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BS69, a Specific Adaptor in the Latent Membrane Protein 1-Mediated c-Jun N-Terminal Kinase Pathway

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We previously demonstrated that the Epstein-Barr virus-encoded latent membrane protein 1 (LMP1) potently activates the cellular c-Jun N-terminal kinase (JNK) pathway by sequentially engaging an unknown adaptor, TRAF6, TAB1/TAK1, and JNKKs. We now show that BS69, a MYND domain-containing cellular protein, is the missing adaptor that bridges LMP1 and TRAF6, as the MYND domain and a separate region of BS69 bind to the carboxyl termini of LMP1 and TRAF6, respectively. While LMP1 promotes the interaction between BS69 and TRAF6, the complex formation between LMP1 and TRAF6 is BS69 dependent. A fraction of LMP1 and BS69 is constitutively colocalized in the membrane lipid rafts. Importantly, knockdown of BS69 by small interfering RNAs specifically inhibits JNK activation by LMP1 but not tumor necrosis factor alpha. Although overexpression of either BS69 or a mutant LMP1 without the cytoplasmic carboxyl tail is not sufficient to activate JNK, interestingly, when BS69 is covalently linked to the mutant LMP1, the chimeric protein restores the ability to activate JNK. This indicates that the recruitment and aggregation of BS69 is a prerequisite for JNK activation by LMP1.

Epstein-Barr virus (EBV) is a transforming DNA virus (45). The main cell types infected by EBV are human B lymphocytes and nasopharyngeal epithelial cells (15, 40, 48). Although up to 95% of world population is EBV positive, most of them will be healthy carriers for the rest of their lives, due to the effective surveillance of their immune systems (41, 48). However, in certain immune system-compromised individuals, the presence of EBV is thought to contribute to several malignancies including Burkitt's lymphoma, Hodgkin's disease, and nasopharyngeal carcinoma (NPC) (41, 45, 48). Although NPC is relatively rare in most part of the world (e.g., <1 per 100,000 Caucasians in Western countries), it is quite prevalent in southern China, including Hong Kong where the incidence rate is >20 per 100,000 (34).

EBV readily transforms quiescent human B cells in vitro, resulting in formation of the immortalized lymphoblastoid cell lines (45). Nine latent viral antigens including six nuclear antigens (EBNA1 to -6) and three membrane proteins (latent membrane protein 1 [LMP1], LMP2A, and LMP2B) are expressed in lymphoblastoid cell lines (45). Among them, LMP1 is most extensively studied and is well established to be an oncogenic protein. LMP1 is a 386-amino-acid (386-aa) viral protein with six transmembrane domains and both its amino and carboxyl tails facing the cytoplasm (Fig. 1A). When overexpressed in fibroblasts and epithelial cells, LMP1 could transform these cells (3, 16, 50). When specifically introduced into epidermis and lymphocytes in transgenic mice, the mice dis-

played epithelial hyperplasia and an increased incidence for lymphoma, respectively (31, 52). In addition, a recombinant EBV with a truncated LMP1 fails to transform resting human B cells in vitro (26, 27). Thus, to understand the molecular mechanisms underlying the EBV-associated pathogenesis, it is crucial for us to first understand the impact of LMP1 in host cells.

Two subregions in the 200-aa (i.e., aa 187 to 386) cytoplasmic carboxyl tail of LMP1, namely, the C-terminal activating region 1 (CTAR1) and CTAR2 (Fig. 1A), play important roles in LMP1-mediated cell transformation and signaling (15, 40, 48). CTAR1 contains a typical tumor necrosis factor (TNF) receptor (TNFR)-associated factor (TRAF)-binding motif; this motif is required for CTAR1 binding to TRAF1, -2, -3, and -5, which are members of an important family of proteins involved in cytokine signaling (7, 15, 40, 48). CTAR1 is capable of activating the phosphatidylinositide-3 kinase/Akt-mediated pathway and, to a lesser extent, the NF-κB pathway (11, 20, 39). In a few selected cell types where TRAF1 is expressed, CTAR1 is also capable of moderately activating the c-Jun N-terminal kinase (JNK) pathway (13). In contrast, CTAR2 is known to be responsible for the majority of the JNK and NF-κB activity induced by LMP1 (15, 40, 48). CTAR2 was found to interact with TNFR-associated death domain protein (TRADD) and receptor-interacting protein, two key proteins indispensable for the TNF-α-mediated NF-κB and JNK pathways (18, 22, 24). In addition, overexpression of the "dominant-negative" TRADD or TRAF2 was found to inhibit the LMP1-induced JNK and NF-κB pathways (12, 23, 25, 29). Thus, LMP1 was previously thought to functionally mimic members of the TNFR superfamily in signaling (15, 40, 48). However, several recent reports argued against a role for

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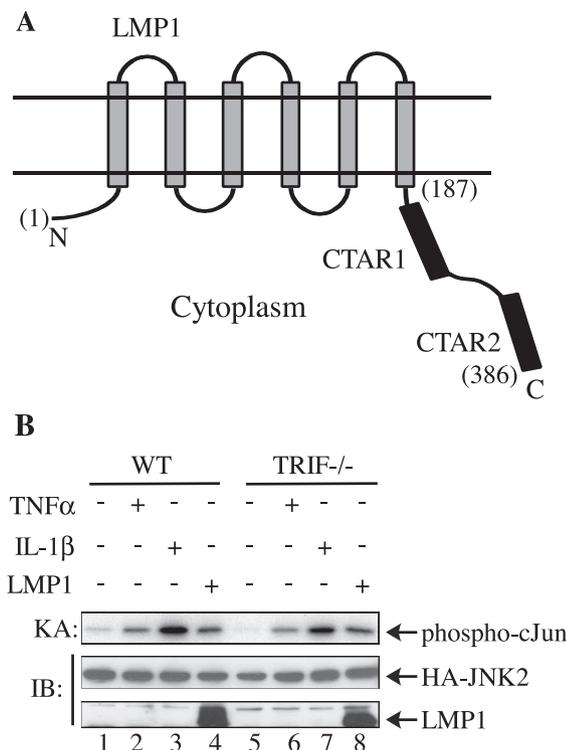† These authors contributed equally to this work.

A

B

FIG. 1. TRIF is not essential in the LMP1-mediated JNK pathway. (A) Schematic representation of LMP1. N and C indicate the amino and carboxyl termini, respectively. The two horizontal parallel lines represent the lipid bilayer of the plasma membrane. The numbers in parenthesis indicate the positions of amino acids. (B) The wild-type (WT) and TRIF$^{-/-}$ MEFs were separately cotransfected with HA-JNK2, with either an empty vector or LMP1. Before harvest, cells were either left untreated or treated with TNF-α or IL-1β (20 ng/ml for 10 min). HA-JNK2 was subjected to immune complex kinase assays (KA). IB, immunoblot.

TRAF2 and TRADD in the LMP1-mediated JNK and NF-κB pathways (29, 37, 49, 54). Using cells derived from different knockout mice and the small interference RNA (siRNA) technique, we recently showed that the LMP1-mediated JNK pathway is distinct from that utilized by members of the TNFR superfamily as LMP1 does not require TRADD, TRAF2, and receptor-interacting protein to activate JNK (49). Instead, LMP1 selectively engages TRAF6, TAB1/TAK1, and JNKK1/2 to activate JNK (49). Although members of the interleukin-1 receptor (IL-1R)/Toll-like receptor superfamily also selectively utilize TRAF6/TAB1/TAK1 to activate JNK (1), LMP1 differs from them in that LMP1 does not require myeloid differentiation factor 88, IL-1 receptor-associated kinase 1 (IRAK1), and IRAK4 to engage TRAF6 (49). As LMP1 does not seem to directly interact with TRAF6 (49), it remains unclear how LMP1 transmits signal to TRAF6.

BS69, a multidomain-containing (i.e., PHD, Bromo, PWWP, and MYND) cellular protein (Fig. 2A), was originally identified as an adenoviral early region 1A (E1A)-interacting protein and shown to inhibit the E1A-mediated transcription (17, 38). The carboxyl MYND domain of BS69 is predicted to adopt a two-zinc-finger-like structure and shown to interact with several target molecules containing the PXLXP motif (2). BRAM1, an alternatively spliced variant of BS69 (Fig. 2A),

retains the full MYND domain and is shown to interact with the BMP receptor 1A (32).

We show here that BS69, but not BRAM1, serves as a specific adaptor directly linking LMP1 and TRAF6. A fraction of LMP1 and BS69 constitutively colocalize in membrane lipid rafts. Furthermore, BS69 is specifically required for LMP1-mediated JNK activation.

MATERIALS AND METHODS

Cell lines, DNA constructs, and reagents. 293T, HeLa, and TRIF$^{-/-}$ (TRIF is TIR domain-containing adaptor protein inducing beta interferon) mouse embryonic fibroblasts (MEFs) were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100-U/ml penicillin, and 100-μg/ml streptomycin in a 37°C incubator with 5% CO$_2$. LMP1, HA-BS69, and HA-BRAM1 were described previously (6, 38). Both the full-length and the truncated BS69 cDNA fragments were inserted in frame into pGADT7 (BD Biosciences) to generate BS69-AD fusion constructs. Yeast bait constructs were constructed by inserting the PCR fragments encoding either the entire carboxyl terminus of LMP1(CT) (aa 187 to 386) or its truncation mutant without the carboxyl terminal 8 aa (aa 187 to 378) [i.e., LMP1(Δ8)] into pGBKT7 (BD Biosciences), respectively. Gal4–DNA-binding domain (DBD)–TRAF6 (Gal4-DBD-TRAF6) and Gal4-DBD-TRAF6(C) were generated by inserting the PCR fragments encoding either the full-length or TRAF(C) domain (aa 351 to 522) of TRAF6 into pGBKT7, respectively. Xpress-tagged TRAF6 (xp-TRAF6) was constructed by inserting the cDNA fragments into pcDNA3.1c. The vector-based BS69 small hairpin RNAs (shRNAs) were constructed by inserting the following two double-stranded oligonucleotides into the pSuper vector between BglII and HindIII sites (5): (i) human BS69 (N terminus, bp 196 to 214) forward, 5' GATCCCC<u>TGC CATTTGCCTGGAGAGG</u>TTCAAGAGA<u>CCTCTCCAGG</u>CAAATGGCAT TTTTGGAAA; (ii) human BS69 (C terminus, bp 1437 to 1455) forward, 5' GATCCCC<u>CATGCAGGGTGAGATGGAC</u>TTCAAGAGA<u>GTCCATCTCAC CCTGCATG</u>TTTTTGGAAA (both sense and antisense BS69 sequences are underlined). The LMP1 (1-186)-BS69 chimera was generated using fusion PCR by linking LMP1 (1-186) with the full-length HA-BS69, and then the PCR product was digested with EcoRI and BamHI and cloned into the PCMV5 vector. All constructs generated above were verified by DNA sequencing. TNF-α and IL-1β were purchased from R&D Systems.

Transfection and cell lysis. Cells were transfected with various plasmids using either Lipofectamine Plus reagents (for 293T and HeLa cells) or Lipofectamine 2000 (Invitrogen) (for MEF cells) according to the manufacturer's instruction. Twenty-four hours after transfection, the cells were lysed in lysis buffer (50 mM HEPES [pH 7.6], 10% glycerol, 1% Triton X-100, 150 mM NaCl, 1 mM EGTA, 1.5 mM MgCl$_2$, 100 mM NaF, 20 mM p-nitrophenyl phosphate, 20 mM β-glycerol phosphate, 2 mM dithiothreitol, 50 μM sodium vanadate, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 0.7 μg/ml pepstatin), followed by removal of insoluble debris with a bench-top centrifuge to obtain whole-cell extracts (WCEs).

Antibodies. Mouse monoclonal antibodies to HA (Santa Cruz), JNK (BD Biosciences), β-tubulin (Sigma), and TRAF6 (Santa Cruz); rabbit polyclonal antibodies to Xpress, caveolin-1 (Santa Cruz), phospho-p38, phospho-JNK, and total p38 (Cell Signaling); and the goat polyclonal antibody to TRADD (Santa Cruz) were used in this study. Monoclonal anti-LMP1 was described previously (6). A rabbit polyclonal antibody to BS69 was raised with an amino-terminal region (aa 1 to 265) of BS69 as an antigen.

Coimmunoprecipitation assays. 293T cells were cotransfected with various plasmids. Thirty-six hours after transfection, the cells were cross-linked with 20-μg/ml of dithiobis(succinimidylpropionate) (Pierce) for 10 min, followed by lysis in RIPA buffer (25 mM HEPES [pH 7.4], 1% NP-40, 0.1% sodium dodecyl sulfate [SDS], 0.5% sodium deoxycholate, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin). Protein A-Sepharose beads were incubated with 400 μg of extracts and 2 μg of appropriate antibodies for 2 h at 4°C. After being washed extensively with RIPA buffer, we eluted the bound proteins by boiling them and subjected them to SDS-polyacrylamide gel electrophoresis and immunoblotting.

Immune complex protein kinase assays. For the JNK kinase assays, we followed the protocols as described previously (53).

Yeast two-hybrid screening. We first transformed the yeast strain AH109 expressing Gal4-DBD-LMP1(CT) (aa 187 to 386) fusion protein with a mouse 17-day-embryo MATCHMAKER cDNA library (no. 638846; BD Biosciences) following the manufacturer's protocol. Half of the cells were plated on triple
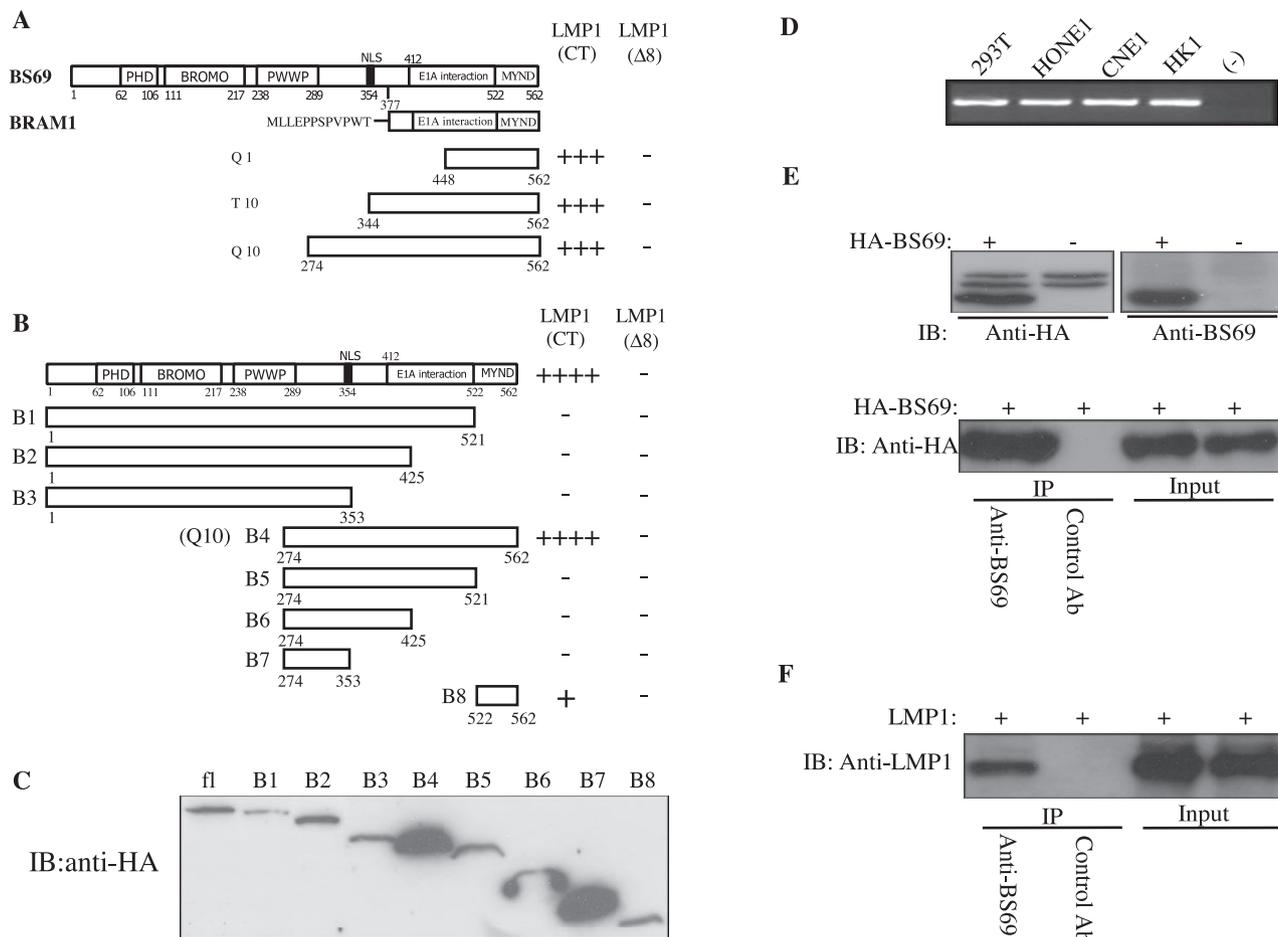
FIG. 2. BS69 interacts with LMP1 in yeast and mammalian cells. (A and B) Schematic representation of BS69, BRAM1, and various truncated fragments of BS69. The numbers underneath each bar indicate the positions of amino acids in BS69. NLS, nuclear localization sequence; CT, carboxyl terminus. The interaction of full-length BS69 and its truncated derivatives with either LMP1(CT) or LMP1(Δ8) was analyzed by yeast two-hybrid assays, and the results are summarized next to each construct on the right. Q1, T10, and Q10 were three BS69 fragments isolated from our yeast two-hybrid screen. ++++ and +++ represent the appearance of yeast colonies in 3 and 5 days, respectively, after being streaked on QDO plates. ++ and + indicate the appearance of yeast colonies in 3 and 5 days, respectively, on TDO plates (these clones did not grow on QDO plates). −, no growth on either QDO or TDO plates. (C) The expression levels of different BS69 fragments in yeast were detected by immunoblotting. fl, full-length. (D) Total RNA was extracted from 293T, HONE1, CNE1, and HK1 cells; an equal amount of RNA was subjected to RT-PCR analysis. (−), negative control without the reverse transcriptase. (E) 293T cells were either mock transfected or transfected with HA-BS69. (Top) WCEs were subjected to direct immunoblot analysis. (Bottom) WCEs were immunoprecipitated separately with either the anti-BS69 antibody or a control antibody (Ab), followed by immunoblotting with an anti-HA antibody. (F) 293T cells were transfected with LMP1. Cells were cross-linked with dithiobis(succinimidylpropionate) for 5 min before harvest. The lysates were immunoprecipitated with either the anti-BS69 antibody or a control antibody, followed by immunoblotting with the anti-LMP1 antibody.

synthetic dropout plates (TDO) lacking leucine, tryptophan, and histidine and supplemented with 5 mM 3-amino-1,2,4-triazole. The other half was plated on quadruple synthetic dropout plates (QDO) lacking leucine, tryptophan, histidine, and adenine. A total of 180 colonies were obtained from primary screening. Yeast fish plasmid DNA were then purified, transformed into *Escherichia coli* DH5α, and recovered on LB-agar plates containing 50 μg/ml ampicillin. Distinctive plasmids (judged by insert size and restriction enzyme digestion pattern) were separately retransformed into AH109 containing either pGBKT7 (empty bait vector), pGBKT7-p53 (negative control), or pGBKT7-LMP1(CT) to confirm their specific interaction with LMP1(CT). Thirty-two LMP1(CT)-specific clones were subjected to DNA sequencing to reveal their identities.

Isolation of lipid rafts by sucrose gradient centrifugation. Lipid rafts were isolated by sucrose gradient centrifugation as described by Yasui et al. with some modifications (56). Briefly, 2×10^7 cells were lysed on ice for 30 min in 0.5 ml of TENT buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 20 mM *p*-nitrophenyl phosphate, 20 mM β-glycerol phosphate, 2 mM dithiothreitol, 50 μM sodium vanadate, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin). Cell lysates were then mixed with 0.5 ml of 90% ice-cold sucrose in TENT. One milliliter of

the mixture was placed at the bottom of a centrifuge tube and overlaid with 1 ml (each) of 30% and 5% sucrose in TENT. After centrifugation in a Hitachi preparative ultracentrifuge (Himac CP80MX) with a Sorvall TST 60.4 rotor at $170,000 \times g$ at 4°C for 18 h, 0.3-ml fractions were aspirated from the top of the gradient and analyzed by SDS-polyacrylamide gel electrophoresis and immunoblotting.

Semiquantitative RT-PCR. Total RNA was extracted from cells using TRI REAGENT (Molecular Research Center, Inc., Cincinnati, Ohio). Reverse transcription-PCR (RT-PCR) was performed as previously described by using AmpliTaq Gold (Applied Biosystems) (44). The PCR program started with an initial denaturation at 95°C for 10 min, followed by 40 cycles (each consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s) of amplification, and ended with a final extension at 72°C for 10 min. The glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH) was used as a control with only 25 cycles of amplification. The sequences of the primers used are as follows: BS69 (forward), 5′ GTCTC GAGTCCACGGTATG; BS69 (reverse), 5′ AACACCTCTCCAGGCAAATG; GAPDH (forward), 5′ ATCTCTGCCCCCTCTGCTGA; GAPDH (reverse), 5′ GGATGACCTTGCCCACAGCC.

RESULTS

TRIF is not involved in the LMP1-mediated JNK pathway. To look for the missing adaptor that may bridge TRAF6 and LMP1, we first focused on a few known TRAF6-interacting proteins including IRAK1, IRAK4, and TRIF (1). As we had already shown that neither IRAK1 nor IRAK4 were involved in the LMP1-mediated JNK pathway (49), we then turned to TRIF, a TRAF6-interacting adaptor molecule mainly involved in toll-like receptor 3/4 signaling (46, 55). The wild-type and TRIF$^{-/-}$ MEFs were separately cotransfected with HA-JNK together with either an empty vector or LMP1. As expected, both the TNF-α- and IL-1β-mediated JNK activation was not affected in TRIF$^{-/-}$ cells (Fig. 1B) (55). Similarly, LMP1 also activated JNK in cells with or without TRIF (Fig. 1B, lanes 4 and 8). This suggests that TRIF is unlikely the adaptor to bridge LMP1 and TRAF6.

BS69 interacts with LMP1 in both yeast and mammalian cells. Next, we performed a yeast two-hybrid screening using the cytoplasmic carboxyl tail of LMP1 (i.e., aa 187 to 386) as bait. Among the 32 positive clones we identified, 12 of them encoded three different but overlapping carboxyl fragments of BS69, a multidomain-containing cellular protein (Fig. 2A) (38). Interestingly, none of the three BS69 fragments interacted with a control LMP1 bait missing the carboxyl-terminal eight amino acids [i.e., LMP1(Δ8)] by yeast two-hybrid assays, suggesting that the carboxyl terminal eight amino acids are necessary for LMP1 binding to BS69.

To map the minimal region on BS69 that binds to LMP1, different truncated BS69 fragments were generated and tested in the yeast two-hybrid assays. Although two longer BS69 fragments with intact carboxyl termini (i.e., full-length and B4) interacted well with LMP1, a short BS69 fragment consisting of only the carboxyl-terminal MYND domain (i.e., B8) also weakly interacted with LMP1 (Fig. 2B). In contrast, all other BS69 fragments without the carboxyl terminal MYND domain (i.e., B1 to B3 and B5 to B7) completely failed to interact with LMP1 (Fig. 2B). As a control, we showed that all BS69 constructs were expressed in yeast cells (Fig. 2C). As several cellular proteins contain the MYND domain, to test whether the MYND of BS69 specifically interacts with LMP1, we also tested the interaction of LMP1 with BLU, an MYND-containing tumor suppressor implicated in several cancers including NPC (44). No such interaction was detected by yeast two-hybrid assays (our unpublished data). Thus, our data above indicate that the MYND domain of BS69 is necessary and sufficient to specifically interact with LMP1.

BS69 was previously shown to be expressed in a few tissues previously examined (17). To find out whether BS69 is expressed in 293T cells and several NPC-derived cell lines (i.e., HONE1, CNE1, and HK1), total RNA was extracted from these cell lines, and RT-PCR was performed. As shown in Fig. 2D, BS69 mRNA was expressed in 293T, HONE1, CNE1, and HK1 cells.

Next, we tested whether LMP1 interacts with the endogenous BS69 in mammalian cells. To facilitate our study, we first raised a polyclonal BS69 antibody recognizing a region at its amino terminus. Although the antibody could not detect the endogenous BS69 in 293T whole-cell extracts in Western blotting, it did recognize the transfected BS69 and could effectively
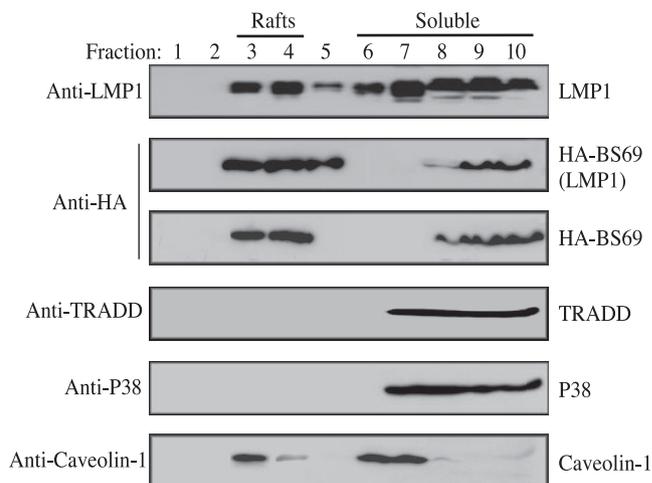

FIG. 3. A fraction of BS69 is localized in membrane lipid rafts. 293T cells were separately transfected with LMP1, HA-BS69, or both. Cells were extracted in TENT buffer, and the lysates were subjected to ultracentrifugation in a sucrose gradient. Samples from each fraction were subjected to immunoblot analysis with various primary antibodies as indicated.

immunoprecipitate it (Fig. 2E). We then transfected LMP1 into 293T cells and prepared the whole-cell extracts. We subjected the whole-cell extracts to immunoprecipitation with either the anti-BS69 antibody or a control antibody. We showed that LMP1 was only coprecipitated by the anti-BS69 antibody but not by the control antibody (Fig. 2F).

A fraction of LMP1 and BS69 constitutively colocalizes in membrane lipid rafts. It has been well established that a fraction of LMP1 resides in membrane lipid rafts (9, 10, 19, 28). We then examined whether BS69 is localized to lipid rafts or not with or without a cotransfected LMP1. As shown in Fig. 3, we confirmed that a fraction of LMP1 was indeed present in the lipid raft fractions, as indicated by the presence of caveolin-1, a known resident of lipid rafts (35). Interestingly, with or without LMP1, a fraction of BS69 was constitutively present in lipid raft fractions (Fig. 3, panels 2 and 3). As a negative control, we showed that neither TRADD nor p38 mitogen-activated protein kinase was present in the lipid raft fractions, in agreement with previous reports (19).

BS69 interacts with TRAF6 in yeast and mammalian cells. We next checked whether BS69 interacts with TRAF6. We first tested whether different BS69 truncation clones (i.e., those used in the experiments shown in Fig. 2B) interacted with either the full-length TRAF6 or its carboxyl-terminal TRAF domain [i.e., TRAF6(C), aa 351 to 522] in yeast two-hybrid assays. As shown in Fig. 4A, all BS69 fragments containing amino acids 274 to 353 bound to TRAF6, with the full-length BS69 and B4 showing the strongest interaction (as manifested by the shorter time it took for yeast cells to grow in quadruple dropout plates). In contrast, those BS69 fragments (i.e., Q1, T10, and B8) missing the region (aa 274 to 353) failed to interact with TRAF6. Furthermore, we found that the full-length BS69 and B4 interacted with TRAF6(C) (Fig. 4A), suggesting that the carboxyl TRAF domain of TRAF6 interacts with BS69. The fact that those BS69 fragments missing the carboxyl terminus interacted only with the full-length TRAF6

FIG. 4. BS69 interacts with TRAF6 in both yeast and mammalian cells. (A) The schematic representation of the full-length and truncated BS69 and a summary of their interaction with either the full-length or the TRAF(C) domain of TRAF6 in yeast. The definitions for the plus and minus signs are the same as that in the legend to Fig. 2. (B to D) 293T cells were cotransfected with various plasmids as indicated. WCEs were immunoprecipitated with either the anti-xp (B), anti-HA (C), or anti-BS69 (D) antibodies, and the coprecipitated proteins were detected by immunoblotting.

but not TRAF6(C) suggested that both the amino terminus of TRAF6 and the carboxyl terminus of BS69 facilitate the interaction between TRAF6(C) and the region in BS69 spanning amino acids 274 to 353.

To further confirm whether BS69 interacts with TRAF6 in mammalian cells, we cotransfected 293T cells with HA-BS69 together with either an xp-TRAF6 or JNKK2 (control). HA-BS69 was specifically coimmunoprecipitated with TRAF6 but not JNKK2 (Fig. 4B). Based on our yeast two-hybrid results, BRAM1, the splice variant of BS69, was not expected to interact with TRAF6, due to its lack of the region spanning amino acids 274 to 353 (Fig. 2A). To test whether this was the case in mammalian cells, we cotransfected 293T cells with xp-TRAF6 together with either BS69 or BRAM1. Indeed, xp-TRAF6 was specifically coprecipitated by BS69 but not by BRAM1 (Fig. 4C). We next tested whether the endogenous BS69 and TRAF6 interacted with each other. Interestingly, we found that the endogenous BS69 and TRAF6 did not significantly interact with each other in cells without LMP1. How-

ever, in the presence of LMP1, BS69 indeed formed a stable complex with TRAF6 (Fig. 4D).

BS69 is required for LMP1 to recruit TRAF6. We previously showed that LMP1 can form a complex with the endogenous TRAF6 in mammalian cells (49). We next tested whether BS69 was essential for the formation of such a complex. We resorted to siRNA to knock down the endogenous BS69 by constructing two vector-based shRNAs, which target separate regions at the amino and carboxyl termini of BS69, respectively (5). Two shRNA clones targeting either end of BS69 were chosen with N3, C2, and C3 (the prefix N and C denoting the amino- and carboxyl-terminal shRNAs, respectively) containing the correct targeting sequences. In contrast, clone N2 contains 2-bp mutations due to errors generated during cloning. When different control and BS69-specific shRNAs were transfected together with HA-BS69 into 293T cells, N3, C2, and C3 shRNA efficiently reduced expression of HA-BS69 (Fig. 5A). In contrast, the empty vector and N2 had no obvious effect (Fig. 5A). When we examined BS69 mRNA by RT-PCR, we also found
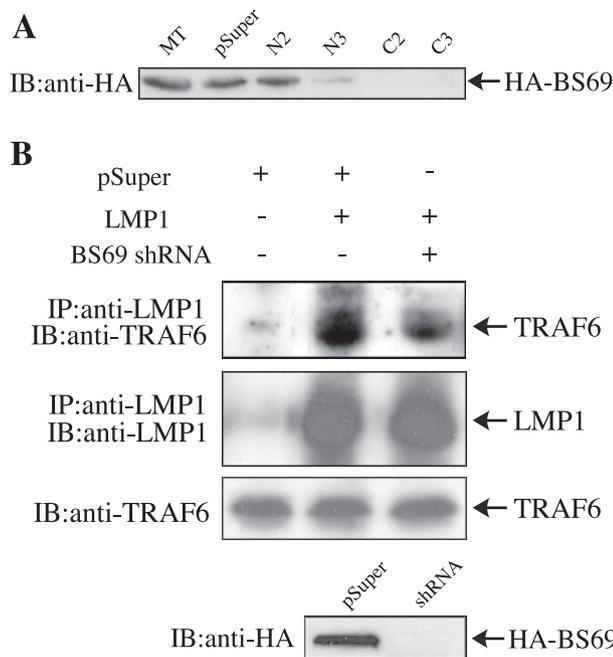
FIG. 5. BS69 is required for the complex formation between LMP1 and TRAF6. (A) The empty shRNA vector (pSuper) or different BS69 shRNA constructs were transfected to 293T cells twice, followed by transfection of an expression vector encoding HA-BS69. WCEs were subjected to immunoblotting with the anti-HA antibody. MT, mock transfected; N2, an ineffective BS69 shRNA clone with 2-bp mutations in its sequence; N3, the correct BS69 shRNA construct targeting a region in the amino terminus of BS69; C2 and C3, two correct clones of BS69 shRNA targeting a region in the carboxyl terminus of BS69. (B) 293T cells were first transfected twice with either pSuper or BS69 shRNA (C3), followed by transfection of an empty vector or LMP1. WCEs were first immunoprecipitated with the anti-LMP1 antibody; both the immunoprecipitated LMP1 and the coprecipitated TRAF6 were sequentially detected by immunoblotting.

FIG. 6. BS69 is specifically involved in the LMP1-meiated JNK pathway. (A) 293T cells were transfected with different shRNAs twice as indicated, followed by transfection of LMP1. WCEs were subjected to immunoblotting with both the anti-phospho-JNK and anti-total JNK antibodies. (B) 293T cells were transfected twice with pSuper or BS69 siRNA. Twenty-four hours after the last transfection, cells were treated with 20-ng/ml TNF-α for 10 min. WCEs were subjected to immunoblotting with anti-phospho-p38, anti-total-p38, anti-phospho-JNK, and anti-total JNK antibodies. Anti-β-tubulin was used here as a loading control. Endogenous JNK1 was also immunoprecipitated from WCEs and subjected to kinase assays (KA).

that N3, C2, and C3 but not N2 led to a significant decrease in BS69 mRNA levels (our unpublished data). To evaluate the role of BS69 in complex formation between LMP1 and TRAF6, we transfected LMP1 into 293T cells with or without a BS69-specific shRNA (C3). Although the endogenous TRAF6 was specifically coprecipitated by LMP1 in the absence of BS69 siRNA, less TRAF6 was coprecipitated by LMP1 in the presence of the BS69-specific shRNA (Fig. 5B). Our data suggest that the complex formation between LMP1 and TRAF6 is BS69 dependent.

BS69 is specifically required for LMP1-mediated JNK activation. Since BS69 scaffolds both LMP1 and TRAF6, we next asked whether it plays any functional role in the LMP1-mediated JNK pathway. We cotransfected 293T cells with different control and BS69-specific shRNAs with or without LMP1. In the presence of either an empty vector (i.e., pSuper) or N2 (the noneffective mutated BS69 siRNA), the endogenous JNK was potently phosphorylated (i.e., activated), as measured by a specific antibody recognizing the dually phosphorylated JNK (Fig. 6A, lanes 2 and 3). However, in the presence of either N3, C2, or C3 (i.e., the effective BS69 shRNAs), the extent of JNK phosphorylation was significantly decreased (Fig. 6A, lanes 4 to
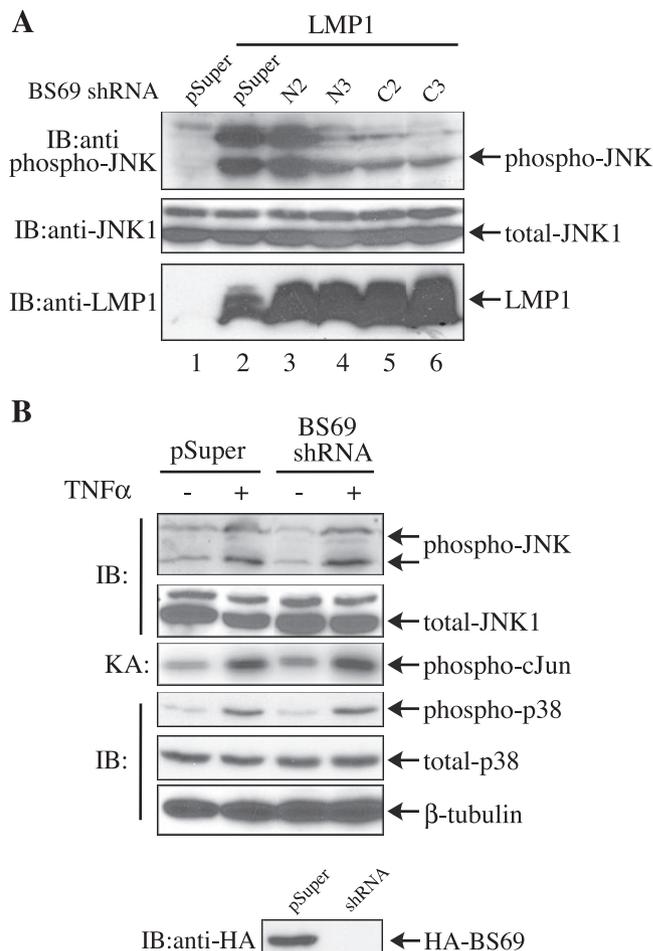
6). Similar results were also achieved in a direct JNK kinase assay (our unpublished data).

We then asked whether BS69 plays any role in the TNF-α-mediated mitogen-activated protein kinase pathways. 293T cells were transfected with either an empty vector (pSuper) or a BS69-specific shRNA (C3) with or without TNF-α treatment. As shown in Fig. 6B, neither the TNF-α-mediated JNK activation (panels 1 and 3) nor p38 activation (panel 4) was affected by the BS69-specific shRNA. Our data suggest that BS69 specifically functions in the LMP1-mediated JNK pathway.

Recruitment and oligomerization of BS69 are required for LMP1-induced JNK activation. Previous studies by several groups including our own clearly showed that the CTAR2 domain is mainly responsible for LMP1-induced JNK activation (14, 30, 49). Our data above showed that a main role of
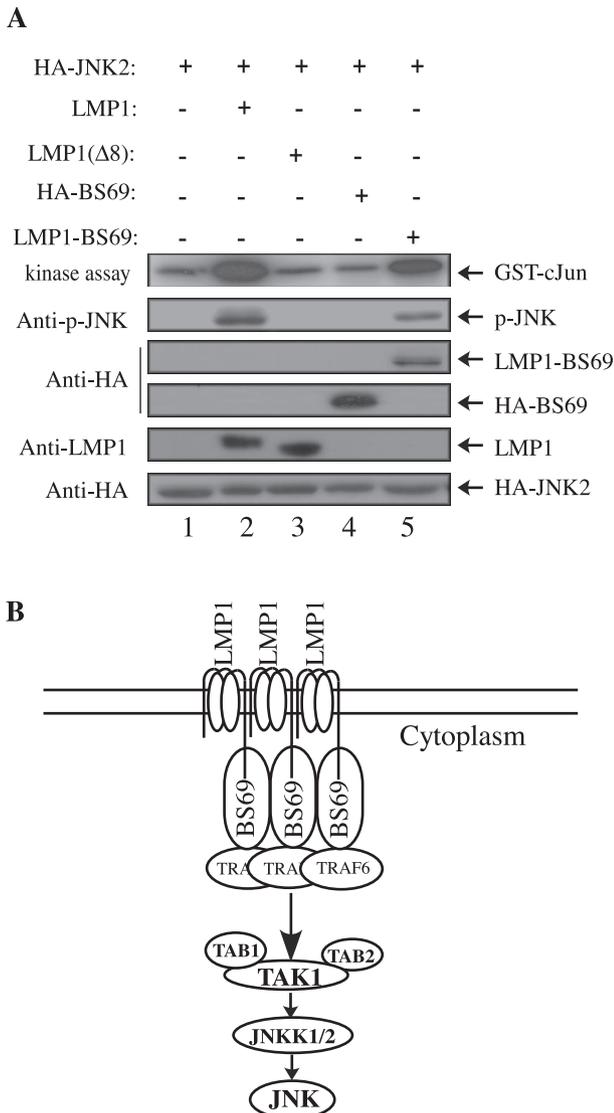
A

B

FIG. 7. Fusion of BS69 to an LMP1 mutant without the cytoplasmic carboxyl terminus restores JNK activation. (A) 293T cells were cotransfected with HA-JNK, together with other expression vectors as indicated. HA-JNK was immunoprecipitated from cell lysates and subjected to in vitro kinase assays. Separately, a part of the cell lysates was also subjected to immunoblotting with various primary antibodies as indicated. (B) The key signal transducers in the LMP1-mediated JNK pathway and the flow of the signal are depicted in the cartoon. Although LMP1, BS69, and TRAF6 are each drawn as a trimer, we do not know for sure whether this is the case (except for TRAF6). We simply imply here that they all need to oligomerize to activate the JNK pathway.

CTAR2 in the LMP1-mediated JNK pathway is to recruit BS69. To test whether BS69 can physically and functionally replace CTAR2 in inducing JNK activation, we started with a mutant LMP1 without the entire cytoplasmic carboxyl tail [i.e., LMP1 (1-186)], which by itself is completely defective in JNK activation (14, 29). We then fused BS69 in frame to this mutant LMP1 (1-186). To test whether the chimeric protein could restore JNK activation, we transfected 293T cells with HA-JNK2, together with either LMP1, LMP1(Δ8), BS69, or the

chimeric LMP1 (1-186)-BS69, as indicated in Fig. 7A. As expected, LMP1 potently activated JNK, whereas LMP1(Δ8) failed to do so (Fig. 7A, compare lanes 2 and 3). Overexpression of BS69 alone was not sufficient to activate JNK either (Fig. 7A, lane 4). Interestingly, the expression of the chimeric LMP1 (1-186)-BS69 in cells restored JNK activation (Fig. 7A, lane 5). This result further confirmed that BS69 participates in the LMP1-mediated JNK pathway and suggested that recruitment and oligomerization of BS69 are prerequisites for JNK activation by LMP1.

DISCUSSION

BS69 but not BRAM1 bridges LMP1 and TRAF6 for JNK activation. Although BS69 was identified a decade ago, its biological role remains largely unknown at present. So far, BS69 has been shown to interact with a few viral proteins (e.g., E1A and EBNA2) and cellular protein (e.g., c-Myb, Ets2, and EMSY) and may function as a transcriptional corepressor (2, 17, 21, 33, 38, 51). We now provide evidence showing that BS69 also serves an important role as an adaptor in viral protein-mediated intracellular signal transduction pathways. It is likely that LMP1 may contribute to EBV-mediated pathogenesis by interfering with the normal cellular function of BS69. Although BS69 already contains several known protein-protein interaction domains (Fig. 2A) (38), in this study, we identified another novel domain of BS69 (i.e., aa 274 to 353) involved in binding TRAF6 (Fig. 4). We also showed that the MYND domain of BS69 is indispensable for its interaction with the carboxyl terminus of LMP1, which is in agreement with a recent finding in which BRAM1, an alternatively spliced form of BS69, was also shown to interact with LMP1 (8). This is not unexpected, since both BS69 and BRAM1 contain the intact MYND domain. Although BRAM1 interacts with LMP1, we show that it does not interact with TRAF6 (Fig. 4). In HEK293 cells, BRAM1 mRNA was barely detected, as judged by RT-PCR with a forward primer annealing to the 5′ end of the gene encoding 12 aa unique to BRAM1 (32), whereas BS69 mRNA could be readily detected (our unpublished data). Thus, BRAM1 may function as a regulatory molecule to fine tune the BS69-mediated cellular signaling pathways in a cell type-dependent manner. We think that it is BS69 (but not BRAM1) that normally participates in the LMP1-initiated signaling pathways. This is further supported by the fact that N3, a BS69-specific shRNA targeting a region present only in BS69 but not in BRAM1, still effectively inhibits LMP1-induced JNK activation (Fig. 6B). Interestingly, although PXLXP and PX-EXX (aromatic-acidic residue) have been defined as the consensus sequences in binding proteins for the MYND domain of BS69 and the TRAF(C) domain of TRAF6, respectively (2, 57), no such motifs are found in LMP1 and BS69. This suggests that the modes of BS69's interaction with both LMP1 and TRAF6 are unique in nature and remain to be structurally elucidated.

As LMP1 also requires TRAF6 to activate NF-κB (37), we initially hypothesized that BS69 is also involved in the LMP1-mediated NF-κB pathway. However, in experiments where the BS69-specific shRNA efficiently reduced the LMP1-mediated JNK activation, we failed to observe a consistent decrease in the LMP1-medited NF-κB activation (our unpublished data).

This could be due to technical reasons, as NF-κB activation by LMP1 is difficult to detect by either the IκB kinase assays or the NF-κB-dependent luciferase reporter assays of several cell lines (e.g., HeLa or MEFs), except in 293T cells (our unpublished data). Differential expression of a key factor in different cell types may account for this. Alternatively, a scaffolding molecule other than BS69 is specifically involved in the LMP1-mediated NF-κB pathway. The exact role of BS69 in the LMP1-mediated NF-κB pathway remains to be further clarified.

Oligomerization of BS69 is a prerequisite for JNK activation by LMP1. Protein oligomerization is an important yet recurring theme in various ligand-induced signal transduction pathways (36, 43, 47). Many receptors including the epidermal growth factor receptor, G-protein-coupled receptors, and TNF receptor form oligomers on cell membrane upon ligand binding, which initiates various downstream signal transduction pathways. Similarly, many intracellular signal transducers are capable of transmitting signals only after oligomerization. For example, members of the TRAF family proteins are known to function by oligomerization (4, 42). BS69, the adaptor in the LMP1-mediated JNK pathway, also seems to function by oligomerization, as expression of BS69 alone fails to activate JNK. When BS69 is covalently linked to the transmembrane domain of LMP1, which is known to promote LMP1 aggregation on cell membranes, the fusion protein significantly activates the JNK pathway (Fig. 7). This result tells us two things. First, oligomerization of BS69 is required to activate JNK. Considering the fact that BS69 recruits TRAF6 to activate JNK and that TRAF6 is known to function as oligomers (4), it is not difficult for us to understand why oligomerization of BS69 is needed. Second, the main function of the CTAR2 domain in the LMP1-mediated JNK pathway is to recruit BS69. Thus, a clear picture of the LMP1-mediated JNK pathway is emerging in which autonomous aggregation of LMP1 on host cell membrane recruits BS69 and facilitates its oligomerization. The oligomerized BS69 in turn recruits and promotes aggregation of TRAF6, eventually leading to TAK1 and JNK activation (Fig. 7B).

In the future, it would be conceivably beneficial to screen for small molecules that disrupt the interaction between LMP1 and BS69. These molecules could be therapeutically useful in interfering with the LMP1-mediated JNK pathway and in inhibiting EBV-mediated pathogenesis.

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