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Citation	Molecular and Cellular Biology, 28(16), 5027-5042 <a href="https://doi.org/10.1128/MCB.00194-08">https://doi.org/10.1128/MCB.00194-08</a>
Issue Date	2008-08
Doc URL	<a href="http://hdl.handle.net/2115/34635">http://hdl.handle.net/2115/34635</a>
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Type	article
File Information	5027.pdf



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# STAP-2 Negatively Regulates both Canonical and Noncanonical NF- $\kappa$ B Activation Induced by Epstein-Barr Virus-Derived Latent Membrane Protein 1<sup>∇</sup>

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Received 7 February 2008/Returned for modification 13 March 2008/Accepted 9 June 2008

**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- $\alpha$  or IKK- $\beta$  and modulates NF- $\kappa$ B signaling in macrophages. In the present study, we found that ectopic expression of STAP-2 inhibited Epstein-Barr virus (EBV) LMP1-mediated NF- $\kappa$ B 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- $\kappa$ B 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.**

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 proliferating lymphoblasts and to provide survival signals for maintaining infected cells.

Among EBV-derived antigens, LMP1 by itself can transform rodent fibroblasts and render them tumorigenic in nude mice (30, 46). Transgenic mice expressing LMP1 under the control of a mouse immunoglobulin H (IgH) enhancer and a V<sub>H</sub> promoter develop B cell lymphomas at an accelerated rate as they age (19). In LMP1 transgenic mice, it has been demonstrated that LMP1 is expressed in lymphoma tissues at high levels and that several NF- $\kappa$ B-activating oncogenes, including A20 and Bcl-2, are upregulated in the LMP1-expressing lymphomas compared with those in LMP1-negative counterparts (19).

Structurally, LMP1 is an integral membrane protein of 386 amino acids (aa) that consists of a short cytoplasmic N-terminal domain (aa 1 to 24), six transmembrane domains (aa 25 to 186), and a long cytoplasmic C-terminal tail (aa 187 to 386) (1, 21, 23). The cytoplasmic C-terminal tail contains two C-terminal activation regions (CTARs), CTAR1 and CTAR2, which are required for LMP1 signaling through tumor necrosis factor receptor (TNFR)-associated factors (TRAFs) (1, 13). CTAR1 contains a consensus TRAF-binding motif (PXQXT) and can bind to TRAF1, -2, -3, and -5 (1, 2, 6, 18, 31). CTAR2 interacts with the TNFR-associated death domain (TRADD) protein, the receptor-interacting protein (RIP) (1, 14, 15), and the BS69 protein (45). LMP1-induced signals through these two domains induce p100 NF- $\kappa$ B (p100/NF- $\kappa$ B2) and p105 NF- $\kappa$ B (p105/NF- $\kappa$ B1) precursors and upregulate their processing into p52 and p50 (13, 20, 25, 29, 32), indicating that LMP1 is involved in both canonical NF- $\kappa$ B upregulation and noncanonical processing of p100 into p52. The binding of LMP1 to TRAFs and TRADD also initiates the formation of a signaling complex that leads to the activation of mitogen-activated protein kinase p38 (7). In addition, the activation of c-Jun N-terminal kinase (JNK) by LMP1 is mediated through CTAR1 and CTAR2 (3, 8). BS69 has been shown to be involved in LMP1-induced JNK activation by interacting with TRAF6 (45).

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∇ Published ahead of print on 23 June 2008.

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 STAT3 through its YXXQ motif and with STAT5 through its PH and SH2-like domains (38). Notably, STAP-2 also plays important roles in LPS/TLR4-induced NF- $\kappa$ B activation via complex formation with MyD88 and IKKs (39). Here, we investigated the influence of STAP-2 on LMP1-induced NF- $\kappa$ B activation.

Our most important findings were that STAP-2 could enhance the formation of LMP1-TRAF3 complexes and downregulate LMP1-induced NF- $\kappa$ B activation. We found, furthermore, that STAP-2 acts a negative modulator of LMP1-induced NF- $\kappa$ B 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.** Doxycycline (Dox) was purchased from MP Biochemicals (Irvine, CA). Expression vectors for TRAF1, TRAF2, TRAF3, TRAF5, TRAF6, TRADD, RIP1, BS69, NF- $\kappa$ B-LUC, and pIL-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( $\Delta$ 232-352)], the GST-fused LMP1 deletion mutants [CTAR1(193-231) and CTAR2(353-386)], and the FLAG-tagged TRAF3 mutants [TRAF3( $\Delta$ 2-92), TRAF3( $\Delta$ 2-190), TRAF3( $\Delta$ 2-367), TRAF3( $\Delta$ 422-568), and TRAF3( $\Delta$ 317-568)] 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); anti-FLAG monoclonal antibody (MAb) (product no. M2) and antihemagglutinin (anti-HA) antibody were from Sigma-Aldrich (St. Louis, MO); anti-I $\kappa$ B $\alpha$  antibody was 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 (IB4 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 (tTA) control and Ramos tTA LMP1 transfectants carrying a tetracycline-regulated control or an LMP1 expression plasmid were prepared as described previously (10). A human cervix carcinoma cell line (HeLa), a human embryonic kidney carcinoma cell line (293T), and mouse embryonic fibroblasts (MEFs) were maintained in Dulbecco's modified Eagle's medium containing 10% FCS. HeLa cells expressing an empty vector (HeLa/pcDNA3) and HeLa cells expressing the STAP-2 wild-type (WT) (HeLa/STAP-2) transfectants were prepared as described previously (38). HeLa cells were transfected by using jetPEI (PolyPlus-transfection, Strasbourg, France) according to the manufacturer's instructions. 293T cells were transfected using a standard calcium precipitation protocol (38). Nucleofection of Raji cells

was performed using a Cell Line Nucleofector V kit (Amaxa Biosystems, Gaithersburg, MD). MEFs ( $2 \times 10^5$  cells/well) in 96-well plates were retrovirally transfected with control or with LMP1 vectors as described previously (44).

**Transfection of siRNAs and luciferase assays.** The small interfering RNAs (siRNAs) targeting STAP-2 and control siRNA used in this study were described previously (37). The siRNAs targeting human TRAF3, TRAF6, TRADD, RIP1, and RIP2 used in this study were as follows: TRAF3 (no. 1), 5'-GGAAGAUU CGCGACUACAATT-3'; TRAF3 (no. 2), 5'-GGAAAUGCCUUCGAAUAAU TT-3'; TRAF6, 5'-GCCUAAUCAUUAUGAUCUATT-3'; TRADD, 5'-GGG UCAGCCUGUAGUGAAUTT-3'; RIP1, 5'-GGGCGAUUUGCAAUA ATT-3'; and RIP2, 5'-GGGACUUGAUGAUGAAAGATT-3'. Raji cells were transiently transfected with siRNAs by electroporation, using a MicroPorator (Digital Bio Technology, Suwon, Korea) according to the manufacturer's instructions. HeLa cells were plated on 24-well plates at  $2 \times 10^4$  cells/well and incubated with an siRNA-Lipofectamine 2000 (Invitrogen, Carlsbad, CA) mixture at 37°C for 4 h, and these steps were followed by the addition of fresh medium containing 10% FCS. HeLa cells were further transfected with or without NF- $\kappa$ B-LUC with or without LMP1, using jetPEI as described in the section above. At 24 h after the cells were transfected, they were harvested and assayed for their luciferase activities, using a dual-luciferase reporter assay system (Promega, Madison, WI) according to the manufacturer's instructions. 293T cells were transfected as described in the section above. The cells were harvested at 36 h after transfection, lysed in 50  $\mu$ l of reporter lysis buffer (Promega), and assayed for their luciferase and  $\beta$ -galactosidase activities according to the manufacturer's instructions. Three or more independent experiments were carried out for each assay.

**Immunoprecipitation and immunoblotting.** Immunoprecipitation and Western blot assays were performed as described previously (38). The immunoprecipitates from cell lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Perkin-Elmer, Boston, MA). The membranes were then immunoblotted with the different primary antibodies. Immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Millipore, Bedford, MA).

**RNA isolation and quantitative real-time PCR.** After cells were harvested, total RNA samples were extracted, using Iso-Gen (Nippon Gene, Tokyo, Japan), and subjected to reverse transcription-PCR (RT-PCR), using an RT-PCR High-Plus kit (Toyobo, Tokyo, Japan). Quantitative real-time PCR analyses for interleukin 6 (IL-6), STAP-2, and control glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA transcripts were carried out using Assays-on-Demand gene-specific fluorescently labeled TaqMan minor groove binder probes in an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA).

**Indirect immunofluorescence microscopy.** To analyze the subcellular localizations of the LMP1 and STAP-2 proteins, we transiently transfected HA-LMP1 and Myc-STAP-2 into HeLa cells by using jetPEI (Polytransfection, Illkirch, France). Immunofluorescence staining procedures were performed as described previously (38). The primary antibodies used were mouse anti-HA and rabbit anti-Myc antibodies. The secondary antibodies used were rhodamine-conjugated anti-mouse IgG and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG antibodies (both from Chemicon). DNA was visualized by staining with 4',6'-diamidino-2-phenylindole (DAPI; Wako Chemicals, Osaka, Japan). The staining was visualized by confocal laser scanning microscopy with an LSM510 model microscope (Carl Zeiss, Thornwood, NY) equipped with an apochromat  $\times 63$ , 1.4 numerical aperture oil immersion objective, using excitation wavelengths of 543 nm (rhodamine) and 488 nm (FITC).

**Cell proliferation assay.** 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  $\mu$ l 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 a microplate reader (Bio-Rad, Hercules, CA).

#### RESULTS

**STAP-2 negatively regulates LMP1-induced NF- $\kappa$ B activation.** We previously demonstrated that STAP-2 plays important roles in LPS/TLR4-mediated NF- $\kappa$ B activation (39). The observations that the EBV-derived protein LMP1 functions like constitutively activated TNFR and transmits signals to activate NF- $\kappa$ B led us to investigate the effects of STAP-2 on

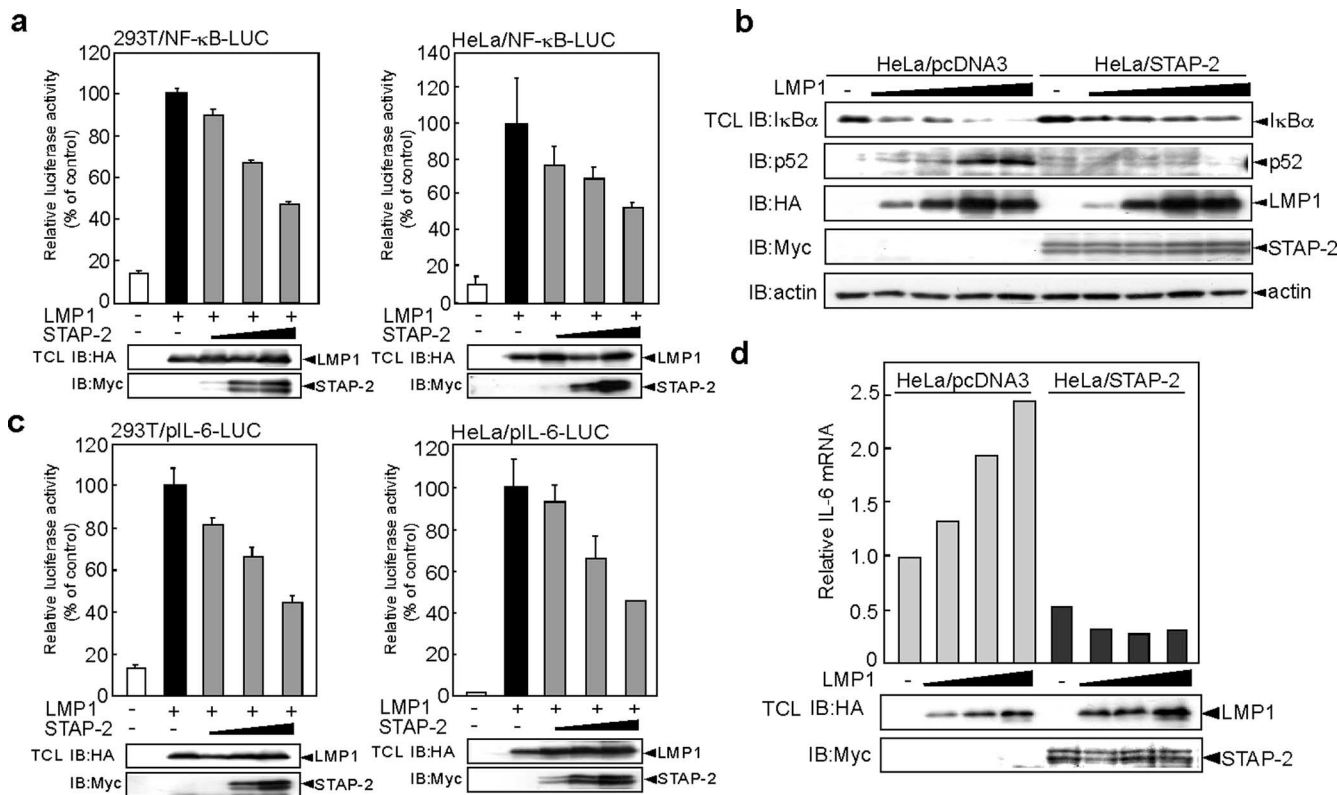


FIG. 1. STAP-2 negatively regulates LMP1-induced NF- $\kappa$ B activation and IL-6 gene expression. (a) 293T cells in 12-well plates were transfected with HA-tagged LMP1 (10 ng) and NF- $\kappa$ B-LUC (100 ng) and/or increasing amounts of Myc-tagged STAP-2 (30, 150, or 300 ng). At 48 h after transfection, the cells were harvested, and the luciferase activities were measured. HeLa cells in 24-well plates were transfected with HA-tagged LMP1 (3 ng) and NF- $\kappa$ B-LUC (200 ng) and/or increasing amounts of Myc-tagged STAP-2, using jetPEI. At 48 h after transfection, the cells were harvested and assayed for their luciferase activities using a dual-luciferase reporter assay system. An aliquot of each total cell lysate (TCL) was analyzed by immunoblotting (IB) with an anti-HA or anti-Myc antibody. (b) HeLa/pcDNA3 and HeLa/STAP-2 in 12-well plates were transfected with or without increasing amounts of HA-tagged LMP1 (0, 0.1, 0.3, 0.5, or 1.5  $\mu$ g), using jetPEI. At 36 or 48 h after transfection, the cells were lysed, and total extracts were immunoblotted with anti-I $\kappa$ B $\alpha$ , anti-p52, anti-HA, anti-Myc, or anti-actin antibodies. (c) 293T cells in 12-well plates were transfected with HA-tagged LMP1 (3 ng) and pIL-6-LUC (400 ng) and/or increasing amounts of Myc-tagged STAP-2 (30, 150, or 300 ng). At 48 h after transfection, the cells were harvested, and the luciferase activities were measured. HeLa cells in 24-well plates were transfected with HA-tagged LMP1 (50 ng) and pIL-6-LUC (100 ng) and/or increasing amounts of Myc-tagged STAP-2, using jetPEI. At 48 h after transfection, the cells were harvested and assayed for their luciferase activities using a dual-luciferase reporter assay system. An aliquot of each TCL was analyzed by immunoblotting with an anti-HA or anti-Myc antibody. (d) HeLa/pcDNA3 and HeLa/STAP-2 in 12-well plates were transfected with or without increasing amounts of HA-tagged LMP1 (0, 0.1, 0.5, or 1.5  $\mu$ g), using jetPEI. At 48 h after transfection, total RNA samples were extracted and analyzed for their IL-6 expression levels by RT and quantitative real-time PCR analyses. Data represent the levels of IL-6 mRNA normalized by that of G3PDH mRNA as an internal control and are expressed relative to the value at time zero. Data represent the means of duplicate PCR determinations, which generally varied by less than 10%. An aliquot of each TCL was analyzed by immunoblotting with an anti-HA or anti-Myc antibody.

LMP1-induced NF- $\kappa$ B activation by using transient reporter assays with NF- $\kappa$ B-LUC. NF- $\kappa$ B-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- $\kappa$ B-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- $\kappa$ B activation, we established stable transformants of HeLa/STAP-2 or HeLa/pcDNA3. First, we examined the effects of STAP-2 on LMP1-induced I $\kappa$ B $\alpha$  degradation, which leads to canonical NF- $\kappa$ B activation. Ectopic expression of LMP1 markedly enhanced I $\kappa$ B $\alpha$  degradation in HeLa/pcDNA3 but failed to induce I $\kappa$ B $\alpha$  degradation in HeLa/STAP-2 (Fig. 1b). Next, we examined the effects of STAP-2 on the processing of LMP1-induced p100/NF- $\kappa$ B2 to

p52, which leads to noncanonical NF- $\kappa$ B activation. Ectopic expression of LMP1 in HeLa/pcDNA3, but not HeLa/STAP-2, markedly enhanced p100/NF- $\kappa$ B2 processing (Fig. 1b). These results indicated that STAP-2 suppressed both the canonical and the noncanonical NF- $\kappa$ B activation induced by LMP1.

NF- $\kappa$ B activation induces the production of inflammatory cytokines, such as IL-6. To confirm the inhibition of LMP1-induced NF- $\kappa$ B 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 ex-

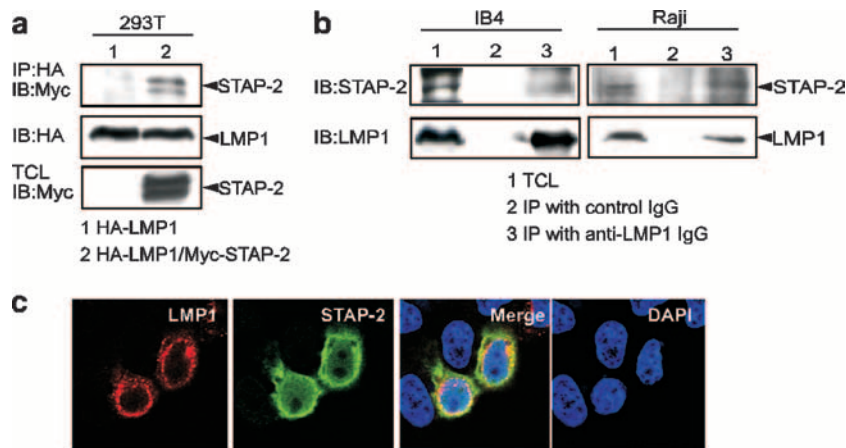


FIG. 2. STAP-2 physiologically associates with LMP1. (a) 293T cells ( $1 \times 10^7$  cells/well) were transfected with HA-tagged LMP1 (10  $\mu$ g) with or without Myc-tagged STAP-2 (5  $\mu$ g). At 48 h after transfection, the cells were lysed, immunoprecipitated with an anti-HA antibody, and immunoblotted (IB) with an anti-Myc or an anti-HA antibody. An aliquot of each total cell lysate (TCL) was immunoblotted with the anti-Myc antibody (bottom panel). (b) Human B lymphoma IB4 and Raji cells ( $2 \times 10^8$  cells/well) were lysed, immunoprecipitated (IP) with control mouse IgG or anti-LMP1 MAbs (S12), and immunoblotted with an anti-STAP-2 antibody or anti-LMP1 MAbs. (c) HeLa cells in 12-well plates were transfected with HA-tagged LMP1 (1  $\mu$ g) and Myc-tagged STAP-2 (1  $\mu$ g), using jetPEI. At 48 h after transfection, the cells were fixed, incubated with anti-HA and anti-Myc antibodies and visualized with FITC- and rhodamine-conjugated secondary antibodies. The same slides were also stained with DAPI to detect the nuclei.

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( $\Delta$ 232-352) were able to bind to STAP-2, although the binding potential of LMP1( $\Delta$ 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

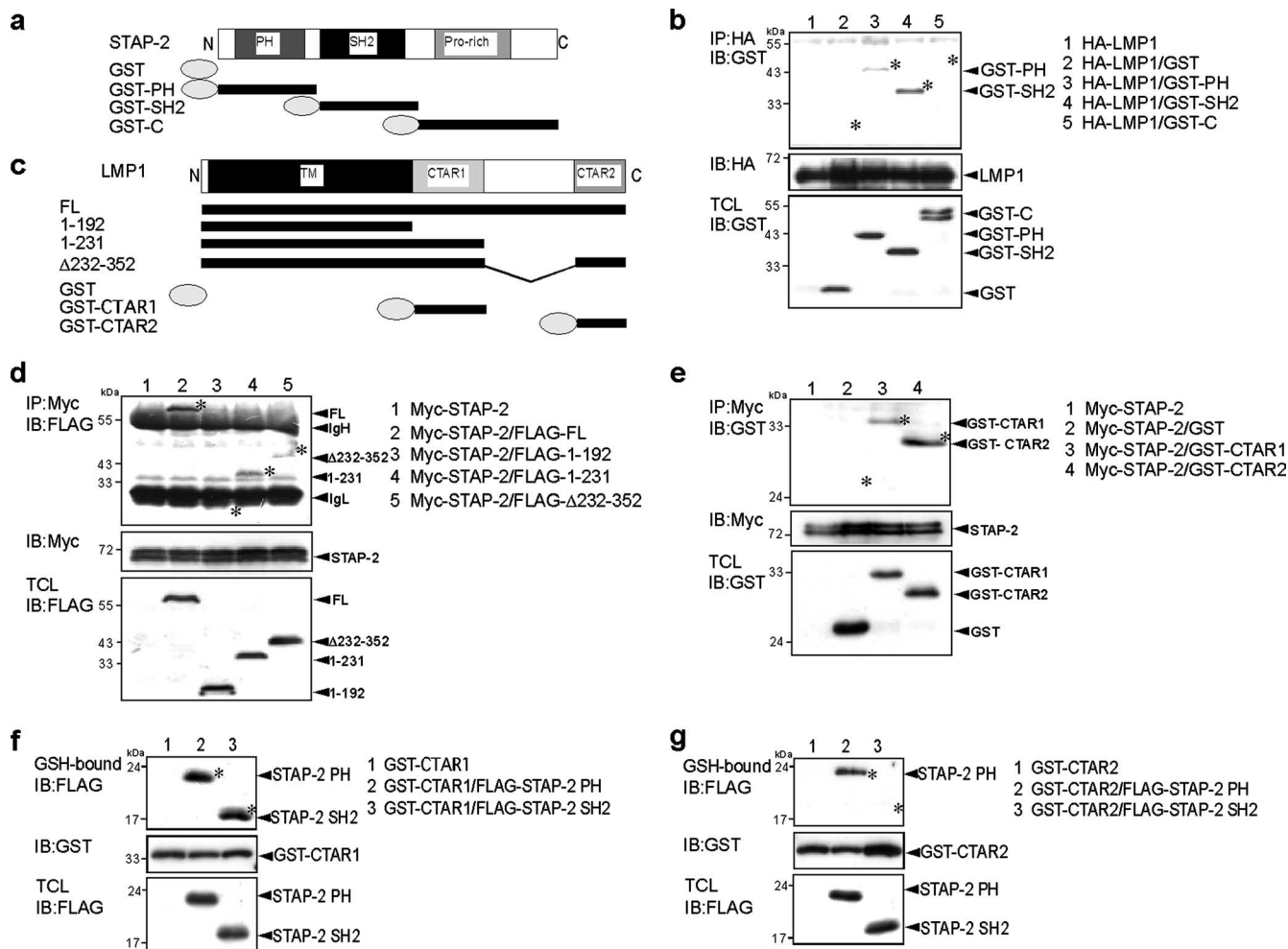


FIG. 3. The PH and SH2-like domains of STAP-2 interact with the CTAR1 and CTAR2 domains of LMP1. (a) Schematic diagram of the domain structures of STAP-2 and its GST-fused mutant fragments. (b) 293T cells ( $1 \times 10^7$  cells/well) were transfected with HA-tagged LMP1 (10  $\mu$ g) with or without GST or GST-fused STAP-2 deletion mutants (8  $\mu$ g). At 48 h after transfection, the cells were lysed, immunoprecipitated (IP) with an anti-HA antibody and immunoblotted (IB) with an anti-GST or anti-HA antibody. An aliquot of each total cell lysate (TCL) was immunoblotted with the anti-GST antibody. (c) Schematic diagram of the domain structures of LMP1 and its mutant fragments. (d) 293T cells ( $1 \times 10^7$  cells/well) were transfected with Myc-tagged STAP-2 (5  $\mu$ g) with or without LMP1 deletion mutants (10  $\mu$ g). 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 ( $1 \times 10^7$  cells/well) were transfected with Myc-tagged STAP-2 (5  $\mu$ g) with or without GST or GST-fused LMP1 deletion mutants (10  $\mu$ g). At 48 h after transfection, the cells were lysed, immunoprecipitated with an anti-Myc antibody, and immunoblotted with an anti-GST or anti-Myc antibody. An aliquot of each TCL was immunoblotted with the anti-GST antibody. (f and g) 293T cells ( $1 \times 10^7$  cells/well) were transfected with GST-fused CTAR1 (f) or CTAR2 (g) (5  $\mu$ g) with or without FLAG-tagged STAP-2 PH or STAP-2 SH2 (10  $\mu$ g). At 48 h after transfection, the cells were lysed, pulled down with glutathione-Sepharose, and immunoblotted with an anti-FLAG or anti-GST antibody. An aliquot of each TCL was immunoblotted with the anti-FLAG antibody. Asterisks indicate the migration positions of the STAP-2 (b, f, and g), LMP1 (d), and GST-fused LMP1 (e) deletion mutants.

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- $\kappa$ B activation, we transfected LMP1 and NF- $\kappa$ B-LUC together with a series of STAP-2 deletion mutants (Fig. 4a) into 293T cells. LMP1-induced NF- $\kappa$ B-LUC activity was significantly reduced by the expression of the STAP-2 full length protein and the STAP-2  $\Delta$ SH2 and STAP-2  $\Delta$ C mutants in a dose-dependent manner (Fig. 4b). However, STAP-2  $\Delta$ PH did not show any influence on the NF- $\kappa$ B activity induced by LMP1. Thus, the PH domain of STAP-2 appears to

play an important role in the control of LMP1-induced NF- $\kappa$ B activation.

We also investigated the effects of STAP-2 on CTAR1- or CTAR2-induced NF- $\kappa$ B activation. A series of LMP1 mutants (Fig. 4c; LMP1 DM, LMP1 ID, and LMP1 3A) and NF- $\kappa$ B-LUC together with STAP-2 were transfected into 293T cells, and their effects on LMP1-induced NF- $\kappa$ B-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- $\kappa$ B activation induced by the LMP1 WT (Fig. 4d). Moreover, LMP1 DM showed only basal NF- $\kappa$ B activation, even when a much higher expression of LMP1 DM was

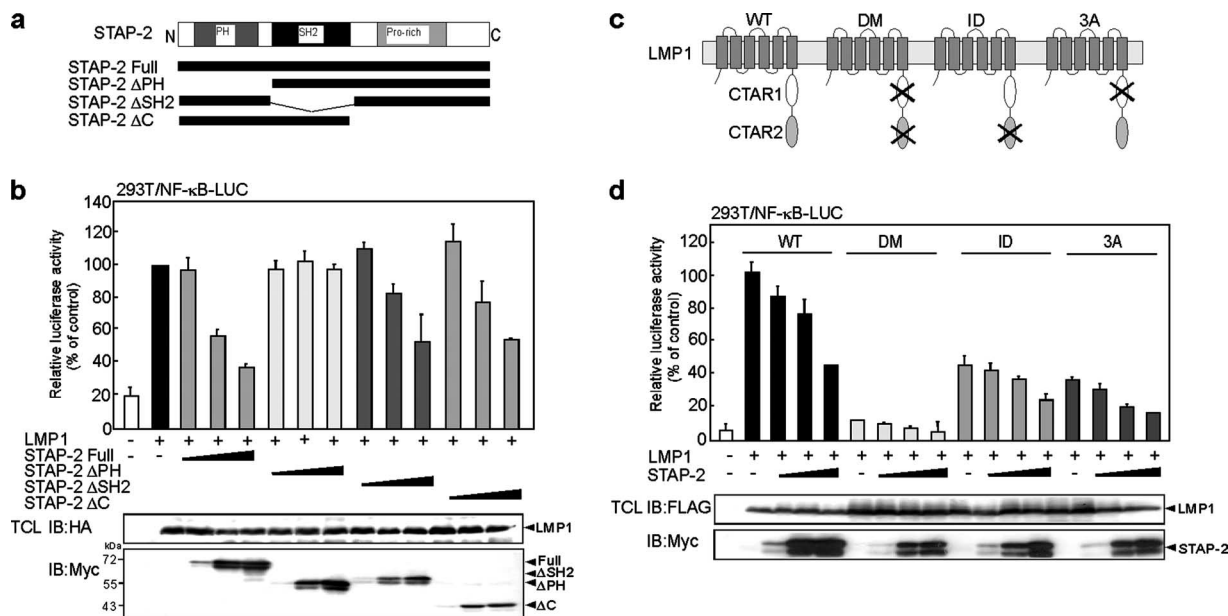


FIG. 4. Functional role of the PH domain of STAP-2 in LMP1-induced signals. (a) Schematic diagram of the domain structures of the STAP-2 deletion mutant fragments. (b) 293T cells in 12-well plates were transfected with HA-tagged LMP1 (10 ng) and NF- $\kappa$ B-LUC (100 ng) and/or increasing amounts of Myc-tagged STAP-2 mutants (30, 150, or 300 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 analyzed by immunoblotting (IB) with an anti-HA or anti-Myc antibody. (c) Schematic diagram of the domain structures of LMP1 with a point mutation. (d) 293T cells in 12-well plates were transfected with FLAG-tagged LMP1 mutants (50 ng) and NF- $\kappa$ B-LUC (100 ng) and/or increasing amounts of Myc-tagged STAP-2 (30, 150 or 300 ng). At 48 h after transfection, the cells were harvested, and the luciferase activities were measured. An aliquot of each TCL was analyzed by immunoblotting with an anti-FLAG or anti-Myc antibody.

induced (Fig. 4d and data not shown). NF- $\kappa$ B 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- $\kappa$ B 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- $\kappa$ B activation.

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

One of the important molecular mechanisms underlying LMP1-induced NF- $\kappa$ B 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. 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 SH2-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- $\kappa$ B 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- $\kappa$ B activation in 293T cells (Fig. 5h).

**Reduction of endogenous STAP-2 or TRAF3 enhances LMP1-induced NF- $\kappa$ B activation.** To determine whether STAP-2 represses LMP1-induced NF- $\kappa$ B 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

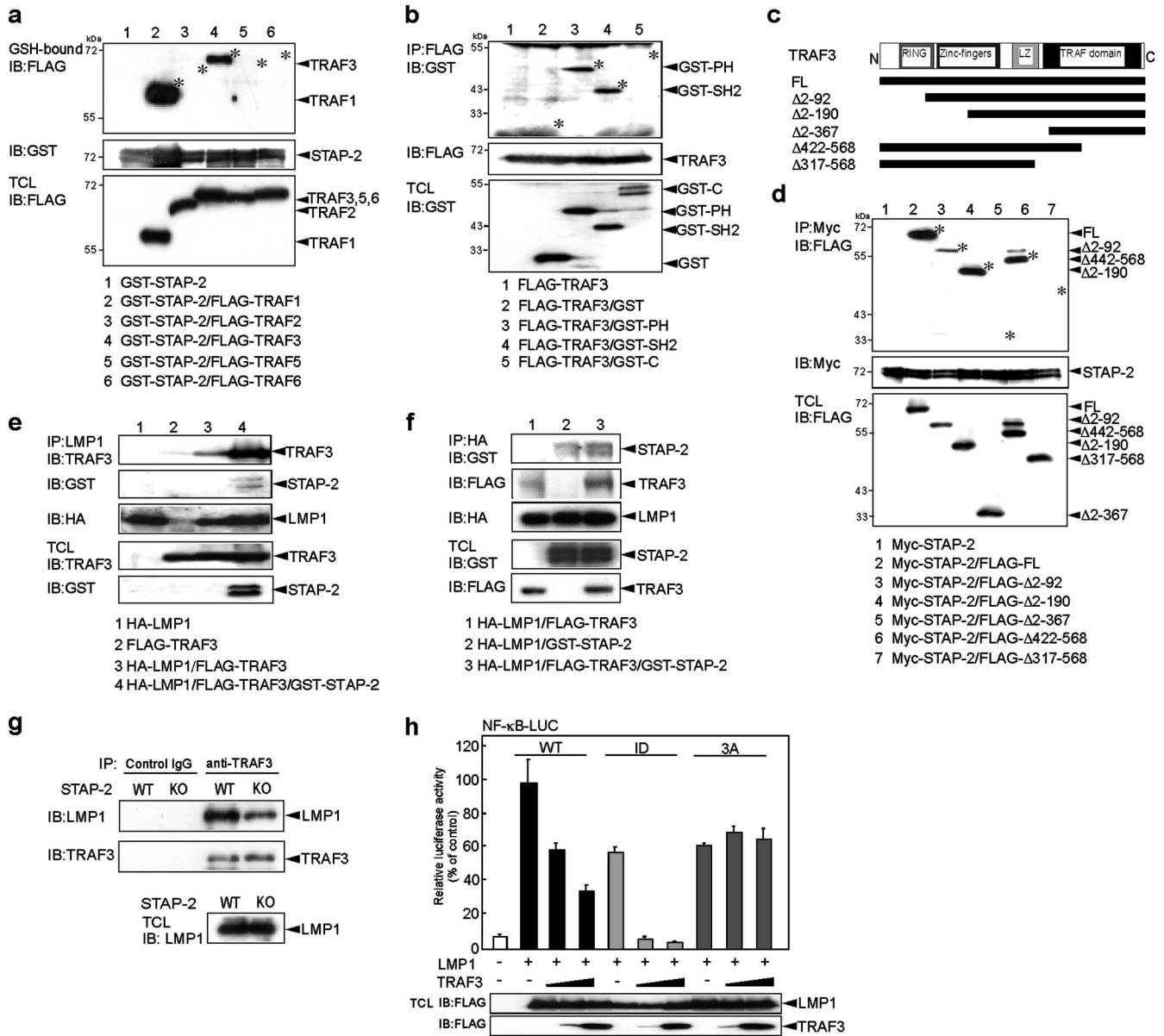


FIG. 5. Molecular interactions among STAP-2, LMP1, and TRAF3. (a) 293T cells ( $1 \times 10^7$  cells/well) were transfected with GST-fused STAP-2 (10  $\mu$ g) with or without FLAG-tagged TRAF1, TRAF2, TRAF3, TRAF5, or TRAF6 (15  $\mu$ g). At 48 h after transfection, the cells were lysed, pulled down 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 ( $1 \times 10^7$  cells) were transfected with FLAG-tagged TRAF3 (8  $\mu$ g) with or without GST or GST-fused STAP-2 deletion mutants (10  $\mu$ g). 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-GST antibody. (c) Schematic diagrams of the domain structures of the TRAF3 deletion mutant fragments. (d) 293T cells ( $1 \times 10^7$  cells/well) were transfected with Myc-tagged STAP-2 (5  $\mu$ g) with or without FLAG-tagged TRAF3 deletion mutants (10  $\mu$ g). 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 ( $1 \times 10^7$ ) were transfected with HA-tagged LMP1 (10  $\mu$ g) or FLAG-tagged TRAF3 (10  $\mu$ g) and/or GST-fused STAP-2 (8  $\mu$ g). At 48 h after transfection, the cells were lysed, immunoprecipitated with anti-LMP1 Mab, and immunoblotted with an anti-TRAF3 or anti-GST antibody or an anti-HA antibody. An aliquot of each TCL was immunoblotted with the anti-TRAF3 or the anti-GST antibody. (f) 293T cells ( $1 \times 10^7$  cells/well) were transfected with HA-tagged LMP1 (10  $\mu$ g) or FLAG-tagged TRAF3 (10  $\mu$ g) and/or GST-fused STAP-2 (8  $\mu$ g). At 48 h after transfection, the cells were lysed, immunoprecipitated with anti-HA antibody, and immunoblotted with an anti-GST or anti-FLAG or anti-HA antibody. An aliquot of each TCL was immunoblotted with the anti-GST or anti-FLAG antibody. (g) 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- $\kappa$ B-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).



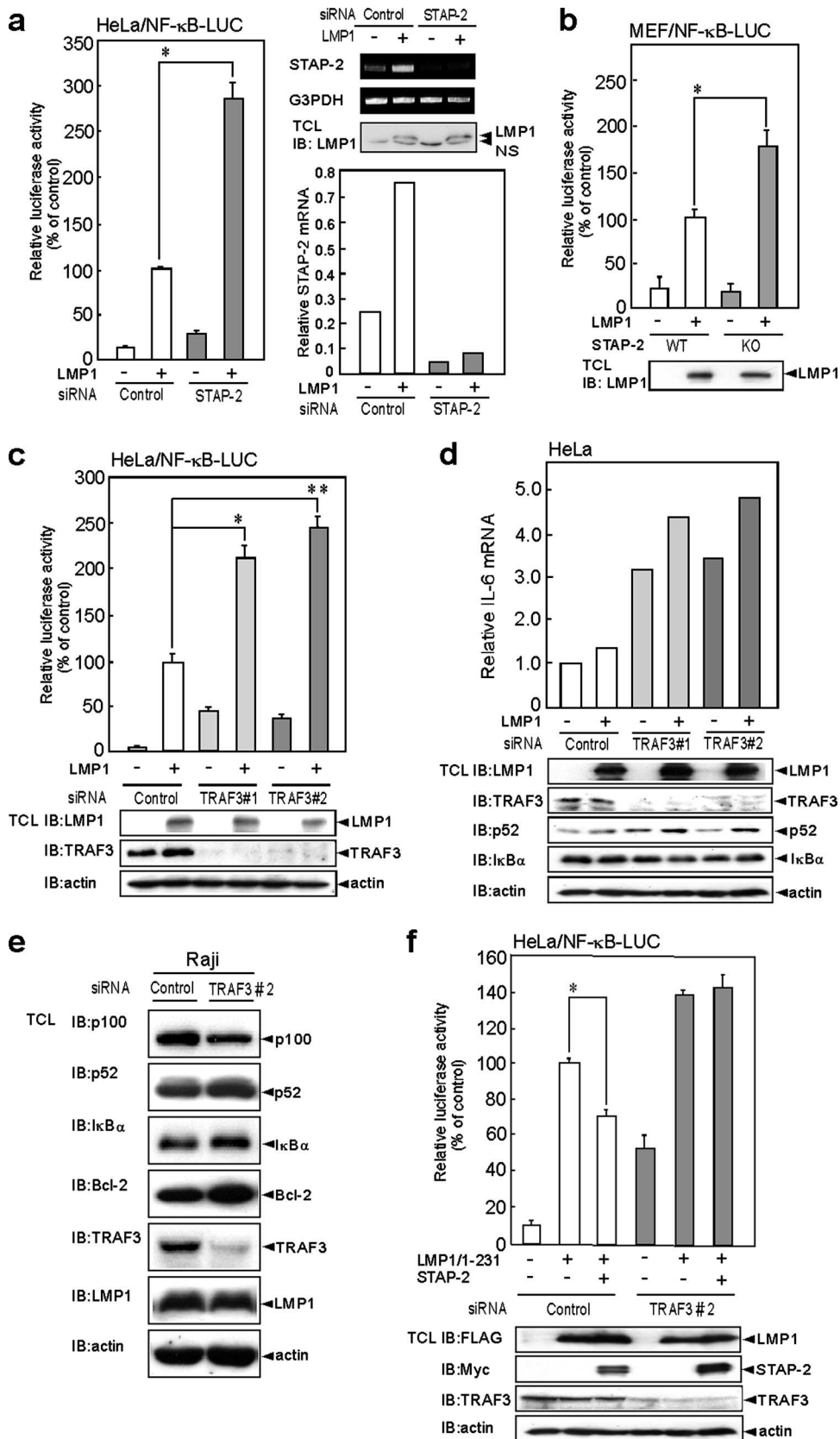


FIG. 6. Reduction of endogenous STAP-2 or TRAF3 enhances LMP1-induced NF-κB activation. (a) HeLa cells in 24-well plates were transfected with a control siRNA or siRNA targeting human STAP-2. The cells were then transfected with HA-tagged LMP1 (3 ng) and NF-κB-LUC (100 ng), using jetPEI. At 48 h after 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- $\kappa$ B 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- $\kappa$ B activation in these cells. Furthermore, we tested LMP1-induced NF- $\kappa$ B activation with the STAP-2 KO MEFs. Importantly, enhanced LMP1-induced NF- $\kappa$ B 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- $\kappa$ B activation. To further verify the roles of TRAF3 in STAP-2-mediated suppression of LMP1-induced NF- $\kappa$ B 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- $\kappa$ B activation in HeLa cells. Furthermore, the TRAF3 knockdown enhanced IL-6 mRNA expression and p100/NF- $\kappa$ B2 processing into p52 but not I $\kappa$ B 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- $\kappa$ B2 processing into p52 via the noncanonical pathway and the Bcl-2 content, although no significant alteration of the I $\kappa$ B content via the canonical pathway was observed (Fig. 6e). These results indicate that TRAF3 may act as a modulator for noncanonical NF- $\kappa$ B activation in HeLa cells and EBV-infected human B cells.

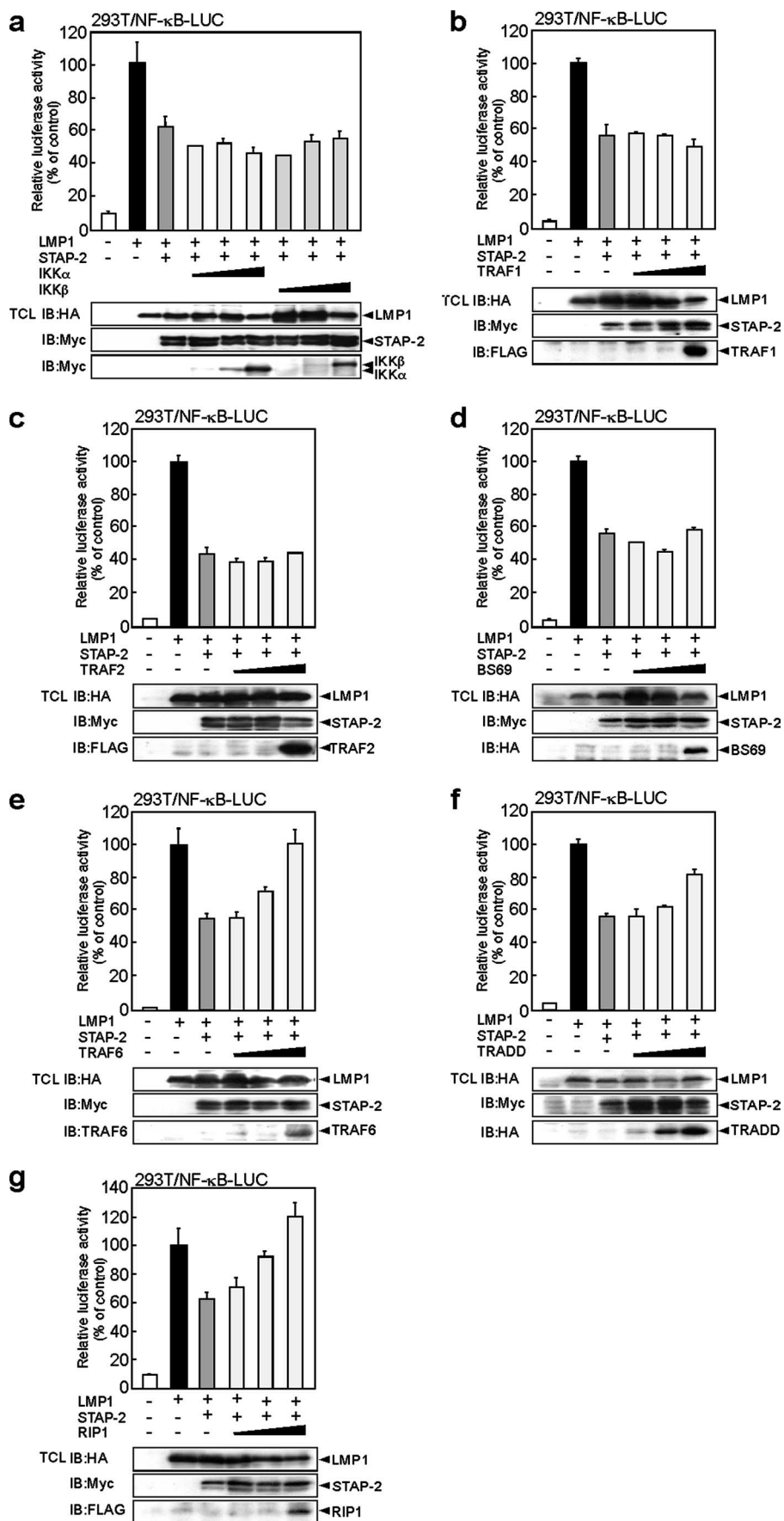
We next examined the effect of reduced TRAF3 expression on STAP-2 suppression of CTAR1-induced NF- $\kappa$ B activation with HeLa cells. As shown in Fig. 6f, reduction of endogenous TRAF3 caused a significant increase in CTAR1-induced NF- $\kappa$ B activation, and STAP-2-mediated suppression of CTAR1-induced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

and is involved in STAP-2-mediated suppression through CTAR1.

**STAP-2 regulates LMP1-induced NF- $\kappa$ B 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- $\kappa$ B activation could be restored by the overexpression of other downstream molecules such as IKK- $\alpha/\beta$ , TRAF1, TRAF2, TRAF6, TRADD, RIP1, and BS69. As shown in Fig. 7a, b, c, and d, overexpression of IKK- $\alpha/\beta$ , TRAF1, TRAF2, or BS69 had no effect on STAP-2-mediated suppression of LMP1-induced NF- $\kappa$ B activation. However, overexpression of TRAF6, TRADD, or RIP1 restored STAP-2-mediated suppression of LMP1-induced NF- $\kappa$ B 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 LMP1-induced NF- $\kappa$ B 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- $\kappa$ B activation in HeLa cells. siRNA-mediated reduced expression of TRAF6 or TRADD resulted in a significant reduction of LMP1-induced NF- $\kappa$ B activation in HeLa cells (Fig. 8a and b), whereas the RIP1 and RIP2 knockdowns had no effect on LMP1-induced NF- $\kappa$ B activation (Fig. 8c and d), indicating that TRAF6 and TRADD, but not RIP1 and RIP2, act as positive regulators of LMP1-induced NF- $\kappa$ B 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- $\kappa$ B activation (14), while RIP is not required for LMP1-induced NF- $\kappa$ B activation (15).

To further delineate the molecular interactions between

independent experiments were carried out for each assay. \*,  $P$  was  $<0.01$ . Total RNA samples isolated from these cells were subjected to RT-PCR analysis using STAP-2 or G3PDH primers. An aliquot of each total cell lysate (TCL) was analyzed by immunoblotting (IB) with an anti-LMP1 antibody. NS, nonspecific band. The STAP-2 expression levels were also quantified by RT and quantitative real-time PCR analysis. Data represent the levels of STAP-2 mRNA normalized by that of G3PDH mRNA as an internal control and are expressed relative to the value at time zero. Data represent the means of duplicate PCR determinations, which generally varied by less than 10%. (b) MEFs in 24-well plates were retrovirally transfected with a control vector or the LMP1 expression vector. The cells were then transfected with NF- $\kappa$ B-LUC (100 ng) using Lipofectamine 2000. 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. \*,  $P$  was  $<0.01$ . (c) HeLa cells in 24-well plates were transfected with a control siRNA or siRNAs targeting human TRAF3 (no. 1 and no. 2). The cells were then transfected with HA-tagged LMP1 (3 ng) and NF- $\kappa$ B-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. \*,  $P$  was  $<0.01$ . An aliquot of each total cell lysate (TCL) was analyzed by immunoblotting with an anti-LMP1, an anti-TRAF3, or an anti-actin antibody. (d) HeLa cells in 12-well plates were transfected with a control siRNA or siRNAs targeting human TRAF3 (no. 1 and no. 2). The cells were then transfected with HA-tagged LMP1 (1.5  $\mu$ g), using jetPEI. At 48 h after transfection, total RNA samples were extracted and analyzed for their IL-6 expression levels by RT and quantitative real-time PCR analysis. Data represent the levels of IL-6 mRNA normalized by that of G3PDH mRNA as an internal control and are expressed relative to data from control siRNA-treated samples without LMP1. Data represent the means of duplicate PCR determinations, which generally varied by less than 10%. An aliquot of each TCL was also analyzed by immunoblotting with the indicated antibodies. (e) Human Raji B cells ( $2 \times 10^6$  cells/well) were transfected with a control siRNA or TRAF3 siRNA (no. 2) using a MicroPporator according to the manufacturer's instructions. The siRNA-transfected Raji B cells were cultured for an additional 36 h and lysed. An aliquot of each TCL was analyzed by immunoblotting with the indicated antibodies. (f) HeLa cells in 24-well plates were transfected with a control siRNA or TRAF3 siRNA (no. 2). The cells were then transfected with NF- $\kappa$ B-LUC (100 ng) with or without FLAG-tagged LMP1 (aa 1 to 231) (100 ng) and Myc-tagged STAP-2 (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. \*,  $P$  was  $<0.01$ . An aliquot of each TCL was analyzed by immunoblotting with an anti-FLAG, an anti-Myc, an anti-TRAF3, or an anti-actin antibody.



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  $\Delta$ PH 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- $\kappa$ B components have revealed essential roles for NF- $\kappa$ B activation in lymphocyte development, activation, proliferation, and survival (22). The target genes of NF- $\kappa$ B 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- $\kappa$ B-activating genes may augment the development of lymphomas. Indeed, aberrant NF- $\kappa$ B 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- $\kappa$ B-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-1 $\beta$ -converting enzyme)-like inhibitory protein (FLIP), designated vFLIP, which can activate the NF- $\kappa$ B 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- $\kappa$ B 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- $\alpha$ , IKK- $\beta$ , TRAF1, TRAF2, and BS69, overcame STAP-2 mediated suppression of LMP1-induced NF- $\kappa$ B activation. (a) 293T cells in 24-well plates were transfected with HA-tagged LMP1 (10 ng) and NF- $\kappa$ B-LUC (100 ng) with or without Myc-tagged STAP-2 (100 ng) and/or increasing amounts of Myc-tagged IKK- $\alpha$  or IKK- $\beta$  (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- $\kappa$ B-LUC (100 ng) with or without Myc-tagged STAP-2 (100 ng) and/or increasing amounts of FLAG-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. (c) 293T cells in 24-well plates were transfected with HA-tagged LMP1 (10 ng) and NF- $\kappa$ B-LUC (100 ng) with or without Myc-tagged STAP-2 (100 ng) and/or increasing amounts of FLAG-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. (d) 293T cells in 24-well plates were transfected with HA-tagged LMP1 (10 ng) and NF- $\kappa$ B-LUC (100 ng) with or without Myc-tagged STAP-2 (100 ng) and/or increasing amounts of HA-tagged BS69 (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 or an anti-Myc antibody. (e) 293T cells in 24-well plates were transfected with HA-tagged LMP1 (10 ng) and NF- $\kappa$ B-LUC (100 ng) with or without Myc-tagged STAP-2 (100 ng) and/or increasing amounts of Myc-tagged TRAF6 (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-TRAF6 antibody. (f) 293T cells in 24-well plates were transfected with HA-tagged LMP1 (10 ng) and NF- $\kappa$ B-LUC (100 ng) with or without Myc-tagged STAP-2 (100 ng) and/or increasing amounts of HA-tagged TRADD (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-Myc or an anti-HA antibody. (g) 293T cells in 24-well plates were transfected with HA-tagged LMP1 (10 ng) and NF- $\kappa$ B-LUC (100 ng) with or without Myc-tagged STAP-2 (100 ng) and/or increasing amounts of FLAG-tagged RIP1 (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.

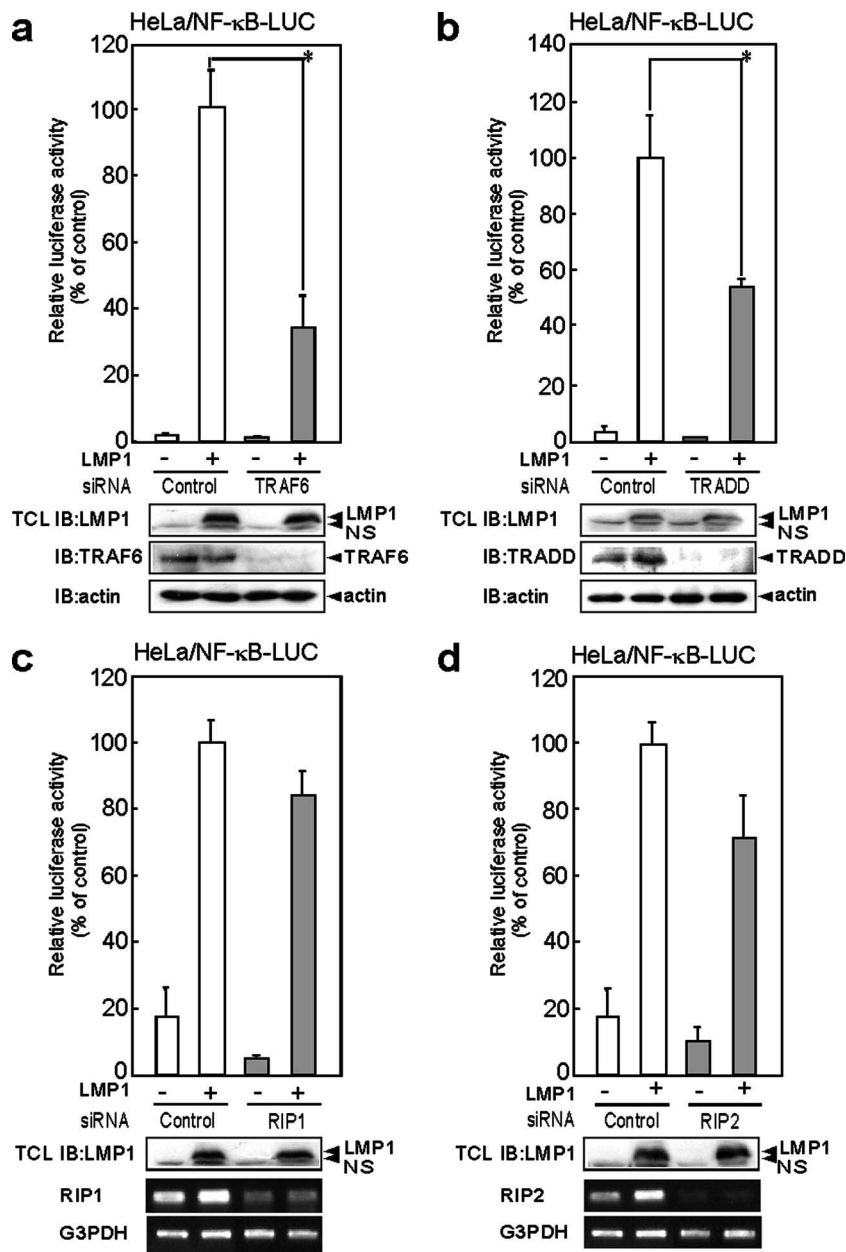


FIG. 8. Reduction of endogenous TRAF6 or TRADD decreases LMP1-induced NF- $\kappa$ B activation. (a to d) HeLa cells in 24-well plates were transfected with a control siRNA or siRNA targeting human TRAF6, TRADD, RIP1, or RIP2. The cells were then transfected with HA-tagged LMP1 (5 ng) and NF- $\kappa$ B-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. \*,  $P$  was  $<0.01$ . 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.

that LMP1-induced NF- $\kappa$ B 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- $\kappa$ B 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-

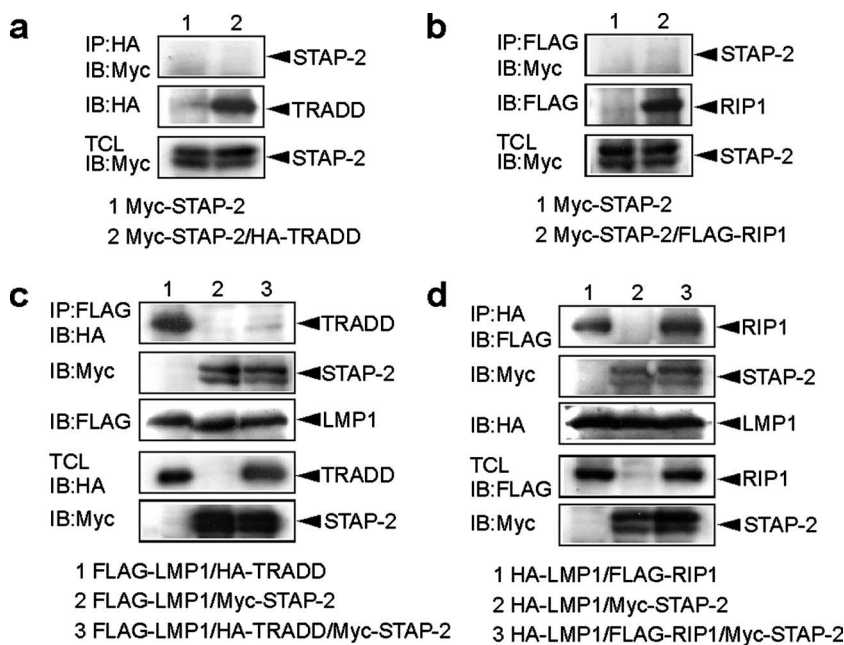


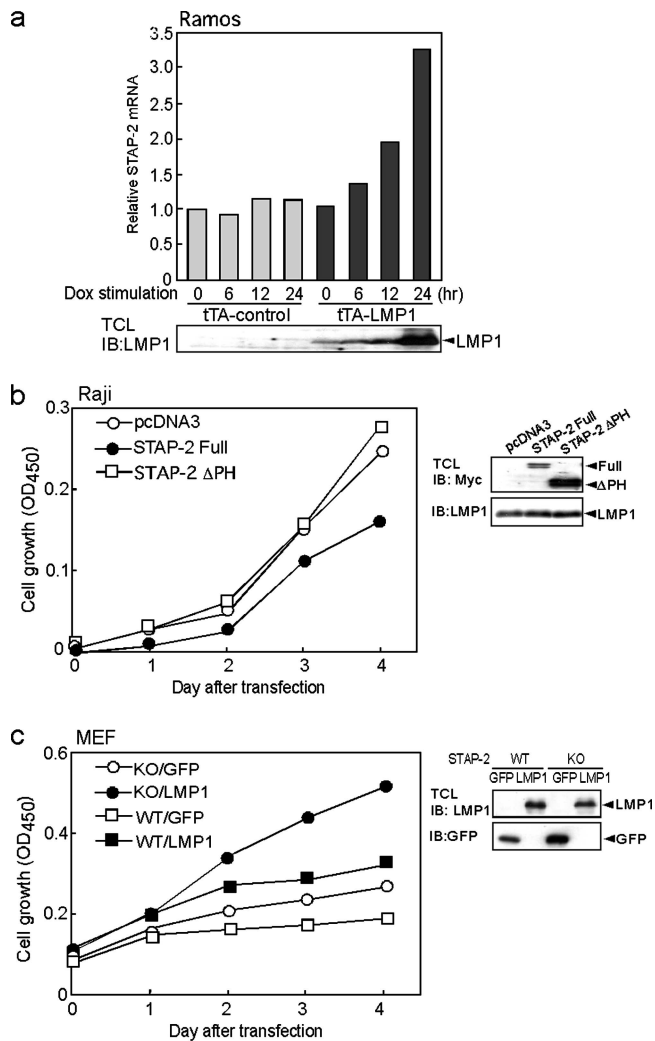
FIG. 9. STAP-2 displaces TRADD from LMP1. (a and b) 293T cells ( $1 \times 10^7$  cells/well) were transfected with Myc-tagged STAP-2 ( $5 \mu\text{g}$ ) with or without HA-tagged TRADD or FLAG-tagged RIP1 ( $10 \mu\text{g}$ ). At 48 h after transfection, the cells were lysed, immunoprecipitated (IP) with an anti-HA (a) or anti-FLAG (b) antibody and immunoblotted (IB) with an anti-Myc or an anti-FLAG antibody. An aliquot of each total cell lysate (TCL) was immunoblotted with the anti-Myc antibody. (c) 293T cells ( $1 \times 10^7$ ) were transfected with FLAG-tagged LMP1 ( $10 \mu\text{g}$ ) or HA-tagged TRADD ( $10 \mu\text{g}$ ) and/or Myc-tagged STAP-2 ( $5 \mu\text{g}$ ). At 48 h after transfection, the cells were lysed, immunoprecipitated with anti-FLAG antibody, and immunoblotted with an anti-HA, an anti-Myc, or an anti-FLAG antibody. An aliquot of each TCL was immunoblotted with the anti-HA or anti-Myc antibody. (d) 293T cells ( $1 \times 10^7$  cells/well) were transfected with HA-tagged LMP1 ( $10 \mu\text{g}$ ) or FLAG-tagged RIP1 ( $10 \mu\text{g}$ ) and/or Myc-tagged STAP-2 ( $5 \mu\text{g}$ ). At 48 h after transfection, the cells were lysed, immunoprecipitated with anti-HA antibody and immunoblotted with an anti-FLAG, an anti-Myc, or an anti-HA antibody. An aliquot of each TCL was immunoblotted with the anti-FLAG or anti-Myc antibody.

tion. 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.

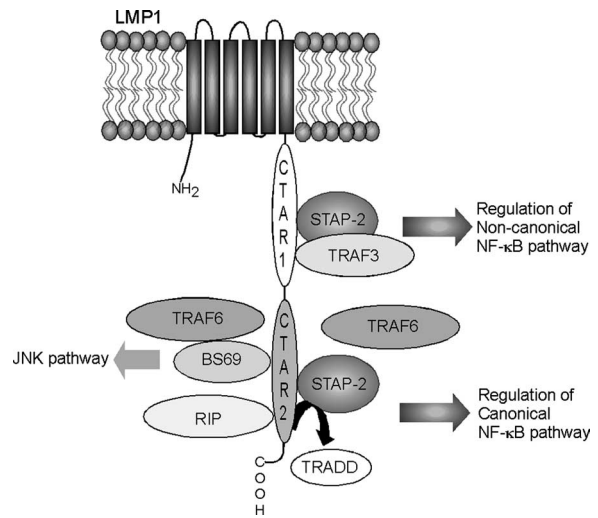
TRAFs are important downstream signaling adaptors for many receptors, such as the TNFR and IL-1 receptor/Toll-like receptor superfamilies, including CD40. With regard to TRAF3, experiments using overexpressed TRAF3 WT have indicated that TRAF3 inhibits NF- $\kappa$ B activation induced by LMP1 (6). For 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- $\kappa$ B 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- $\kappa$ B 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. TRAF3 is also known to interact with the TRAF family member-associated NF- $\kappa$ B activator (TANK) (5, 18). TANK possesses both stimulatory and inhibitory properties upon NF- $\kappa$ B activation (5). For example, TANK functions as an inhibitor of TANK-binding kinase 1 (TBK1)-independent pathways and as a cofactor for stimulation in a TBK1-dependent pathway (33). TANK has also been demonstrated to inhibit LMP1-induced NF- $\kappa$ B 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- $\kappa$ B activation through TANK. The interactions between STAP-2 and TRAF3 may influence the bifunctional property of TANK on LMP1-induced NF- $\kappa$ B activation, although more detailed investigations will be required.

LMP1 has two putative domains, CTAR1 and CTAR2, which initiate signals toward the activation of NF- $\kappa$ B. CTAR1 interacts with TRAF1, -2, -3, and -5 (6), while CTAR2 interacts with TRADD and RIP (14, 15). TRAF6 participates in the



**FIG. 10.** LMP1 induces STAP-2 mRNA expression in human B cells, and STAP-2 acts as an endogenous negative regulator of LMP1-mediated signaling. (a) Human Ramos B cells expressing tTA-control or tTA-LMP1 ( $1 \times 10^7$  cells/well) were incubated with or without Dox ( $1 \mu\text{g/ml}$ ) for the indicated periods. The STAP-2 expression levels were quantified by RT and quantitative real-time PCR analysis. Data represent the levels of STAP-2 mRNA normalized by that of G3PDH mRNA as an internal control and are expressed relative to the values at time zero. Data represent the means of duplicate PCR determinations, which generally varied by less than 10%. An aliquot of each total cell lysate (TCL) was analyzed by immunoblotting (IB) with an anti-LMP1 antibody. (b) Human Raji B cells ( $2 \times 10^6$  cells/well) were nucleofected with empty vector, the Myc-tagged STAP-2 WT, or the STAP-2  $\Delta\text{PH}$  construct ( $2 \mu\text{g}$ ), using  $100 \mu\text{l}$  of V solution (Amaxa Biosystems) according to the manufacturer's instructions. Nucleofected Raji cells ( $3 \times 10^3$  cells/well) were cultured in 96-well plates for the indicated periods, and the final numbers of cells were measured using a Cell Counting Kit-8. Data are the means of triplicate experiments, which generally varied by less than 10%. Similar results were obtained in three independent experiments. An aliquot of each TCL was analyzed by immunoblotting with an anti-Myc or an anti-LMP1 antibody. (c) MEFs ( $2 \times 10^3$  cells/well) in 96-well plates were retrovirally transfected with a control GFP vector or an LMP1 expression vector and cultured for the indicated periods. The cell numbers were measured using a Cell Counting Kit-8. Data are the means of triplicate experiments, which generally varied by less than 10%. Similar results were obtained in three independent experiments. An aliquot of each TCL was analyzed by immunoblotting with an anti-LMP1 or an anti-GFP antibody.



**FIG. 11.** Schematic showing the proposed function of STAP-2 in the suppression of LMP1-induced NF- $\kappa$ B activation. LMP1 consists of an N-terminal tail, six transmembrane domains, and a long cytoplasmic C-terminal tail which contains two NF- $\kappa$ B activating domains, CTAR1 and CTAR2. STAP-2 directly interacts with LMP1 and suppresses LMP1-induced NF- $\kappa$ B activation through both CTAR1 and CTAR2. STAP-2 suppresses CTAR1-mediated noncanonical NF- $\kappa$ B activation through direct interactions with TRAF3. Alternatively, STAP-2 regulates CTAR2-mediated canonical NF- $\kappa$ B activation by displacing TRADD from LMP1. LMP1-induced JNK activation is mediated by BS69 and TRAF6.

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- $\kappa$ B activation (14), whereas RIP is not required for LMP1-induced NF- $\kappa$ B 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- $\kappa$ B activation through CTAR1, but not CTAR2, was found to be dependent on TRAF3, since TRAF3 inhibited LMP1 ID-induced, but not LMP1 3A-induced, NF- $\kappa$ B activation. On the other hand, STAP-2-mediated suppression of LMP1-induced NF- $\kappa$ B activation was restored by overexpression of TRAF6, suggesting that STAP-2 also affected LMP1-mediated NF- $\kappa$ B 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- $\kappa$ B activation, which interacted directly with LMP1 through CTAR2. These facts suggest that STAP-2-mediated suppression of LMP1-induced NF- $\kappa$ B 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- $\kappa$ B activation through direct or indirect modulation of interactions among signaling molecules, and our proposed mechanisms are now

illustrated in Fig. 11. We have also shown that LMP1 expression upregulates STAP-2 expression, suggesting that STAP-2 plays a role in the defense of host cells against EBV infection. In addition, our data suggest the possibility that STAP-2 may be a novel candidate for antiviral drug development, to regulate EBV LMP1-induced signaling.

#### ACKNOWLEDGMENTS

We thank J. Inoue, H. Kobayashi, N. Inohara, H. Shibuya, T. Fujita, and S. Akira for their kind gifts of reagents.

This study was supported in part by the Sankyo Foundation of Life Science and a Grant-in-Aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

We have no competing financial interests.

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