Negative Regulation of Epstein-Barr Virus Latent Membrane Protein 1-mediated Functions by the Bone Morphogenetic Protein Receptor IA-binding Protein, BRAM1*

Pei-Jung Chung‡‡, Yu-Sun Chang§§, Chih-Lung Liang¶, and Ching-Liang Meng‡**

From the ‡Graduate Institute of Life Sciences, National Defense Medical Center, Taipei 114, and the §Graduate Institute of Basic Medical Sciences, Chang-Gung University School of Medicine, Kwei-Shan, Taoyuan 333, Taiwan, Republic of China

The latent membrane protein 1 (LMP1) of Epstein-Barr virus causes cellular transformation and activates several intracellular signals, including NF-κB and c-Jun N-terminal kinase. Using yeast two-hybrid screening with the LMP1 C-terminal sequence as bait, we demonstrate that BRAM1 (bone morphogenetic protein receptor-associated molecule 1) is an LMP1-interacting protein. BRAM1 associates with LMP1, both in vitro and in vivo, as revealed by confocal microscopy, glutathione S-transferase pull-down, and co-immunoprecipitation assays. This association mainly involves the C-terminal half of BRAM1 comprising the MYND domain and the CTAR2 region of LMP1, which is critical in LMP1-mediated signaling pathways. We show that BRAM1 interferes with LMP1-mediated NF-κB activation but not the JNK signaling pathway. Because the CTAR2 region interacts with the tumor necrosis factor (TNF-α receptor-associated death domain protein, it is interesting to find that BRAM1 also interferes with NF-κB activation mediated by TNF-α. BRAM1 interferes LMP1-mediated and TNF-α-induced NF-κB activation by targeting IκBα molecules. Moreover, BRAM1 inhibits the resistance of LMP1-expressing cells to TNF-α-induced cytotoxicity. We therefore propose that the BRAM1 molecule associates with LMP1 and functions as a negative regulator of LMP1-mediated biological functions.

Latent membrane protein 1 (LMP1)1 of Epstein-Barr virus (EBV) is one of the viral proteins expressed in EBV-associated tumors. EBV efficiently infects and immortalizes resting B-cells in vitro. LMP1 is indispensable in this immortalization process (1). The protein transforms rodent fibroblast cells, indicating genuine oncogenicity (2, 3). Transgenic mice containing an Ig heavy chain promoter/enhancer-driven LMP1 gene develop lymphomas with a high incidence in vivo (4). In B-cells, LMP1 also activates the expression of Bel-2 and protects cells from apoptosis (5). In epithelial cells, LMP1 down-regulates cytokeratin levels (6), induces the anti-apoptotic protein, A-20, inhibits epithelial cell differentiation (7), and induces cell migration activity (8, 9).

LMP1 is an integral membrane protein with a predicted short N-terminal cytoplasmic domain, six membrane-spanning domains, and a large C-terminal cytoplasmic domain. These regions are potentially important for LMP1-mediated functions, including cellular transformation and signaling. LMP1 associates with tumor necrosis factor receptor-associated factor (TRAF), as originally determined by the yeast two-hybrid assay (10), implying involvement in the signal transduction pathway mediated by the TNF receptor superfamily. Similar to TNF signaling, LMP1 activates NF-κB activity and induces phenotypic changes in a wide range of cell lines (11, 12). Deletion mutant analyses led to the mapping of two regions in the C-terminal domain of LMP1 (CTAR1 and CTAR2) that mediate NF-κB activation. The CTAR2 domain accounts for ~70% total NF-κB induction capacity, whereas the remaining 30% is derived from CTAR1 (13). Further functional analyses demonstrate that amino acids 204–208 of CTAR1 comprise the consensus motif for TRAF binding (14). CTAR2 does not directly interact with TRAFs but recruits the TNF-associated death domain protein (TRADD) through a TRADD-specific N-terminal sequence (15). Interactions between LMP1 and TRADD activate the c-Jun N-terminal kinase (JNK) cascade, resulting in JNK-mediated phosphorylation of c-Jun and concomitant up-regulation of AP-1 transcriptional transactivation (16, 17). Therefore, LMP1 and TNF receptor appear to function through similar mechanisms.

The transmembrane protein LMP1 contains a long cytoplasmic domain that mediates protein function. High levels of LMP1 may be cytotoxic to cells (18). To date, it is still not clear how LMP1-mediated functions can be balanced in a cell. Recent reports additionally indicate that at least 30% LMP1 proteins are membrane-bound (19). We therefore hypothesize that LMP1 (a constitutively activated receptor-like protein) signaling is coordinately regulated. Specific regulators may be identified by examining the molecules associated with this protein.

In this study, we identified a negative regulator, BRAM1 (BMP receptor-associated molecule 1) (20) by yeast two-hybrid screening using the LMP1 C-terminal sequence as bait. Here
we present data showing that BRAM1 interacts with LMP1 in \textit{vitro} and \textit{in vivo}. Moreover, CTAR2 and the last three amino acids (YDY) of LMP1 are critical for interactions between these two proteins. The region of BRAM1 that associates with LMP1 is within the 116 amino acids of the C terminus. Interactions between LMP1 and BRAM1 result in the inhibition of LMP1-mediated NF-κB activation but not JNK activation. BRAM1 interferes LMP1-mediated and TNF-α-induced NF-κB activation by targeting the posttranslational modifications of IκBα molecules. Furthermore, BRAM1 also reduces the resistance of LMP1-expressing cells to TNF-α-induced cytotoxicity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Cell lines including BALB/c3T3, 293, and NPCTW02 were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml streptomycin, and 100 units/ml penicillin.

**Yeast Two-hybrid Screening**—Yeast transformation and β-galactosidase detection in \textit{Saccharomyces cerevisiae} Y190 was performed according to the manufacturer’s instructions (Clontech). A BALB/c3T3 cDNA library constructed with the pGAD10 plasmid (comprising 1.5 × 10\(^6\) independent DNA clones) was used to screen for proteins interacting with the C terminus of LMP1 in the Y190 yeast strain. β-Galactosidase-positive clones were recovered. Among these, a cDNA insert of BRAM1 (corresponding to amino acids 83–198) was identified and designated BRAM1/C116. The full-length mouse BRAM1 cDNA was generated by PCR fragment containing the sequence resulting from mRNA derived from BALB/c3T3 cells using the reverse transcription-PCR method with BRAM1-specific primers. The 611-bp PCR fragment containing the EcoRI and BamHI enzyme sites was inserted into the identical enzyme sites of the cloning vector pFLAG-CMV2. To examine the interactions between LMP1 and BRAM1, full-length mouse BRAM1 (denoted WT) and its deletion mutant constructs BRAM1/Δ58, BRAM1/Δ48, and BRAM1/Δ116 were cloned into pGAD10. DNA fragments encoding LMP1 C-terminal amino acids were fused to the DNA-binding domain of the Gal4 protein. The Ura+ His+ Leu+ transformants were further selected and tested on X-gal plates.

**Transfection and Luciferase Assays**—All of the transfection experiments were carried out with cells grown to 70–80% confluency. Transfection of BALB/c3T3 cells was performed by electroporation at 960 V/cm at 250 μF. The cell lysates were measured with 10% SDS-PAGE, and phosphorylated IκBα were identified by autoradiography.

**Western blot analysis of endogenous IκBα molecules was carried out by incubation of cell lysates with monoclonal anti-phospho-antibody (B-9) and polyclonal anti-IκBα antibody (C-21), respectively. Both antibodies were purchased from Santa Cruz Biotechnology Inc.**

**Confocal Immunofluorescence Microscopy**—NPCTW02 cells were cultured on glass coverslips and transfected with 5 μg each of FLAG-BRAM1, FLAG-TRAF2, and GFP-LMP1. The cells were rapidly washed with phosphate-buffered saline, fixed in cold methanol for 30 min at 20°C, and incubated with anti-FLAG antibody M2 (Kodak) for 1 h at room temperature, followed by anti-mouse TRITC-conjugated antibody (Jackson Immuno Research, West Grove, PA) for 30 min at room temperature. The resulting coverslips were mounted with Vectashield (Vector Labs) and examined under a confocal microscope (Leica TCS-NT, Leica Lasertecnch GmbH, Heidelberg, Germany).

**Immunocomplex Kinase Assays**—For the IKK activity assay, 293 cells were transiently transfected with BRAM1 and LMP1 expression vectors individually. After transfection (24 h), the cells were lysed in solution B (20 mM Tris-HCl at pH 7.5, 0.5 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride). To immunoprecipitate endogenous JNK1, 500 μg of cleared cell extracts were incubated with anti-JNK1 (C-7) antibody (Santa Cruz Biotechnology) at 4°C for 2 h and immobilized to protein G-Sepharose beads. The beads were washed three times and 1 ml of solution B (20 mM Tris-HCl at pH 7.5, 0.5 mM dithiothreitol) containing 0.5 M NaCl and once with kinase reaction buffer (20 mM Tris-HCl at pH 7.5, 20 mM MgCl\(_2\), 0.5 μM dithiothreitol, and 0.2 μM ATP). In vitro kinase reactions to assay the activity of JNK1 were performed using kinase reaction buffer in the presence of 10 μCi of \([γ\text{-}32\text{P}]\text{ATP}\) and 2 μg of GST-c-Jun (1–79). The reaction was stopped by the addition of 4× Laemmli sample buffer and boiling for 5 min. The samples were analyzed by electrophoresis with 10% SDS-PAGE. Phosphorylated GST-c-Jun (1–79) (37 kDa) was identified by autoradiography.

For assaying the endogenous IKK activity, BALB/c3T3 cells were transfected with BRAM1 expression vector or the control vector individually. After TNF-α treatment, the cells were lysed with lysis buffer (25 mM HEPES at pH 7.6, 0.3 M NaCl, 1.5 mM MgCl\(_2\), 0.2 mM EDTA, 0.1% Triton X-100, 20 mM glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml aprotinin). The cell lysates were diluted with three volumes of dilution buffer (20 mM HEPES at pH 7.6, 2.5 mM MgCl\(_2\), 0.1 mM EDTA, 0.05% Triton X-100, 20 mM glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml aprotinin) and incubated on ice for 10 min. After centrifugation, the cleared cell extracts were immunoprecipitated with the anti-IKK-α antibody (M-110, Santa Cruz Biotechnol-
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**Fig. 1.** BRAM1 interacts with LMP1 in vitro and in vivo. A schematic representation of full-length BRAM1(WT) and its deletion mutants. Construction of these expression vectors was described under Experimental Procedures. BRAM1(C116) is a cDNA clone isolated by yeast two-hybrid screening. B, identification of the interacting domains of BRAM1 and LMP1 by the yeast two-hybrid assay. The C-terminal fragments of LMP1 were fused to the Gal4 DNA-binding domain, whereas BRAM1 and fragments were fused to the Gal4-activating domain. The β-galactosidase activity values for each interaction represent the averages of three independent transformants. The positive control (transformants containing pTD1 and pVA3) of the interaction was averaged as 187 ± 36 β-galactosidase units, whereas the negative control (transformants containing pAS2.1 and pGAD10) was 0.5 ± 0.3. The constructs are described under Experimental Procedures. Lower panel, for the filter assay of the interacting proteins G4AD-BRAM1(C116) and G4BD-NLMP1 (186–381) were co-transfected into the Y190 yeast strain. Interactions between BRAM1(C116) and LMP1 were identified from the blue colonies (representing β-galactosidase activity) 1 h after reaction with the X-gal. The colonies containing pTD1 and pVA3 were positive controls (blue), whereas those containing pAS2.1 and pGAD10 were negative controls (white). C, GST-BRAM1 pull-down assay. Cleared cell lysates from BALB/c3T3 cells or BALB/c3T3 cells stably expressing LMP1 (3T3-LMP1) were incubated at room temperature with immobilized GST-BRAM1c or GST control. The bound proteins were analyzed by immunoblotting using the anti-LMP1 antibody S12. The amount of LMP1 protein in 5% 3T3-LMP1 cell lysates is shown as 381 (complete C-terminal sequence), specifically BRAM1(378–381) interacting with full-length BRAM1(WT) and C-terminal BRAM1(C116) but not with BRAM1 constructs lacking the C-terminal sequence, specifically BRAM1(C116), NLMP1 (amino acids 186–381), and BRAM1(Δc48). NLMP1 (amino acids 186–378), an LMP1 construct without the last three amino acids (YDY), did not interact with either BRAM1(WT) or BRAM1(C116). It is worth noting that a corresponding C-terminal sequence of LMP1 derived from the B95-8 strain also interacted with BRAM1 (data not shown). Our results indicate that interactions between LMP1 and BRAM1 are mediated by the CTAR2 domain of the former and the C-terminal region of the latter protein. We additionally demonstrate in this study that the MYND domain of BRAM1 is critical for association with LMP1, because the deletion of this domain (constructs BRAM1(Δc48) and BRAM1(Δc98)) resulted in a failure of interaction between the two proteins.

**Fig. 2.** BRAM1 and LMP1 are co-localized in cells. A–C, subcellular distribution patterns of LMP1 and BRAM1 in NPC-TW02 cells. After transfection with GFP-LMP1 and FLAG-BRAM1, the cells were subjected to anti-FLAG immunofluorescence staining and observed with either a conventional fluorescence microscope (A) or a confocal microscope in XY scanning (B) or XZ scanning mode (C). GFP signals were displayed in green, whereas BRAM1 proteins were stained red. Both signals were superimposed in the MERGE panels (yellow). D, co-localization of LMP1 and TRAF2 was examined by transfection with GFP-LMP1 and FLAG-TRAF2 plasmids. Images were observed under similar conditions as in B.

The C-terminal 116-Amino Acid Sequence of BRAM1 Specifically Interacts with CTAR2 of LMP1—The cytoplasmic domain of LMP1 interacting with BRAM1 consists of two key regions, CTAR1 and CTAR2, separated by an intervening sequence. These two regions contribute to most of the known functions mediated by LMP1. We mapped the critical region of LMP1 required for interactions with BRAM1 using the yeast two-hybrid assay. As shown in Fig. 1B, the NLMP1 C-terminal sequences comprising amino acids 186–381 (complete C-terminal sequence), 241–381 (CTAR2 and the intervening sequence), 186–247 (CTAR1 region), 186–323 (CTAR1 and the intervening sequence), and 186–378 (the complete C-terminal sequence excluding the last three amino acids, YDY) were individually cloned into the Gal4-AD parent vector. Each of these constructs was co-transformed into yeast cells with the Gal4-AD vector containing complete or partial BRAM1 sequences. The extent of interaction was scored by the resulting β-galactosidase activity. As summarized in Fig. 1B, LMP1 containing the complete CTAR2 region (amino acids 186–381 and 241–381) interacted with full-length BRAM1(WT) and C-terminal BRAM1(C116) but not with BRAM1 constructs lacking the C-terminal sequence, specifically BRAM1(Δc98) and BRAM1(Δc48). NLMP1 (amino acids 186–378), an LMP1 construct without the last three amino acids (YDY), did not interact with either BRAM1(WT) or BRAM1(C116). It is worth noting that a corresponding C-terminal sequence of LMP1 derived from the B95-8 strain also interacted with BRAM1 (data not shown). Our results indicate that interactions between LMP1 and BRAM1 are mediated by the CTAR2 domain of the former and the C-terminal region of the latter protein. We additionally demonstrate in this study that the MYND domain of BRAM1 is critical for association with LMP1, because the deletion of this domain (constructs BRAM1(Δc48) and BRAM1(Δc98)) resulted in a failure of interaction between the two proteins.

**BRAM1 Associates with LMP1 in Vitro and in Vivo**—To further confirm the physical association between LMP1 and BRAM1, a GST pull-down assay with purified BRAM1-GST fusion protein and lysates from BALB/c3T3 cells stably expressing LMP1 was performed. A GST protein fused to the C-terminal 116 amino acids of BRAM1 was overexpressed in E. coli and purified with glutathione agarose beads. The cell extracts were precipitated with glutathione-agarose beads, and bound LMP1 was analyzed by immunoblotting with anti-LMP1.
antibody. As shown in Fig. 1C, LMP1 co-precipitated with GST-purified BRAM1 but not GST alone, suggesting that the two proteins are physically associated.

To examine whether LMP1 and BRAM1 interact *in vivo*, 293 cells were co-transfected with a FLAG-tagged construct containing full-length BRAM1 (FLAG-BRAM1) and LMP1 expression plasmids. The cell extracts were immunoprecipitated with either anti-FLAG or anti-LMP1 antibodies, and the precipitated complex was examined by immunoblotting. The results shown in Fig. 1D demonstrate that LMP1 and BRAM1 co-precipitate, as expected for *in vivo* associations.

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**BRAM1 Inhibits LMP1-mediated NF-κB activation**—Because BRAM1 associates with LMP1 via the CTAR2 domain, which contributes to ~70% potency in NF-κB activation (13), it is possible that this cytoplasmic protein is involved in this function of LMP1. To investigate this possibility, we co-transfected BALB/c3T3 cells with the LMP1 expression vector and varying amounts (0.5, 1, 2, and 3 μg) of BRAM1 expression vector, followed by treatment with 20 ng/ml of TNF-α or the reagent control for 6 h. The cells were harvested at 24 h after transfection. Using the luciferase activity obtained from cells transfected with the reporter plasmid 4xκB-Luc as 1, the TNF-α-induced NF-κB activity obtained from cells transfected with the vector control was elevated more than ~150-fold (or 100% stimulation). The fold activation was determined by dividing the luciferase activity of each transfection with the value obtained from cells transfected with the vector control without TNF-α treatment. The percentage of repression marked at the top of each bar from the TNF-α-treated samples was determined by using the value from TNF-α-treated samples as 100%. The data shown here are representative of three independent experiments.
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A.

FIG. 5. BRAM1 interferes with the phosphorylation and degradation of IκBα in response to LMP1 and TNF-α. A. BALB/c3T3 cells were transfected with B-LMP1 expression vector and/or BRAM1 expression vector or control. The cells were harvested 24 h after transfection, and the cell lysates were subjected to Western blot analysis using anti-IκBα-specific antibody (C-21) and an anti-phospho-IκBα antibody (B-9), as described under "Experimental Procedures." B. BALB/c3T3 cells were transiently transfected with BRAM1 expression vector or the control vector. Twenty-four hours after transfection, the cells were treated with TNF-α for 0, 5, 10, 15, and 20 min and then harvested. Total and phospho-IκBα molecules were detected by the specific antibodies described in A. C, reduction in the relative ratio of phosphorylated IκBα (p-IκBα) to the total IκBα by BRAM1. The relative ratio of phosphorylated IκBα to the total IκBα in cells transfected with BRAM1 expressing vector and the control vector in response to TNF-α shown in B was obtained by dividing the intensity (determined by the densitometer) of phosphorylated IκBα with that of total IκBα at each time point and normalized to the value from 0 min (without TNF-α treatment) samples. D. attenuation of TNF-α-induced IKK activity by BRAM1. BALB/c3T3 cells were transiently transfected with BRAM1 expression vector or the control vector. After 24 h, the cells were stimulated with recombinant TNF-α (20 ng/ml) for 10 and 20 min. Lane 0, untreated. The endogenous IKK activity was determined by an immunocomplex kinase assay using anti-IKKα antibody (M-110) as described under "Experimental Procedures." In vitro phosphorylation reactions were carried out with bacterially expressed IκBα and [γ-32P]ATP. The endogenous IKKα in each immunocomplex was detected by immunoblotting with anti-IKKα antibodies with long time exposure (as shown in the lower panel). The "fold" shown at the bottom of the gel pattern was measured by the personal densitometer SI (Molecular Dynamics) using the untreated control (intensity at 0 min) as 1.
Degradation Induced by LMP1 and TNF-α—NF-κB activation induced by LMP1 involves the phosphorylation and degradation of IκB molecules (23, 24). Thus, BRAM1 may abrogate NF-κB activation by preventing the phosphorylation and degradation of IκB. As shown in Fig. 5A, LMP1-induced phosphorylation of IκBα (detected by monoclonal anti-phospho-IκBα antibody) concomitant with degradation IκBα (detected by polyclonal anti-IκBα antibody) was observed in cells transfected with LMP1 expressing vector but not in cells transfected with the control vector. Thus, BRAM1 prevented LMP1-induced IκBα phosphorylation and the subsequent degradation.

TNF-α-induced NF-κB activation also requires phosphorylation and degradation of IκBα (25, 26). Thus, the role of BRAM1 in the inhibition of phosphorylation and degradation of IκB in response to TNF-α treatment was tested in BALB/c3T3 cells. As shown in Fig. 5B, TNFα induced IκBα phosphorylation in 5 min (−8-fold as compared with the untreated), which was more evident in 10 min (−12-fold). The level of phosphorylated IκBα remained at a relative high level at 15 and 20 min. Total IκBα degradation (−30% reduction as compared with that of the untreated) was detected in 5 min. Prolonged treatment (15 and 20 min) resulted in −60% reduction of the total IκBα. In the presence of BRAM1, TNF-α induced IκBα phosphorylation in 5 min (−12-fold) and 10 min (−9-fold). The phosphorylated IκBα was reduced greatly (−60%; activation dropped from 9- to 3-fold stimulation) in 15 min. Prolonged treatment (20 min) showed a similar level of reduction. The degradation of total IκB protein was delayed by BRAM1 as compared with the rate of degradation in the vector control group. No obvious IκBα degradation was detected at the 5-min time point, and −60% of IκBα was detected in cells treated with TNF-α for 15 or 20 min. The effect of BRAM1 represented by the relative ratio of phosphorylated IκBα to total IκBα was semi-quantitatively estimated. As shown in Fig. 5C, BRAM1 significantly reduced the IκBα response to TNF-α treatment, and this effect was more evident in cells treated for 10, 15, and 20 min.

Phosphorylation and degradation of IκBα is modulated by the NIK-IKK complex (26–29). Thus, BRAM1 may interfere with the phosphorylation of IκBα by its kinase IKK. To explore this possibility, we next assessed the IKK kinase activity to phosphorylate IκBα. BALB/c3T3 cells were transfected with BRAM1 expressing vector or vector control, followed by TNF-α treatment of indicated time. The cell lysates were collected for immunoprecipitation with anti-IKK-α antibody. The immunoprecipitates were assayed for the endogenous IKK activity by measuring the phosphorylation of the substrate protein GST-IκBα (1–317) in vitro kinase assays. As demonstrated in Fig. 5D, TNF-α induced −2-fold activation of IKK activity. In the presence of BRAM1, phosphorylation of GST-IκBα (1–317) induced by TNF-α was inhibited. The results collectively suggest that BRAM1 interferes with LMP1- and TNF-α-induced NF-κB activation by targeting the posttranslational modifications of IκBα molecules.

**BRAM1 Inhibits the Resistance of LMP1-expressing Cells to TNF-α-induced Cytotoxicity**—LMP1 is a transforming protein that prevents the apoptosis of cells by inducing agents. To further examine the inhibitory role of BRAM1, 293 cells were transfected with LMP1- and/or BRAM1-expressing vectors or the vector control, respectively, and treated with or without the inducing agent, TNF-α (20 ng/ml), in the presence of cycloheximide (10 μg/ml). Forty hours after treatment, the cells were subjected to flow cytometry to analyze the cell population in the sub-G1 phase (representing apoptotic cells). The results presented in Fig. 6 show that LMP1 alone prevents cells from entering the sub-G1 phase (18% without TNF-α treatment, compared with 23% with treatment). Co-transfection of cells with BRAM1 and LMP1 enhanced the sub-G1 population from 18 to 35%, and treatment with TNF-α further increased the apoptotic cell population to 46%. BRAM1 alone and the vector control showed similar level of cytotoxicity; −16% of cells in the sub-G1 phase without TNF-α treatment as compared with −40% with TNF-α treatment. It also interesting to note that cells co-transfected with BRAM1- and LMP1-expressing vectors showed a relative strong cytotoxic response even without the TNF-α treatment. Nevertheless, our data indicate that BRAM1 interferes with the anti-apoptotic effect of LMP1. Similar results were observed in cell clones stably expressing LMP1 (data not shown).
LMP1 has been considered to be a constitutively activated oncogene that is involved in various cellular signaling pathways. The complicated interactions between LMP1 and cellular factors may result in both stimulatory and inhibitory activities. At the physiological level, LMP1 inhibits EBV and cellular gene expression moderately. However, at high levels of expression, LMP1 is cytotoxic (18, 30), presumably by enforcing the inhibition of both the steady-state RNA synthesis and protein synthesis (30). The mechanisms that regulate the constitutive activity of LMP1 have not been clearly demonstrated. In this study, we present data showing that LMP1 interacts with a cytoplasmic protein BRAM1, which down-regulates the LMP1-mediated NF-κB activation and promotes cells entering apoptosis under TNF-α treatment. We also demonstrate that BRAM1 interferes the TNF-α-mediated NF-κB activation. Our findings collectively suggest that BRAM-1 plays a negative role in LMP1-mediated signaling and promoting cell death induced by TNF-α and cycloheximide that is normally repressed by LMP1 by inhibiting the survival pathways.

BRAM1 protein, a cytoplasmic protein and an alternative spliced form of an adenovirus E1A-associated protein BS69, shares the C-terminal 186 amino acids including a MYND domain with BS69 (Fig. 1). BS69, like other MYND-containing proteins DEAF-1 (31) and MTGS/ETO (32), is primarily a nuclear protein and functions as a negative regulator for transcription by interacting with components of transcriptional repressor complexes including co-repressors Sin3, N-CoR, SMRT, and histone deacetylase (33–37). BS69 can bind viral and cellular nuclear transcription factors such as adenovirus E1A, EBV EBNA2, and the Myc-related cellular transcription factor MGA (38) through the PXLXP motif of these proteins. The MYND domain of BRAM1 is also critical for BRAM1 to interact with LMP1, because the deletion of MYND domain (constructs BRAM1(Δc48) and BRAM1(Δc98)) failed to interact with LMP1 (Fig. 1B). However, LMP1, similar to two other BRAM1-interacting proteins, BMP type IA receptor (BMPR-IA) (20) and BIP (39), does not contain the PXLXP motif for binding. This discrepancy remains to be investigated.

The negative role of the cytoplasmic BRAM1 has recently been observed in two homologs of the human BRAM1 in Caenorhabditis elegans, bra-1 and bra-2 (40). BRA-1 associates with the type I receptor and functions as a negative regulator of DAF-7 transforming growth factor-β signaling. The expression pattern of BRA-2 is similar to another type I receptor; therefore it is possibly involved in another transforming growth factor-β signaling in C. elegans. Whether BRAM1 plays a similar role in BMP signaling requires further investigation. Similar to TRADD, BRAM1 preferentially interacts with the CTAR2 region of LMP1, and the last three amino acids of LMP1 are critical for the binding. Different from TRADD, which acts as a positive mediator in LMP1- and TNF-mediated NF-κB activation, BRAM1 functions as a negative regulator. TRADD contains a death domain in its C terminus that determines the interaction of TRADD with TNF receptor. However, TRADD interacts with CTAR2 of LMP1 through its N terminus. BRAM1, on the other hand, contains no death domain, and its C-terminal MYND is critical for interaction with CTAR2. In the co-immunoprecipitation experiments,2 BRAM1 can co-precipitate with a small amount of TRADD, indicating that these three proteins can be present within a complex. Currently, we are examining the interaction among LMP1, BRAM1, and TRADD in LMP1-mediated NF-κB activation.

BRAM1 represses LMP1-mediated NF-κB activation by reducing IκBα phosphorylation and delaying IκBα degradation. This is consistent with the current report indicating that LMP1-mediated NF-κB activation is modulated through NIK (a mitogen-activated protein kinase kinase kinase)-mediated phosphorylation of IκBα kinase (IKK) complex (24). Activation of IKK through phosphorylation at Ser-176 by NIK results in activation of downstream NF-κB signaling. This is followed by phosphorylation of IκBα serines 32 and 36. NF-κB typically resides in the cytoplasm as an inactive form by association with its inhibitory protein IκB. Phosphorylation of IκBα by IKK results in ubiquitination and degradation of IκBα and, subsequently, NF-κB translocation to the nucleus (27–29). The present results strongly suggest that BRAM1 inhibits NF-κB activation by interfering the activation of the upstream NIK-IKK-IκB complex.

LMP1-expressing cells are more resistant to apoptosis induced by cytotoxic agents. Induction of NF-κB activity is considered to be one of the survival pathways in LMP1-expressing cells. In this study, we show that BRAM1 interferes LMP1-mediated NF-κB activation and promotes the cell death induced by apoptosis inducing agents, suggesting that BRAM1 plays a critical biological role in LMP1-mediated functions.

NF-κB activation is involved in LMP1-mediated transformation and tumorigenesis of Rat-1 fibroblasts (41). NF-κB induces the expression of a variety of genes involved in cell proliferation and cell cycle progression, including the c-Myc gene (42) and the cyclin D1 gene (43, 44). Therefore, inhibition of NF-κB may be a means of killing LMP1 expressing cells. Inhibitors of NF-κB activation such as glucocorticoids are wildly used as immune and inflammatory suppressants (45–48) and as supplements for therapy for certain hematological malignancies (49). Here, results demonstrate BRAM1 is a negative regulator for LMP1-mediated and TNF-α-induced NF-κB activation. Thus, BRAM1 may be used as a therapeutic molecule in treating EBV-associated, LMP1-positive cancers.

Although it is unclear what direct impact LMP1-mediated NF-κB activation has in virus-induced oncogenesis, identification of BRAM1 as an upstream modulator that affects IκBα activity should potentially be useful for studying signal pathways that can mediate LMP1-induced oncogenesis.

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