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The MYND domain-containing protein BRAM1 inhibits lymphotoxin beta receptor-mediated signaling through affecting receptor oligomerization

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ABSTRACT

MYND (myeloid-Nervy-DEAF-1) domains exist in a large number of proteins that are functionally important in development or associated with cancers. We have previously demonstrated that a MYND domain-containing protein, the bone morphogenesis protein receptor-associated molecule 1 (BRAM1), is able to interact with Epstein-Barr virus-encoded latent membrane protein 1 (LMP1), which acts as a constitutively activated tumor necrosis factor receptor (TNFR). Herein we further demonstrated that BRAM1 additionally associates with the TNFR-superfamily member, the lymphotoxin beta receptor (LTβR), and hence inhibits LTβR-mediated function. Using the yeast two-hybrid assay, we demonstrated that BRAM1 interacts with LTβR mainly through the self-association domain of LTβR (aa 336–398). The co-immunoprecipitation experiment further revealed that BRAM1 as well as MYND domain-containing proteins, MTG8 and DEAF-1, interacts with LTβR *via* their MYND domains. The BRAM1-LTβR interaction impedes the self-association of LTβR and the recruitment of TNFR-associated factors 2 and 3 (TRAF2 and TRAF3), leading to abolishment of LTβR-induced NF-κB signaling, JNK activation, and caspase-dependent cell death. In sum, our data demonstrate that the MYND-containing protein BRAM1 abrogates LTβR function through a protein–protein interaction. These findings may provide a direction for the treatment of dysregulation of LTβR-mediated signaling.

1. Introduction

The evolutionarily conserved MYND domain is present in the AML1 chromosomal translocation partner ETO/MTG8 [1] or in several developmentally important proteins, such as Nervy [2], DEAF1 [3], and BS69 [4]. The MYND domain of ETO/MTG8 recruits a histone deacetylase complex and mediates gene repression [1,5]. Similarly, the MYND domain of BS69 has been suggested to be involved in gene repression through N-CoR recruitment [6]. BS69 also binds to adenovirus E1A and Epstein-Barr virus nuclear protein 2 (EBNA-2) through the MYND domain, thereby inhibiting the trans-activation potential of these oncoviral proteins [7,8].

An alternatively spliced BS69 isoform, termed BRAM1, encompasses the MYND domain of BS69 and resides in cytosol [9]. BRAM1, as well as its orthologs in zebrafish and *Caenorhabditis elegans*, has been proposed to participate in BMP and TGFβ signaling pathways [9–11]. In addition, BRAM1 is able to interact with Epstein-Barr virus-encoded latent membrane protein 1 (LMP1) [12], which mimics a member of the TNFR superfamily by constitutively activating TNFR-associated factor (TRAF)-mediated signaling [13]. BRAM1 interaction with LMP1 requires the BRAM1 MYND domain, and negatively regulates LMP1-induced NF-κB activation [12]. While the cytoplasmic domains of the TNFR-superfamily proteins exhibit little sequence homology, they all bind to a related set of signaling molecules, namely TRAFs. Thus, it is conceivable to speculate that BRAM1 may be capable of interacting with and functionally affecting other members in the TNFR superfamily, such as lymphotoxin beta receptor (LTβR). Notably, LTβR encompasses a PXLXP motif, which is also present in BS69-interacting partners with preference to interact with the BS69 MYND domain [8].

LTβR is prominent on the surface of most of the cell types, including epithelial cells [14–16]. The genetic ablation studies demonstrate that LTβR plays a central role in the development of lymphoid organs [17,18]. LTβR consists of 435 amino acids, with the

Abbreviations: BMP, Bone morphogenesis protein; BRAM1, BMP receptor-associated molecule 1; DEAF-1, Deformed epidermal autoregulatory factor-1; EBV, Epstein-Barr virus; JNK, c-Jun N-terminal kinase; LMP1, Latent membrane protein 1; LTβR, Lymphotoxin beta receptor; MYND domain, myeloid-Nervy-DEAF-1 domain; NF-κB, Nuclear factor kappa B; TGFβ, Tumor growth factor β; TNFR, Tumor necrosis factor receptor; TRAF, TNFR-associated factor.

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C-terminal 194 amino acid tail presented toward the intracellular space [19]. As with most TNFR-superfamily members, LTβR signaling is initiated by receptor aggregation resulting from ligand binding, and sequentially activates multiple signaling pathways, including those involving NF-κB activation, JNK signaling, and cell death [20–22]. LTβR mediates most of its functions by interacting with TRAF2, 3, 4 and 5, but not TRAF6 [23,24]. The sub-region of C-terminal LTβR (aa 336–398) that is essential for the receptor self-association encompasses a minimal TRAF3 binding region (^{389}PEEGDPG^{395}) [19] and mediates cell death [19,22,25]. This sub-region also overlaps with the region responsible for NF-κB activation [19], which is TRAF2 or TRAF5 recruitment essential [23].

Subtle differences exist in the structure–function relationship among LTβR and other TNFR-superfamily members. The cytoplasmic domains of TNFRI and Fas self-associate *via* their death domains, thereby prompting signaling events in the TNFRI and Fas pathways [26,27]. In contrast, the cytoplasmic domains of LTβR and TNFRII do not contain death domains [27,28]. Instead, LTβR self-associates through a non-death domain, leading to cell apoptosis in the absence of ligand binding [22].

In this study, we aimed to assess whether BRAM1 with the MYND domain binds to LTβR and affects LTβR-mediated function. Using the yeast two-hybrid and co-immunoprecipitation assays, we verified the interaction between LTβR and BRAM1, which primarily depends on the self-association domain of LTβR rather than the PXLXP motif as expected. The functional studies further revealed that BRAM1–LTβR interaction results in inhibition of LTβR-induced signaling and cell death. These findings advance understanding of the structural constraints for the protein–protein interaction involving MYND domain, and may provide a direction for the therapeutic intervention of LTβR signaling dysregulation.

2. Materials and methods

2.1. Plasmids

The plasmid pCMV-LTβR encoding the full-length LTβR has been described previously [29]. The Myc-tagged and HA-tagged LTβR constructs were generated by subcloning the LTβR coding region from pCMV-LTβR into the pCMV-Myc and pCMV-HA (Clontech), respectively. For generation of pGFP-LTβR, a cDNA fragment encoding the full-length GFP was amplified by polymerase chain reaction (PCR) using the pEGFP-C3 (Clontech) as template, and primers 5′-AACTAGTTAGTTATTAATAG-TAATCAAT-3′ (sense) and 5′-AACTAGTAAAAAA TTTCGTTCATTTT-3′ (antisense). PCR was carried out as follows: 35 cycles of 95 °C, 30 s; 55 °C, 1 min; and 72 °C, 1 min. The 1.5-kb GFP PCR product was subcloned into the *Spe*I site of pCMV-LTβR to produce pGFP-LTβR. The HA-tagged and Flag-tagged BRAM1 constructs were generated by insertion of the cDNA fragments for BRAM1 into the pCMV-HA and pFLAG-CMV-2 (Sigma), respectively. The pFLAG-cMTG8, encoding the C-terminal 173 amino acids of MTG8 (aa 405–577), and the pFLAG-cDEAF-1, encoding the C-terminal 162 amino acids of DEAF-1 (aa 404–565), were also generated using pFLAG-CMV-2.

The shRNA for BRAM1 (shBRAM1) was generated as previously described [30]. In brief, a double-strand oligonucleotide encompassing a sequence derived from the open reading frame of human BRAM1 (nucleotides 109–127) was designed in forward and reverse orientation and separated by a 9-base-pair spacer region (ttcaagaga) to allow formation of the hairpin structure in the expressed oligo-RNA: sense strand, 5′-gatccccAAGAAGTTAAGTGCCTCTTttcaagagaAAGAGGCACT-TAACTTCTTtttttggaaa; antisense strand, 5′-agcttttccaaaaaAAGAAGT-TAAGTGCCTCTTctcttgaaAAGAGGCACTTAACTTCTTgggg. The resulting double-stranded oligonucleotide was constructed into the *Bgl* II and *Hind* III sites of the pSUPER vector for expression under the control of the H1 RNA promoter. A scrambled sequence derived from the nucleotides 109–127 was used as a control shRNA (shCtrl).

2.2. Cell culture and transfection

HeLa cells (ATCC No. CCL2), HEK293 cells (ATCC No. CRL-1573), and 293T cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml streptomycin, and 100 units/ml penicillin. All transfection experiments were carried out with 70–80% confluent cells. Transfection of HeLa cells was performed by electroporation at 960 μF and 0.23 kV, using an electrophoresis power supply and an electroporation chamber (Bio-Rad). Transient transfection in HEK293 cells and 293T cells was carried out with the calcium phosphate precipitation or the lipofectamine method.

2.3. Yeast two-hybrid assay

Yeast transformation and β-galactosidase detection in *S. cerevisiae* Y190 was performed according to the manufacturer's instructions (PT1113-1; Clontech). To examine the interaction between LTβR and BRAM1, we fused the cDNA fragments encoding the full length or the C-terminal 116 amino acids of BRAM1 (C116) to the activation domain of the Gal4 protein (denoted as Gal4-AD) in the pGAD10 vector (Clontech). cDNA fragments encoding the LTβR cytoplasmic domain were fused to the DNA-binding domain of the Gal4 protein (denoted as Gal4-BD) in pAS2.1 vector (Clontech). A series of cDNA fragments encoding the cytoplasmic domains of LTβR was subcloned into pAS2.1, producing plasmids designated LTβR aa 249–435, aa 249–255, aa 249–335, aa 336–397 and aa 398–435, respectively. The Ura$^+$ Trp$^+$ His$^+$ Leu$^+$ transformants were further selected and confirmed *via* 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) assays.

2.4. Preparation of cell lysates and immunoblotting

Cells were washed thrice with PBS; cell pellets were resuspended in 5 volumes of lysis buffer (10 mM Tris–HCl, pH 7.1, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin) and incubated in ice for 20 min and followed by sonication. Immunoblotting was carried out as previously described [12]. Briefly, cell extracts were resolved by 10% SDS-PAGE and electrophoretically transferred onto PVDF membranes (Millipore). Membranes were probed with the following antibodies, respectively: anti-FLAG antibody (M2, Kodak), anti-Myc polyclonal antibody (sc-789, Santa Cruz Biotechnology), anti-HA monoclonal antibody (sc-40, Santa Cruz Biotechnology), and anti-TRAF3 polyclonal antibody (sc-948, Santa Cruz Biotechnology). Western blot analysis of endogenous I-κBα molecules was carried out by incubation of cell lysates with monoclonal anti-phospho antibody (B-9; Santa Cruz Biotechnology), and polyclonal anti-I-κBα antibody (C-21; Santa Cruz Biotechnology). Polyclonal anti-BRAM1 antibody was generated by immunizing rabbits with a synthetic peptide corresponding to the last 15 amino acids of BRAM1, and purified by using an affinity column. Immunoreactive protein bands were detected using an enhanced chemiluminescence detection kit (Santa Cruz Biotechnology).

2.5. Co-immunoprecipitation assay

Immunoprecipitation assays were performed using HEK293 or 293T cells. For the anti-FLAG immunoprecipitation assay, cells were lysed in 200 μl of lysis buffer (25 mM Tris–HCl, pH 7.0, 300 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin). For the anti-HA and the anti-Myc immunoprecipitation assays, cells were lysed in lysis buffer containing 20 mM Tris–HCl, pH 8.0, 137 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin. Cleared lysates were incubated at 4 °C for 2 h with anti-FLAG M2 affinity gel (Kodak), anti-HA affinity