Bim1, a MAGUK Family Member Linking Protein Kinase C Activation to Bcl10-mediated NF-κB Induction*

Linda M. McAllister-Lucasa,b,c,d, Naohiro Inoharae,b,c, Peter C. Lucasb,c,e, Jürgen Rulandf, Adalberto Benitoa,b,e, Qiutang Liht, Shu Chenb, Felicia F. Chenb, Shoji Yamaoa, Inder M. Vermaa, Tak W. Makf, and Gabriel Núñezb,j

From the 1Department of Pediatrics and Communicable Diseases and 4Department of Pathology and Comprehensive Cancer Center, The University of Michigan Medical School, Ann Arbor, Michigan 48109, the 3Amgen Institute and Ontario Cancer Institute and Departments of Medical Biophysics and Immunology, University of Toronto, Toronto, Ontario M5G 2C1, Canada, the 2Laboratory of Genetics, The Salk Institute, La Jolla, California 92037, and the 1Department of Microbiology, Tokyo Medical and Dental University, School of Medicine, Yushima 1-5-45, Bunkyo-ku, Tokyo 113-8519, Japan

Bcl10 and MALT1, products of distinct chromosomal translocations in mucosa-associated lymphoid tissue lymphoma, cooperate in activating NF-κB. Mice lacking Bcl10 demonstrate severe immunodeficiency associated with failure of lymphocytes to activate nuclear factor κB (NF-κB) in response to antigen receptor stimulation and protein kinase C activation. We characterize Bim1, a new signaling protein that binds Bcl10 and activates NF-κB. Bim1-mediated NF-κB activation requires Bcl10 and IκB kinases, indicating that Bim1 acts upstream of these mediators. Bim1, Bcl10, and MALT1 form a ternary complex, with Bcl10 bridging the Bim1/MALT1 interaction. A dominant negative Bim1 mutant inhibits NF-κB activation by anti-CD3 ligation, phorbol ester, and protein kinase C expression. These results suggest that Bim1 links surface receptor stimulation and protein kinase C activation to Bcl10/MALT1, thus leading to NF-κB induction.

B-cell lymphomas of mucosa-associated lymphoid tissue (MALT)1 are the most common form of lymphoma arising in extranodal sites. MALT lymphoma most commonly occurs within the stomach where it is strongly associated with chronic Helicobacter pylori infection (1). Until recently, little has been known about the molecular events that lead to the development of MALT lymphoma. However, studies have now identified several genes that are involved in recurrent chromosomal translocations in MALT lymphoma. The t(1;14)(p22;q32) results in the placement of the entire coding region of the Bcl10 gene adjacent to the strong immunoglobulin heavy chain locus transcriptional enhancer, leading to deregulated overexpression of Bcl10 (2, 3). A second recurrent chromosomal translocation, t(11;18)(q21;q21), is seen in up to 50% of MALT lymphomas. This translocation results in the creation of a fusion gene that encodes a chimeric protein consisting of the N-terminal portion of the inhibitor of apoptosis, c-IAP-2 (also known as AP12) linked to the C terminus of a novel protein, MALT1 (4–6).

Bcl10 (also known as CIPER, mE10, c-CARMEN, CLAP, and c-E10) is a caspase-recruitment domain (CARD)-containing protein that activates the transcription factor NF-κB (2, 7–11). MALT1, the other target of chromosomal translocation in MALT lymphomas, is a novel protein composed of a death domain, two immunoglobulin-like domains, and a C-terminal caspase-like domain (5, 6). Recent studies have shown that MALT1 binds specifically to Bcl10 and enhances the ability of Bcl10 to activate NF-κB (12, 13). Moreover, Bcl10 mediates the oligomerization of MALT1, an event that is sufficient for NF-κB activation (5). In addition, the c-IAP-2-MALT1 fusion protein resulting from the t(11;18)(q21;q21) chromosomal translocation strongly activates NF-κB (12, 13). These observations suggest that MALT1 functions as a downstream effector in a Bcl10-MALT1 complex, and that Bcl10 and MALT1, although independent targets of chromosomal translocation, act in a common NF-κB signaling pathway to promote MALT lymphoma.

Analysis of mutant mice lacking Bcl10 have shown that this protein plays a critical role in lymphoid activation and proliferation (14). In addition, Bcl10 is important for neural development, because a significant percentage of mice deficient in Bcl10 exhibit a defect in neural tube closure (14). Bcl10 −/− lymphocytes fail to activate NF-κB in response to CD3, CD3/CD28, and IgM ligation, indicating that Bcl10 is a signal transducer between lymphoid antigen receptors and NF-κB (14). One class of signaling molecules that has been implicated in mediating both T-cell receptor and B-cell receptor signaling is the family of protein kinase C (PKC) enzymes. Mounting evidence suggests that the PKA-activated isofoms such as PKCγ and PKCβ are important in CD3/CD28 and IgM receptor-mediated NF-κB induction, respectively (15–18). Bcl10 −/−...
lymphoid cells retain normal Ca\(^{2+}\) mobilization but are defective in NF-\(\kappa\)B stimulation induced by PMA/Ca\(^{2+}\) ionophore, suggesting that Bcl10 acts downstream of PKC in the antigen receptor-mediated signaling pathway (14).

Until now, proteins operating upstream of the Bcl10-MALT1 signaling complex have not been definitively identified. MALT1, a proposed downstream effector of Bcl10, does not contain a CARD, and therefore cannot participate in a CARD/CARD interaction with Bcl10. Recently, the protein CARD9 was identified as a CARD-containing protein that interacts selectively with the CARD of Bcl10, but the physiological significance of this binding has not yet been explored (19). Furthermore, the mechanisms by which surface receptors and PKC activation are linked to Bcl10 are unknown. Here we characterize a new Bcl10-binding molecule, Bimp1 (Bcl10-interacting MAGUK protein), a CARD-containing protein with homology to CARD9. Unlike CARD9, Bimp1 contains Src homology (SH3), Z0–1 homologous (PDZ), and guanylate kinase (GUK)-like domains. Bimp1 is a member of the MAGUK (membrane-associated guanylate kinase) family, a class of proteins that function as molecular scaffolds in assembling multiprotein complexes at the plasma membrane (20, 21). We show that Bimp1 is a component of a novel NF-\(\kappa\)B signaling pathway that links surface receptor signaling and PKC activation to Bcl10, MALT1, and the I\(\kappa\)B kinases (IKKs).

**EXPERIMENTAL PROCEDURES**

**Identification of Bimp1 and Bimp2 cDNAs**—The nucleotide sequence of cDNAs encoding peptides with homology to the CARD of CARD9 (GenBank™ accession number AF311287) were found in the EST data bases of GenBank™ using the TBLASTN program. cDNAs encoding a full-length coding region corresponding to Bimp1 and Bimp2 were obtained from Incyte Genomics Inc. (St. Louis, MO). The authenticity of the full-length coding region corresponding to Bimp1 and Bimp2 were obtained by digestion with Pml

**Construction of Expression Plasmids**—The expression plasmid pcDNA3-FLAG and pcDNA3-HA were created using a polymerase chain reaction method. For Bimp1, the cDNA insert of EST clone ES278025 was used as a template for a polymerase chain reaction using Bimp1-specific primers to generate wild-type Bimp1, Bimp1-CARD (residues 1–116), and Bimp1CARD (residues 117–1021). The inserts were subcloned into the pcDNA3-FLAG and pcDNA3-HA expression vectors. For Bimp2, the cDNA insert of EST clone BF100075 was used as a template for a polymerase chain reaction to generate a fragment that was ligated into pcDNA3-HA. All inserts were verified by DNA sequencing.

**Transfections and NF-\(\kappa\)B Activation Assays**—2 \(\times\) 10\(^6\) HEK293T cells were plated in standard 12-well culture dishes and transfected with the reporter constructs pEF1-BOS-\(\beta\)-gal, pBIVX-Luc, pcDNA3-FLAG, pcDNA3-HA, pcDNA3-IKK\(\alpha\) and pcDNA3-IKK\(\beta\). The cells were harvested 24 h after transfection, and the lysates were assayed for NF-\(\kappa\)B activation as described above. In experiments using the T-cell hybridoma cell line 68-41, 1 \(\times\) 10\(^5\) cells were transfected with 20 ng of pBIVX-Luc and 20 ng of pEF1-\(\beta\)-gal (Promega), 10 ng of pcDNA3-p35 plus 50 ng of indicated expression plasmids, and 50 ng of pcDNA3-HA plus the indicated DEAE-dextran method, and NF-\(\kappa\)B induction was assessed using the dual luciferase assay according to the manufacturer’s instructions (Promega). 10 ng/ml of TNF\(\alpha\) was added to the medium 4 h prior to harvest when indicated. For cells treated with PMA/Ca\(^{2+}\) ionophore, 50 ng/ml of PMA and 0.7 \(\mu\)g/ml of Ca\(^{2+}\) ionophore A23187 were added to the medium 4 h prior to harvest. 68-41 cells were stimulated with 5 \(\mu\)l/ml anti-CD3 2C11 coated onto 12-well plates 4 h prior to harvest when indicated.

Bcl10 \(\pm\), Bimp1 \(\pm\), and Bimp2 \(\pm\) MEFs were generated as described (14). For transfections of Bcl10 \(\pm\), Bimp1 \(\pm\), and Bimp2 \(\pm\), 1 \(\times\) 10\(^5\) cells/well were plated 24 h prior to transfection. 750 ng of pEF1-BOS-\(\beta\)-gal and 250 ng of pBIVX-Luc plus indicated expression plasmids were transfected using the Superfect (Qiagen) system. The cells were lysed and assayed 48 h after transfection using the luciferase assay system (Promega) according to the manufacturer’s instructions. MEFs from IKK\(\alpha\)-, IKK\(\beta\)-, and double IKK\(\alpha\)/\(\beta\)-deficient mice were generated as described (28) and transfected as indicated above for Rat-1 cells.

**Immunoprecipitations and Western Blotting**—HEK293T cells were harvested 24 h following transfection and lysed in 0.2% Nonidet P-40 lysis buffer (29). FLAG-tagged Bimp1 and its binding partners were immunoprecipitated by combining 25 \(\mu\)l of a 50% suspension of anti-FLAG M2 affinity gel (Sigma) with 1 ml of soluble cell lysate and incubated for 3 h at 4 \(\circ\)C. Immunoblotting was performed using mouse monoclonal anti-FLAG (Sigma), rabbit polyclonal anti-Myc (Santa Cruz), mouse monoclonal anti-HA 12CA5 (Roche Molecular Biochemicals), or rabbit polyclonal anti-HA (Santa Cruz) antibodies.

**RESULTS**

**Identification of Bimp1 cDNA and Analysis of Amino Acid Sequence**—In an effort to identify potential binding partners that could participate in a CARD/CARD interaction with Bcl10, we searched public data bases of ESTs for clones with homology to the CARD of CARD9 (19). Sequence analysis of one of the ESTs obtained from our search revealed a mouse cDNA that encodes a 1021-amino acid protein with a predicted molecular mass of 114 kDa. This protein was designated Bimp1 (Fig. 1A). A search for a human orthologue revealed several overlapping partial cDNAs that could be constructed to form a single open reading frame encoding a 1032-amino acid protein with 90% identity to the mouse sequence. The corresponding human gene maped to chromosome 22q13 (GenBank™ accession numbers AL022315 and AL049851). In our search, we also identified a second mouse cDNA encoding a protein with significant homology to Bimp1 that we have designated Bimp2 (Fig. 1A). Bimp2 is 1000 amino acids in length with a predicted molecular mass of 113 kDa.

Analysis of the amino acid sequence of Bimp1 revealed that its structure is comprised of five putative functional domains: an N-terminal CARD, a central coiled-coil domain, and a C-terminal structure comprised of SH3, PDZ, and GUK-like domains (Fig. 1B). The presence of the PDZ, SH3, and GUK domains identifies Bimp1 and Bimp2 as members of the growing MAGUK family of proteins (20, 21). These proteins serve as molecular scaffolds for signaling pathway components by utilizing multiple protein/protein interaction domains to cluster receptors, adaptor proteins, and cytosolic signaling proteins at the cell membrane (20, 21). The central coiled-coil structure in Bimp1 is predicted to participate in protein oligomerization (30). The CARD of Bimp1 does share some degree of sequence homology with the CARD of a variety of cell death pathway components (Fig. 1C), but it is most strikingly similar to the CARD of CARD9 (47% identity) and Bimp2 (47% identity) (19) (Fig. 1C).

**Bimp1 Is Expressed in Multiple Tissues**—We performed Northern blot analysis to determine the distribution of Bimp1 transcripts in various mouse tissues. A \(~6.8\)-kilobase Bimp1
transcript was detected in all tissues examined including heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testes (Fig. 1D). The transcript was most abundant in heart and kidney. A smaller ~3.8-kilobase Bimp1 transcript was also detected in brain but was not detected in any of the other tissues sampled (Fig. 1D). The significance of this alternative form of Bimp1 mRNA is not currently known.

**Bimp1 Activates NF-κB—Several CARD-containing proteins, including Bcl10, have been shown to induce NF-κB activation (8, 22). To test whether Bimp1 activates NF-κB, a Bimp1 expression plasmid was co-transfected with an NF-κB luciferase reporter plasmid. HA-tagged Bimp1 induced NF-κB activity in a dose-dependent manner to a maximum of ~250-fold induction as compared with empty vector (Fig. 2A). Transfection of untagged Bimp1 expression plasmid also resulted in NF-κB activation, showing that this activity cannot be attributed to the epitope tag (data not shown).

**The Bimp1 CARD Is Essential but Not Sufficient for NF-κB Activation—**To test the importance of the CARD of Bimp1 in NF-κB activation, we utilized two mutants of Bimp1, one that lacked the entire CARD (Bimp1ΔCARD) and one that contained only the CARD (Bimp1CARD) (Fig. 2B). Deletion of the CARD completely abolished NF-κB activation, indicating that the CARD is essential for Bimp1-mediated NF-κB signaling (Fig. 2B). However, the Bimp1CARD mutant was also inactive when expressed by itself, suggesting that Bimp1 domains other than the CARD are also required for NF-κB-inducing activity. Immunoblotting confirmed that the mutant forms of Bimp1 were expressed as effectively as wild-type Bimp1 (Fig. 2B, inset).

**Bimp1 and Bimp2 Act through the IKK Complex to Activate NF-κB—**In response to certain stimuli, IκB is phosphorylated by the IKK complex, which includes two IκB kinases, IKKα and β, and one regulatory subunit, IKKγ. The phosphorylation of IκB leads to its ubiquitination and degradation, thus allowing NF-κB to translocate to the nucleus where it activates transcription of target genes (31). To investigate the role of the catalytic subunits of the IKK complex in Bimp1 signaling, we tested the ability of Bimp1 to induce NF-κB activation in wild-type MEFs and in MEFs deficient in IKKα or IKKβ subunit or both (28). Bimp1 induced comparable levels of NF-κB activation in wild-type MEFs and MEFs deficient in IKKβ but reduced levels in cells deficient in IKKα or both (28). Bimp1 induced comparable levels of NF-κB activation in wild-type MEFs and MEFs deficient in IKKβ but reduced levels in cells deficient in IKKα or IKKβ subunits (Fig. 3A). No detectable Bimp1-mediated NF-κB induction was observed in MEFs deficient in both IKKα and IKKβ subunits (Fig. 3A). Transfection of Nod1, a molecule known to activate NF-κB through the IKK complex yielded similar results (Fig. 3A) (22). In control experiments, expression of IKKβ effectively induced NF-κB activation in cells lacking IKKα/IKKβ, showing that NF-κB signaling machinery downstream of the IKK complex is intact in these MEFs (data not shown). To determine whether the IKKγ regulatory subunit is important for Bimp1-mediated NF-κB activation, we co-expressed Bimp1 with a dominant negative form of IKKγ that lacks amino acids 1–134 and is therefore unable to bind IKKα and β (23). Co-expression of the dominant negative IKKγ resulted in effective inhibition of Bimp1-mediated...
...treatment of cells with TNFα...

Experimental Procedures. CARD-FLAG, along with pEF1-BOS-empty vector, whereas in Bcl10-deficient 5R cells (Fig. 3B). While in the parental Rat-1 cells Bimp1 effectively activates NF-κB in Bcl10−/− cells (Fig. 4A). Together, these results provide strong evidence that Bimp1 and Bimp2 operate upstream of Bcl10 in a pathway leading to NF-κB activation.

Bimp1 Binds to Bcl10 through a CARD/CARD Interaction—The CARD is an α-helical domain that links signaling components through homophilic CARD/CARD interactions (33). To determine whether Bimp1 binds to the CARD of Bcl10, Bimp1 was co-expressed with Bcl10, cell extracts were prepared, and Bimp1 was immunoprecipitated with anti-FLAG antibody. Immunoblotting revealed that Bcl10 co-immunoprecipitated with Bimp1 (Fig. 4B). The binding of Bimp1 to Bcl10 was dependent on the N-terminal CARD of Bcl10, because Bimp1 failed to co-precipitate a variant of Bcl10 containing a point mutation (L14Q) that disrupts CARD/CARD interactions (8) (Fig. 4B). The association between Bimp1 and Bcl10 was specific in that RICK, another CARD-containing protein (23), did not co-precipitate with Bimp1 (Fig. 4B). In reciprocal experiments, Bcl10 co-immunoprecipitated with wild-type and mutant Bimp1 containing the CARD alone but not with a mutant of Bimp1 lacking the CARD (Fig. 4C). These results indicate that Bimp1 and Bcl10 associate through a CARD/CARD homotypic interaction.

Bimp1, Bcl10, and MALT1 Can Form a Ternary Protein Complex—Bcl10 has been shown to bind to MALT1 and to mediate MALT1 oligomerization, an event that is sufficient for the activation of NF-κB (12, 13). Because Bimp1 binds Bcl10 and operates upstream of Bcl10 in an NF-κB signaling pathway, we tested whether Bimp1 can associate with MALT1 through Bcl10. Because wild-type MALT1 is expressed at very low levels in our transfection system, we used a truncated mutant, MALT1(1–330), which retains full binding to Bcl10 and can be expressed at higher levels than the wild-type MALT1 (13). When MALT1 was co-expressed with Bimp1, in the absence of Bcl10, the MALT1 protein did not co-precipitate with Bimp1 (Fig. 5A). However, MALT1 did co-precipitate with Bimp1 when Bcl10 was also included in the transfection (Fig. 5A). These results support the notion that Bimp1, Bcl10, and MALT1 can form a multimeric protein complex, with Bcl10 forming a bridge between Bimp1 and MALT1. In an effort to...
Bimp1, a MAGUK Protein Acting Upstream of Bcl10

Bimp1 fails to activate NF-κB in IKKα/β-deficient cells. MEFs were transfected with pBViX-Luc, pEF1-BOS-β-gal, and 0.35 μg of the indicated expression plasmid (pcDNA3-Bimp1-HA or pcDNA3-Flag-IKKi) as described under “Experimental Procedures.” The cells were harvested 24 h after transfection, and NF-κB activity was determined as described under “Experimental Procedures.” B, dominant negative IKKγ blocks Bimp1-mediated NF-κB activation. 293T cells were transfected with either 25 μg of pcDNA3-Bimp1-HA or 12.5 μg of pcDNA3-Flag-IKKi in the absence or presence of 75 ng of expression plasmids encoding dominant negative IKKγ (pcDNA3-HA-IKKγ(134–419)), IkBo (RSV-Mad-3MSS(IkBo-S32A/S36A)), or MyD88 (pcDNA3-MyD88DN(1–109)). 24 h posttransfection, cells were harvested, and NF-κB induction was measured. C, Bimp1 fails to activate NF-κB in IKKγ-deficient cells. Rat-1 or 5R cells were transfected with 0.35 μg of indicated expression plasmid. For cells treated with TNFα, 10 ng/ml of TNFα was added to the medium 2 h prior to harvest. The cells were harvested 24 h after transfection, and NF-κB activity was measured. For A–C, all experiments were done in triplicate, and the error bars represent the standard deviations. WT, wild type.

Further confirm that Bcl10 can mediate the interaction between Bimp1 and MAL1, we utilized mutant versions of Bcl10, which disrupt either Bimp1/Bcl10 or Bcl10-MAL1 binding. Bcl10 (L41Q) contains a point mutation in the CARD, which prevents binding to Bimp1 (Fig. 4B). When Bcl10 (L41Q) replaced wild-type Bcl10 in our co-immunoprecipitation experiments, MAL1 did not co-precipitate with Bimp1, indicating that disruption of the Bimp1/Bcl10 interaction destroyed the ability of Bimp1 to recruit MAL1 to a binding complex (Fig. 5A). The Bcl10 (Δ107–119) mutant is deficient in MAL1 binding and in NF-κB activation (13). This Bcl10 mutant bound to Bimp1, suggesting that its overall structural integrity is maintained and that the CARD is intact (Fig. 5A). However, MAL1 did not co-precipitate with Bimp1 when Bcl10 (Δ107–119) was included in the transfection in place of wild-type Bcl10 (Fig. 5A). These results suggest that disruption of Bcl10-MAL1 binding abolishes the ability of Bimp1 to recruit MAL1 to a binding complex. Taken together, our results indicate that Bimp1, Bcl10, and MAL1 can form a multimeric signaling complex in which Bcl10 forms a molecular bridge between Bimp1 and MAL1.

Bimp1 Cooperates with Bcl10 and MAL1 in NF-κB Induction—MAL1 specifically enhances Bcl10-mediated NF-κB activation, suggesting that Bcl10 and MAL1 cooperate in a common signaling pathway leading to NF-κB activation (13). To determine whether Bimp1 cooperates with Bcl10 and MAL1 in this same signaling pathway, we tested whether Bcl10 and MAL1 could enhance Bimp1-mediated NF-κB activation. Because overexpression of Bcl10 induces potent NF-κB activation, we transfected cells with low amounts of Bcl10 expression plasmid and assessed the effect of Bcl10, in the absence or the presence of MAL1, on Bimp-1-mediated NF-κB induction. Transfection of 2.5 ng of Bcl10, with or without MAL1, resulted in no appreciable activation of NF-κB (Fig. 5B). However, co-transfection of 2.5 ng of Bcl10 with Bimp1 resulted in an approximately 2-fold enhancement of NF-κB activation compared with Bimp1 alone (Fig. 5B). Furthermore, co-transfection of Bimp1 with the combination of Bcl10 plus MAL1 resulted in an even greater enhancement (3.5-fold) of NF-κB activation compared with Bimp1 alone (Fig. 5B). The cooperative effects of Bcl10 and MAL1 were specific for Bimp1, because co-transfection with Bcl10, with or without MAL1, had no effect on NF-κB activation by RICK (Fig. 5B). Immunoblotting confirmed that neither Bimp1 nor RICK levels were affected by co-expression of Bcl10 and MAL1, indicating that Bcl10 and MAL1 were specifically influencing the function of the Bimp1 signaling pathway (Fig. 5B). These results provide evidence that Bcl10 and MAL1 can cooperate with Bimp1 in a signaling pathway leading to NF-κB activation. To further verify the specificity of the functional cooperation of these proteins, we utilized a Bcl10 variant, L41Q, which contains a single point mutation in the CARD that abolishes binding to Bimp1 as well as the ability of Bcl10 to activate NF-κB (8) (Fig. 4B). When Bcl10 (L41Q) was co-transfected with Bimp1, either in the absence or the presence of MAL1, absolutely no enhancement of NF-κB activation was seen (Fig. 5C). Again, immunoblotting showed that the levels of Bimp1 expression were unaffected by co-transfection with Bcl10 (L41Q) and MAL1. These results support the hypothesis that Bimp1 is able to cooperate in a signaling pathway with Bcl10 and MAL1 through a CARD-mediated mechanism.

A Mutant of Bimp1 Lacking the CARD Blocks NF-κB Activation Induced by CD3 Ligation, PMA/Ionomycin, and PKC—Unlike normal T-cells, Bcl10 deficient T-cells are unable to activate NF-κB in response to CD3/CD28 (14). Bcl10-deficient lymphoid cells also fail to activate NF-κB in response to PMA/
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Fig. 4. Bimp1 activates NF-κB through a Bcl10-dependent mechanism and binds to Bcl10 through CARD/CARD interaction. A, Bimp1-mediated activation of NF-κB is dependent on Bcl10. Bcl10 +/- or Bcl10 +/- MEFs were transfected with 900 ng of the indicated expression plasmid: pcDNA3-Bimp1-HA, pcDNA3-Bimp2-HA, or pcDNA3-Nod1-HA. 48 h after transfection, the cells were lysed, and NF-κB induction was assayed as described under “Experimental Procedures.” The data shown are representative of four separate experiments. Transfections and assays were done in triplicate, and the error bars represent the standard deviations. B, Bimp1 binds specifically to the CARD of Bcl10. 293T cells were transfected with indicated combinations of expression plasmids encoding Myc-tagged Bcl10 (pcDNA3-Bcl10-Myc, 2 μg) or Bcl10 mutant (pcDNA3-Bcl10(L41Q)-Myc, 2 μg), FLAG-tagged Bimp1 (pcDNA3-Bimp1-FLAG, 4 μg), and Myc-tagged RICK (pcDNA3-RICK-Myc, 3 μg). 24 h post transfection, the cells were lysed, and FLAG-tagged proteins were immunoprecipitated as described under “Experimental Procedures.” The products were resolved by 10% SDS-PAGE and visualized by Western blot with indicated antibodies. C, the Bimp1 CARD binds to Bcl10. 293T cells were transfected with indicated expression plasmids: pcDNA3-Bimp1-FLAG, 4 μg; pcDNA3-Bimp1CARD-FLAG, 4 μg; pcDNA3-BimpCARD-FLAG, 4 μg; and pcDNA-Bcl10-Myc, 2 μg. 24 h after transfection, the cells were lysed, and FLAG-tagged proteins were immunoprecipitated as described. The products were resolved by 8–15% SDS-PAGE and visualized by Western blot with indicated antibodies. WB, Western blot; *, phosphorylated form of Bcl10.

Fig. 5. Bimp1 recruits MALT1 to a multimeric binding complex and cooperates with Bcl10-MALT1 in NF-κB activation. A, Bimp1 recruits MALT1 to a multimeric binding complex in which Bcl10 functions as a molecular bridge. 293T cells were transfected with indicated combinations of expression plasmids: pcDNA3-Bimp1-FLAG, 4 μg; pcDNA3-Bcl10-Myc, 2 μg; pcDNA3-Bcl10(L41Q)-Myc, 2 μg; pcDNA3-BimpCARD-FLAG, 4 μg; and pcDNA-MALT1-Myc, 500 ng. NF-κB induction was measured as described under “Experimental Procedures.” The data shown are representative of four separate experiments. B, co-transfection of Bcl10 and MALT1 with Bimp1 leads to enhanced NF-κB induction. 293T cells were transfected with the indicated expression plasmids: (pcDNA3-Bcl10-Myc, 2.5 ng; pcDNA3-MALT1-Myc, 500 ng; pcDNA3-Bimp1-HA, 125 ng; and pcDNA3-MALT1-(1-330)-HA, 6 μg; and pcDNA MALT1-(1-330)-HA, 6 μg; and pcDNA-Bcl10-Myc, 2 μg; and pcDNA-Bcl10(L41Q)-Myc, 2 μg; and pcDNA3-BimpCARD-FLAG, 4 μg; and pcDNA-Bcl10-Myc, 2 μg). 24 h after transfection, the cell extracts were prepared, and FLAG-tagged proteins were immunoprecipitated as described under “Experimental Procedures.” The data shown are representative of four separate experiments. The experiments were done in triplicate, and the error bars represent the standard deviations. C, the synergy between Bimp1, Bcl10, and MALT1 is dependent on an intact CARD in Bcl10. The synergy between Bimp1, Bcl10, and MALT1 is dependent on an intact CARD in 293T cells and found that CARD alone (Bimp1CARD) had no inhibitory effect on NF-κB induction. The experiments were done in triplicate, and the error bars represent the standard deviations. The cell extracts were subjected to immunoblotting with mouse monoclonal anti-HA antibody to assess the level of expression of Bimp1 or with rabbit polyclonal anti-Myc antibody to assess the level of RICK expression. C, the synergy between Bimp1, Bcl10, and MALT1 is dependent on an intact CARD in Bcl10. The expression plasmid pcDNA-Bcl10(L41Q)-Myc, which encodes a mutant Bcl10 with a defective CARD, was utilized in place of the wild-type plasmid pcDNA3-Bcl10-Myc. Immunoblotting was performed as described above.

ionomycin treatment (14), suggesting that Bcl10 functions downstream of PKC in a pathway leading to NF-κB activation. To investigate the role of Bimp1 in this pathway, we utilized the mutant version of Bimp1, which lacks the CARD (Bimp1CARD) and therefore cannot bind to Bcl10 (Fig. 4C). We tested whether this mutant could function as a dominant negative by inhibiting anti-CD3, PMA/Ca²⁺ ionophore, and PKC-mediated NF-κB activation. First, we evaluated the effect of expression of Bimp1CARD in 293T cells and found that Bimp1CARD expression resulted in effective inhibition of PMA/Ca²⁺ ionophore A23187-induced NF-κB activation (Fig. 6A). In contrast, the mutant of Bimp1 that consists of the CARD alone (Bimp1CARD) had no inhibitory effect on NF-κB induction by PMA/Ca²⁺ ionophore (Fig. 6A). In control experiments, a dominant negative form of MyD88, a key factor in a different NF-κB signaling pathway that is triggered by Toll/
interleukin-1 receptors, did not block PMA-induced NF-κB activation, whereas a dominant negative form of IKKβ, which operates downstream of Bimp1 and Bcl10, did inhibit activation (Fig. 6A). The effect of the Bimp1 dominant negative was specific in that cells treated with TNFα or transfected with an expression plasmid encoding a constitutively active mutant Toll-like receptor 2 (34) displayed full activation of NF-κB. A model of signaling pathway linking antigen receptor, PKC, Bimp1, and Bcl10-MALT1 to NF-κB activation is shown (Fig. 6A). We next examined the ability of the Bimp1ΔCARD to function as a dominant negative mutant in inhibiting NF-κB activation triggered by anti-CD3 in the T-cell hybridoma cell line 68-41 (35). In this system, Bimp1ΔCARD expression blocked both anti-CD3 antibody and PMA/Ca2+ ionophore-induced NF-κB activation but had no effect on NF-κB induction by TNFα (Fig. 6B). Again, expression of the Bimp1ΔCARD mutant did not inhibit NF-κB induction triggered by CD3 ligation or PMA (Fig. 6B). We propose that Bimp1 and Bimp2 represent a subfamily of MAGUK proteins that are distinguished

**DISCUSSION**

We have identified a novel protein, Bimp1, that binds Bcl10 and activates NF-κB. Bimp1 is homologous to Bimp2, another protein that also activates NF-κB in a Bcl10-dependent manner. The domain structures of Bimp1 and Bimp2 identify these proteins as members of the MAGUK family, a class of proteins as members of the MAGUK family, a class of proteins that are composed of multiple discreet modules, including the SH3, PDZ, and GUK-like domains, which mediate protein-protein interaction. We propose that Bimp1 and Bimp2 represent a subfamily of MAGUK proteins that are distinguished...
from other subfamilies by the presence of an N-terminal CARD. As this manuscript was completed, Bertin et al. (37) published a report identifying two CARD-containing MAGUK proteins that they named CARD11 and CARD14. CARD14 may represent the human orthologue of Bimp2. These authors showed that CARD11 and CARD14 bind to Bcl10 and activate NF-κB. However, this report does not establish whether CARD11 and CARD14 operate upstream of Bcl10, nor does it address the relationship of CARD11, CARD14, and Bcl10 to T-cell receptor triggering and PKC activation (37).

To our knowledge, Bimp1 is the first molecule to be definitively identified as operating upstream of Bcl10 and as linking PKC activation to Bcl10-mediated NF-κB induction. Based on our data, we propose a model for a Bimp-mediated signaling pathway leading to NF-κB activation (Fig. 6D). In this proposed pathway, surface receptor stimulation leads to activation of PKC, and a Bimp protein functions as a critical link between PKC and Bcl10. Because the Bimp proteins are members of the MAGUK family, it is likely that Bimps function as molecular scaffolds in recruiting molecules such as plasma membrane receptors, phosphoplaspe C, PKC, Bcl10, MALT1, and other as yet unidentified molecules to a large signaling complex, thereby holding together the signaling components that allow NF-κB activation to occur (Fig. 6D). The Bimps may, like many MAGUK proteins, anchor to the cytoplasmic tail of surface receptors through PDZ domain interaction (20). Our data suggest that Bimp1 operates immediately upstream of Bcl10 with the CARD/CARD interaction between Bcl10 and Bimp1 being essential for NF-κB signaling. Bimp1, Bcl10, and MALT1 can form a ternary complex whereby Bcl10 links Bimp1 to MALT1. The formation of this multiprotein complex has functional significance because Bimp1, Bcl10, and MALT1 can cooperate to activate NF-κB. Recent studies have shown that MALT1 binds to Bcl10 through its immunoglobulin domains (12, 13). Furthermore, Bcl10 mediates the oligomerization of MALT1, an event that is sufficient for NF-κB activation, thus suggesting that MALT1 is a downstream effector of Bcl10 (13). It is not known whether MALT1 is the only downstream effector of Bcl10 or whether other molecules also function downstream of Bcl10. Bimp1/Bcl10-mediated activation of NF-κB occurs through a mechanism that requires the regulatory subunit IKKγ and the kinase subunits IKKα or IKKβ of the IKK complex. The molecular events that link oligomerization and activation of the MALT1 caspase-like domain to activation of the IKK complex remain unknown.

Recent knockout studies revealed that the Bcl10-mediated NF-κB activation pathway plays an essential role in antigen receptor signaling in B- and T-cells (14). In addition, the diacylglycerol/PMA-sensitive PKC isoforms have also been implicated as critical components of NF-κB signaling in lymphoid cells (15–18). For example, antigen receptor stimulation in T-cells activates PKCδ, a step that is required for NF-κB induction (18). Likewise, PKCβ has been shown to be essential for antigen receptor signaling in B-cells (15). We have now identified a molecule, Bimp1, which appears to link PKC activation to the Bcl10-dependent pathway of NF-κB induction. Importantly, the role of Bimp1 in lymphocytes is specifically demonstrated by our studies of CD3 signaling in T-cell hybrids. In these cells, activation of NF-κB by anti-CD3 is completely blocked by a dominant negative mutant of Bimp1. The notion that PKC, Bimp1, and Bcl10 participate in a common NF-κB signaling pathway is supported by the fact that mice deficient in PKCδ and PKCβ demonstrate defects in lymphoid activation that are similar to those observed in mice lacking Bcl10 (15, 18).

Although our results implicate Bimp1 in linking T-cell receptor stimulation and PKC activation to Bcl10, it is possible that Bimp1 and Bimp2 may also be involved in PKC-dependent NF-κB signaling pathways in a wide array of other physiologic systems including the developing neural system. Embryos from mice lacking Bcl10 as well as embryos lacking both IKKα and IKKβ exhibit defects in neural tube closure associated with enhanced apoptosis in the hindbrain neuroepithelium (14, 28). These observations suggest that Bcl10-mediated NF-κB activation acts in a survival pathway to protect developing neurons. It will be important to determine whether PKC and Bimp proteins act upstream of Bcl10 in this neuronal survival pathway.

The importance of Bimps in linking PKC activation to NF-κB signaling may extend to cellular processes other than lymphocyte activation and central nervous development. Recent studies suggest that PKC isoforms other than PKCθ and PKCβ may mediate the induction of NF-κB in a variety of physiologic systems. For example, PKCe activation is a critical step in the induction of NF-κB by platelet-derived growth factor (38). Also, the induction of NF-κB that occurs in protective ischemic preconditioning in cardiomyocytes is mediated by PKCe (39). PKCα has been implicated as an upstream activator of NF-κB in both human osteosarcoma and lung epithelial cell lines (40, 41). The finding that dominant negative Bimp1 inhibits NF-κB activation induced by both PKCα and PKCe suggests that Bimp1 may be involved in linking multiple classical and novel PKC isoforms to NF-κB activation in a variety of cells. These events could be mediated by either Bimp1 and/or Bimp2, because these CARD/MAGUK proteins are highly homologous and are likely to participate in similar signaling pathways. Future studies will be aimed at exploring the potential role of the PKC/Bimp/Bcl10-MALT1 pathway in a variety of physiologic systems, identifying the upstream signals that trigger activation of PKC and characterizing the mechanism(s) by which PKC activation is linked to Bimp1/Bcl10-MALT1 proteins in this novel NF-κB signaling pathway.

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REFERENCES
1. Zucca, E., Bertoni, F., Roggero, E., and Cavelli, F. (2000) Blood 96, 410–419
2. Willis, T. G., Jadayel, D. M., Du, M.-Q., Peng, H., Perry, A. R., Abdul-Rauf, M., Price, H., Karran, I., Majekodunmi, L., Wlodarska, I., Pan, L., Crook, T., Friend, S., Isakov, R., Isakov, S., and Denhardt, D. T. (2000) Blood 95, 1544–1549
3. Zhang, Q., Siebert, R., Yan, M., Hinzmann, B., Cui, X., Xue, L., Rakestraw, K. M., Naeye, C. W., Beckman, G., Weisenburger, D. D., Sanger, W. G., Nowatny, H., Vesely, M., Callet-Bauchu, E., Saites, G., Dixit, V. M., Rosenthal, A., Schlegelberger, B., and Morris, S. W. (1999) Nat. Genet. 22, 63–69
4. Akagi, T., Motegi, M., Tamura, A., Suzuki, R., Hoshawaka, Y., Suzuki, H., Ota, H., Nakamura, S., Morishima, Y., Taniwaki, M., and Soto, M. (1999) Oncogene 18, 5785–5794
5. Dierlamm, J., Baens, M., Wlodarska, I., Stefanova-Ozounova, M., Hernandez, J. M., Hsu, S. S., DeWolf-Peeters, C., Hagemeijer, A., Vanden Bergh, H., and Maryn, P. (1999) Blood 93, 3601–3609
6. Morgan, J. A., Yin, Y., Borowsky, A. D., Kuo, F., Nourmand, N., Koontz, J. I., Rehfeld, C., Storger, L., Griffin, C. A., Graeme-Cook, F., Harris, N. L., Weisenburger, D., Pinkus, G., Fletcher, J., and Sklar, J. (1999) Cancer Res. 59, 6205–6213
7. Costanzo, A., Guiet, C., Vito, P. (1999) J. Biol. Chem. 274, 21207–21212
8. Koski, T., Inohara, N., Chen, S., Carrio, R., Merino, J., Hottiger, M. O., Nabel, G., and Nunez, G. (1999) J. Biol. Chem. 274, 9965–9961
9. Srivinivasula, S. M., Ahmad, M., Lin, J., Poyet, J.-L., Fernandes-Alnemri, T., and Alnemri, E. S. (1999) J. Biol. Chem. 274, 17964–17968
10. Thome, M., Martinon, F., Hofmann, K., Rubio, V., Steiner, V., Schneider, P., Mattmann, C., and Tschopp, J. (1999) J. Biol. Chem. 274, 9962–9968
11. Yan, M., Lee, J., Schilbach, S., Goddard, A., and Dixit, V. (1990) J. Biol. Chem. 265, 10297–10292
12. Uren, A. G., O’Rourke, K., Aravind, L., Pisabarro, M. T., Seshagiri, S., Koonin, E. V., and Dixit, V. M. (1999) J. Biol. Chem. 274, 9965–9961
13. Lucas, P. C., Yoneyama, M., Inohara, N., McAllister, S., Lucas, L. M., Azaebed, M. E., Chen, F. F., Yamazaki, E., and Dixit, V. M. (2000) Mol. Cell. 6, 961–967
14. Roland, J., Duncan, G. S., Elia, A., de Barco Barrantes, I., Nguyen, L., Flyte,
15. Leitges, M., Schmedt, C., Guinamard, R., Davoust, J., Schaal, S., Stabel, S., and Tarakhovsky, A. (1996) *Science* **97**, 788–791
16. Coudronniere, N., Villalba, M., Englund, N., and Altman, A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 3394–3399
17. Lin, X., O'Mahony, A., Mu, Y., Geleziunas, R., and Greene, W. C. (2000) *Mol. Cell. Biol.* **20**, 2933–2940
18. Sun, Z., Arendt, C. W., Ellmeier, W., Schaeffer, E. M., Sunshine, M. J., Gandhi, L, Annes, J., Schwartzberg, P. L., and Littman, D. R. (2000) *Nature* **404**, 402–407
19. Bertin, J., Guo, Y., Wang, L., Srinivasula, S. M., Jacobson, M. D., Poyet, J.-L., Merriam, S., Du, M.-Q., Dyer, M. J. S., Robison, K. E., Distefano, P. S., and Alnemri, E. S. (2000) *J. Biol. Chem.* **275**, 41082–41086
20. Dimitratos, S. D., Woods, D. F., Stathakis, D. G., and Bryan, P. J. (1999) *BioEssays* **21**, 912–921
21. Fanning, A. S., and Anderson, J. M. (1999) *Curr. Opin. Cell Biol.* **11**, 432–439
22. Inohara, N., Koseki, T., del Peso, L., Hu, Y., Yee, C., Chen, S., Carrio, R., Merino, J., Liu, D., Ni, J., and Núñez, G. (1999) *J. Biol. Chem.* **274**, 14560–14567
23. Inohara, N., Koseki, T., Lin, J., del Peso, L., Lucas, P. C., Chen, F. F., Ogura, Y., and Núñez, G. (2000) *J. Biol. Chem.* **275**, 27823–27831
24. Laflèna, M.-J., Din-Meco, M. T., Bren, G., Paya, C. V., and Moscat, J. (1999) *Mol. Cell. Biol.* **19**, 2180–2188
25. Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Gosh, S., and Janeway, C. A. (1998) *Mol. Cell* **2**, 253–258
26. Perkins, N. D., Felzien, L. K., Betts, J. C., Leung, R., Beach, D. H., and Nabel, G. J. (1997) *Science* **275**, 523–527
27. Shimada, T., Kawai, T., Takeda, K., Matsumoto, M., Inoue, J., Tatsumi, Y., Kanamaru, A., and Akira, S. (1999) *Int. Immunol.* **11**, 1357–1362
28. Li, Q., Estepa, G., Memet, S., Israel, A., and Verma, I. M. (2000) *Genes Dev.* **14**, 1729–1733
29. Oltvai, Z. N., and Korsmeyer, S. J. (1994) *Cell* **79**, 189–192
30. Lupas, A. (1996) *Trends Biochem. Sci.* **21**, 375–382
31. Karin, M., and Ben-Neriah, Y. (2000) *Annu. Rev. Immunol.* **18**, 621–663
32. Yamaoka, S., Courtois, G., Bessia, C., Whiteside, S. T., Weil, R., Aou, F., Kirk, H. E., Kay, R. J., and Israel, A. (1998) *Cell* **93**, 1231–1240
33. Hofmann, K., and Bucher, P. (1997) *Trends Biochem. Sci.* **22**, 155–156
34. Medzhitov, R., Preston-Hurlburt, P., Janeway, C. A., Jr. (1997) *Nature* **388**, 384–397
35. Tokoyoda, K., Takemoto, Y., Nakayama, T., Arai, T., and Kubo, M. (2000) *J. Biol. Chem.* **275**, 11728–11734
36. Cohen, O., Feinstein, E., and Kimchi, A. (1997) *EMBO J.* **16**, 998–1008
37. Bertin, J., Wang, L., Guo, Y., Jacobson, M. D., Poyet, J.-L., Srinivasula, S. M., Merriam, S., Distefano, P., and Alnemri, E. (2001) *J. Biol. Chem.* **276**, 11877–11882
38. Tojima, Y., Fujimoto, A., Delhase, M., Chen, Y., Hatakeyama, S., Nakayama, K., Kaneko, Y., Minura, Y., Motoshima, N., Ikeda, K., Karin, M., and Nakanishi, M. (2000) *Nature* **404**, 778–782
39. Li, R. C., Png, P., Zhang, J., Wead, W. B., Cao, X., Gao, J., Zheng, Y., Huang, S., Han, J., and Bolli, R. (2000) *Am. J. Physiol.* **279**, H1679–H1689
40. Chen, C. C., Sun, Y. T., Chen, J. J., and Chiu, K. T. (2000) *J. Immunol.* **165**, 2719–2728
41. Vertegeal, A. C. O., Kuiperij, H. B., Yamaoka, S., Courtois, G., van der Eb, A. J., Zantema, A. (2000) *Cell. Signal.* **12**, 759–768
42. Qin, H., Srinivasula, S. M., Wu, G., Fernando-Alnemri, T., Alnemri, E. S., and Shi, Y. (1999) *Nature* **399**, 549–557
Bimp1, a MAGUK Family Member Linking Protein Kinase C Activation to Bcl10-mediated NF-κB Induction

Linda M. McAllister-Lucas, Naohiro Inohara, Peter C. Lucas, Jürgen Ruland, Adalberto Benito, Qiutang Li, Shu Chen, Felicia F. Chen, Shoji Yamaoka, Inder M. Verma, Tak W. Mak and Gabriel Núñez

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