the luciferase activities were measured. An aliquot of each TCL was immunoblotted with an anti-HA, an anti-Myc, or an anti-FLAG antibody. (f) 293T cells in 24-well plates were transfected with HA-tagged LMP1 (10 ng) and NF-κB-LUC (100 ng) with or without Myc-tagged STAP-2 (100 ng) and/or increasing amounts of Myc-tagged IKK-α or IKK-β (1.0, 10, or 100 ng). At 48 h after transfection, the cells were harvested, and the luciferase activities were measured. An aliquot of each TCL was immunoblotted with an anti-HA, an anti-Myc, or an anti-FLAG antibody. (g) 293T cells in 24-well plates were transfected with HA-tagged LMP1 (10 ng) and NF-κB-LUC (100 ng) with or without Myc-tagged STAP-2 (100 ng) and/or increasing amounts of Myc-tagged IKK-α or IKK-β (1.0, 10, or 100 ng). At 48 h after transfection, the cells were harvested, and the luciferase activities were measured. An aliquot of each TCL was immunoblotted with an anti-HA, an anti-Myc, or an anti-FLAG antibody.
that LMP1-induced NF-κB activation is downregulated by STAP-2. This observation indicates that STAP-2 may be important in limiting EBV infection. Taken together with the finding that the STAP-2 expression is greatly enhanced by LMP1, it is possible that humans have acquired STAP-2 as a host defense system against EBV infection. It will be interesting to investigate whether KSHV and/or HTLV-1 infection induces inhibitory adaptor proteins, such as STAP-2.

We have demonstrated here that STAP-2 suppresses both canonical and noncanonical NF-κB activation induced by LMP1. With regard to the mechanisms for these effects, we found that STAP-2 enhanced the association of LMP1 with TRAF3. STAP-2 can bind to both LMP1 and TRAF3, and we showed a similar intracellular distribution to LMP1. In the reverse situation, expression of TRAF3 also enhanced the interaction between STAP-2 and LMP1. These results suggest that STAP-2 likely functions as a bridge between LMP1 and TRAF3 in vivo. It is well known that the interaction between LMP1 and TRAF3 is strong and direct. However, our data clearly indicated that STAP-2 further enhances their associa-

FIG. 8. Reduction of endogenous TRAF6 or TRADD decreases LMP1-induced NF-κB activation. (a to d) HeLa cells in 24-well plates were transfected with a control siRNA or siRNA targeting human TRAF6, TRADD, RIP1, or RIP2. The cells were then transfected with HA-tagged LMP1 (5 ng) and NF-κB-LUC (100 ng), using jetPEI. At 48 h after transfection, the cells were harvested, and the luciferase activities were measured. At least three independent experiments were carried out for each assay. *, P < 0.01. An aliquot of each total cell lysate (TCL) from TRAF6 or TRADD siRNA-treated cells was analyzed by immunoblotting (IB) with the respective antibodies. Total RNA samples isolated from RIP1 or RIP2 siRNA-treated cells were subjected to RT-PCR analysis using RIP1, RIP2, or G3PDH primers.
The PH and SH2-like domains of STAP-2 are responsible for the associations with both LMP1 and TRAF3. On the other hand, STAP-2 interacts with the CTAR1 and CTAR2 domains in LMP1, as well as with a narrow region beside the TRAF-binding domain in TRAF3. These multiple binding sites on each molecule could be the reason why STAP-2 enhanced the interaction between LMP1 and TRAF3.

TRAFs are important downstream signaling adaptors for many receptors, such as the TNFR and IL-1 receptor/Toll-like receptor superfamilies, including CD40. With regard to TRAF3, experiments using overexpressed TRAF3 WT have indicated that TRAF3 inhibits NF-κB activation induced by LMP1 (6). For example, TRAF3 negatively regulated LMP1 signaling by displacing TRAF1 and TRAF2 from CTAR1 (6). However, recent works indicated that LMP1 signaling was markedly defective in TRAF3 KO B cells (47, 48). In addition, TRAF3 was shown to mediate LMP1 signaling through direct interactions with CTAR1 and indirect interactions with CTAR2 (47). Thus, TRAF3 is likely to be both positive and negative for LMP1-mediated NF-κB activation. There are some possible reasons why the different role was concluded for TRAF3. The effects of TRAF3 might be dependent on cell specificity. However, our siRNA knockdown of TRAF3 in EBV-infected human Raji B cells resulted in an enhancement of the noncanonical NF-κB pathway, although Xie et al. showed that LMP1 signaling was markedly defective in the TRAF3 KO murine B cell lines generated by a somatic cell gene targeting (47, 48). Another possibility might come from diverse experimental paradigms. Although an overlap of the binding sites for TRAF1, -2, -3, and -5 on LMP1 makes it difficult to evaluate how each TRAF molecule participates in signaling, a quantitative balance among TRAFs might be important for the determination of positive or negative roles for TRAF3. To date, the role for TRAF3 in LMP1-mediated signaling is still controversial, and further analysis of each individual situation will be needed to clarify molecular mechanisms to determine positive or negative signaling.

TANK has also been demonstrated to inhibit LMP1-induced NF-κB activation by displacing TRAF2 from LMP1 (18). Importantly, TANK and LMP1 bind to the same binding crevice on TRAF3 (9). Therefore, there might be the possibility that TRAF3 acts as a positive or negative regulator on LMP1-induced NF-κB activation through TANK. The interactions between STAP-2 and TRAF3 may influence the bifunctional property of TANK on LMP1-induced NF-κB activation, although more detailed investigations will be required.

LMP1 has two putative domains, CTAR1 and CTAR2, which initiate signals toward the activation of NF-κB. CTAR1 interacts with TRAF1, -2, -3, and -5 (6), while CTAR2 interacts with TRADD and RIP (14, 15). TRAF6 participates in the
active LMP1 signaling complex by an indirect mechanism involving both CTAR1 and CTAR2 (36). Importantly, the LMP1 CTAR2 mutant that fails to interact with TRADD is defective in LMP1-induced NF-κB activation (14), whereas RIP is not required for LMP1-induced NF-κB activation (15). Similar results were also obtained from our experiments. Furthermore, we have shown that CTAR1 binds to both the PH and SH2-like domains of STAP-2, while CTAR2 recognizes only the PH domain. Importantly, STAP-2-mediated suppression of LMP1-induced NF-κB activation through CTAR1, but not CTAR2, was found to be dependent on TRAF3, since TRAF3 inhibited LMP1 1D-induced, but not LMP1 3A-induced, NF-κB activation. On the other hand, STAP-2-mediated suppression of LMP1-induced NF-κB activation was restored by overexpression of TRAF6, suggesting that STAP-2 also affected LMP1-mediated NF-κB activation in a TRAF6-dependent manner.

Although several reports have demonstrated the participation of TRAF6 in LMP1-induced signaling through indirect interactions with LMP1 (24, 36), the detailed effects of STAP-2 on TRAF6 actions remain unknown, since STAP-2 does not bind to TRAF6. It is noteworthy that STAP-2 displaced TRADD from LMP1, thus indicating that TRADD might be another positive regulator of LMP1-induced NF-κB activation, which interacted directly with LMP1 through CTAR2. These facts suggest that STAP-2-mediated suppression of LMP1-induced NF-κB activation through CTAR2 is partly dependent on TRADD.

In the present paper, we have provided evidence that STAP-2 can inhibit EBV LMP1-mediated NF-κB activation through direct or indirect modulation of interactions among signaling molecules, and our proposed mechanisms are now
factor 2 is a mediator of NF-κB activation by beta-actin and EBV latent membrane protein 1, the Epstein-Barr virus transforming protein. Proc. Natl. Acad. Sci. USA 93:11085–11090.