Corrections

**BIOPHYSICS.** For the article “Structure of the coat protein in fd filamentous bacteriophage particles determined by solid-state NMR spectroscopy,” by Ana Carolina Zeri, Michael F. Mesleh, Alexander A. Nevzorov, and Stanley J. Opella, which appeared in issue 11, May 27, 2003, of Proc Natl Acad Sci USA (100:6458–6463; first published May 15, 2003; 10.1073/pnas.1132059100), the authors note that Fig. 4 appeared incorrectly. The corrected figure and its legend appear below.

![Fig. 4](image1)

**CELL BIOLOGY.** For the article “Bcl10 plays a critical role in NF-κB activation induced by G protein-coupled receptors,” by Donghai Wang, Yun You, Pei-Chun Lin, Liqian Xue, Stephan W. Morris, Hu Zeng, Renren Wen, and Xin Lin, which appeared in issue 1, January 2, 2007, of Proc Natl Acad Sci USA (104:145–150; first published December 19, 2006; 10.1073/pnas.0601894104), the authors note that the affiliations for Donghai Wang and Xin Lin appeared incorrectly. The corrected author and affiliation lines, and related footnotes, appear below. The online version has been corrected.


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**EVOLUTION.** For the article “The first fossil leaf insect: 47 million years of specialized cryptic morphology and behavior,” by Sonja Wedmann, Sven Bradler, and Jes Rust, which appeared in issue 2, January 9, 2007, of Proc Natl Acad Sci USA (104:565–569; first published December 29, 2006; 10.1073/pnas.0606937104), due to a printer’s error, in Materials and Methods, line 7, *Clarias brachysoma* should have appeared as *Chitoniscus brachysoma*. The online version has been corrected.

![Fig. 4](image2)
For the article “Tracking germinal center B cells expressing germ-line immunoglobulin γ1 transcripts by conditional gene targeting,” by Stefano Casola, Giorgio Cattoretti, Nathalie Uyttersprot, Sergei B. Koralov, Jane Segal, Zhenyue Hao, Ari Waisman, Angela Egert, Dvora Ghitza, and Klaus Rajewsky, which appeared in issue 19, May 9, 2006, of Proc Natl Acad Sci USA (103:7396–7401; first published May 1, 2006; 10.1073/pnas.0602353103), the authors note that the author name Jane Segal should have appeared as Jane Seagal. The corrected author line appears below. The online version has been corrected.

Stefano Casola, Giorgio Cattoretti, Nathalie Uyttersprot, Sergei B. Koralov, Jane Seagal, Zhenyue Hao, Ari Waisman, Angela Egert, Dvora Ghitza, and Klaus Rajewsky

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For the article “Identification of NVP-TAE684, a potent, selective, and efficacious inhibitor of NPM-ALK,” by Anna V. Galkin, Jonathan S. Melnick, Sungjoon Kim, Tami L. Hood, Nanxin Li, Lintong Li, Gang Xia, Ruo Steensma, Greg Chopik, Jiqing Jiang, Yongqin Wan, Peter Ding, Yi Liu, Fangxian Sun, Peter G. Schultz, Nathanael S. Gray, and Markus Warmuth, which appeared in issue 1, January 2, 2007, of Proc Natl Acad Sci USA (104:270–275; first published December 21, 2006; 10.1073/pnas.0609412103), the authors note that the first two authors, Anna V. Galkin and Jonathan S. Melnick, contributed equally to this work.

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Bcl10 plays a critical role in NF-κB activation induced by G protein-coupled receptors

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G protein-coupled receptors (GPCRs) play pivotal roles in cell proliferation, differentiation, and survival. Although many studies indicate that the stimulation of GPCRs leads to NF-κB activation, the molecular mechanism by which GPCRs induced NF-κB activation remains largely unknown. Bcl10 is an essential adaptor molecule connecting antigen receptor signaling cascades to NF-κB activation in lymphocytes. However, the function of Bcl10 in nonlymphoid cells remains to be determined. In this study, we demonstrated that the deficiency of Bcl10 resulted in the defect in NF-κB activation induced by either expressing the constitutively active mutant of G protein or stimulation of cells with lysophosphatidic acid or endothelin-1, which activate their GPCR. In contrast, TNF-α, LPS-, and integrin-induced NF-κB activation was not affected in Bcl10-deficient cells. Together, our results provide genetic evidence showing that Bcl10 is a key signaling component mediating NF-κB activation induced by GPCRs in nonlymphoid cells.

lyophosphatidic acid | signal transduction | endothelin-1

G protein-coupled receptors (GPCRs) constitute a large family of cell surface receptors and play important roles in regulating cell migration, differentiation, proliferation, and survival (1). GPCRs transduce environmental signals across the plasma membrane through their ability to stimulate guanine nucleotide exchange by heterotrimERIC G proteins (2). Exchange of GDP for GTP results in activation of the Gα subunits and dissociation of the Gβγ subunits. The Gα subunits contain several subgroups, including Gq, Gi, G12, and G13. These G proteins can independently activate their downstream signaling cascades composed of scaffold/adaptor molecules and effector enzymes, leading to activation of various transcription factors, including NF-κB (3).

The NF-κB family of transcription factors plays critical roles in controlling expression of survival factors, cytokines, and proinflammatory molecules (4). Stimulation of various cell surface receptors, including receptors for proinflammatory cytokines, Toll-like receptors, antigen receptors, and GPCRs, induces different signaling pathways, leading to activation of the IκB kinase (IKK) complex. The activated IKK phosphorylates the inhibitory molecules, IκBs, triggering the rapid ubiquitination and proteolysis of IκBs, which unmaps the nuclear localization sequence of NF-κB. NF-κB is then rapidly translocated from the cytoplasm into the nucleus, where it regulates the transcription of its target genes (5). Although many GPCRs, such as the receptors for lysophosphatidic acid (LPA), endothelin-1 (ET-1), and angiotensin-II, are shown to effectively activate NF-κB, the molecular mechanism by which GPCRs activate NF-κB remains largely unknown.

LPA is a potent bioactive lipid that elicits a wide variety of biological actions, particularly as an inducer of cell proliferation, migration, and survival. It regulates tissue remodeling, neurogenesis, myelination, olfactions, immunity, and reproductive functions (6). In addition to its physiological functions, LPA is involved in pathophysiological processes such as wound healing, cancer, and atherosclerosis (7). LPA binds to its cognate GPCRs, LPA1–4, on the cell surface. These GPCRs initiate several signaling pathways through Gq, Gi, and G12/13, leading to the activation of various transcription factors, including NF-κB (8).

It has been shown that NF-κB is the key transcription factor mediating LPA-induced expression of cytokines and prosurvival factors (9). Although the molecular mechanism by which LPA induces NF-κB activation remains to be determined, earlier studies on signaling pathways induced by LPA receptors and other GPCRs suggest that PKC mediates GPCR-induced NF-κB activation (9, 10). However, how PKC activates the IKK complex after the stimulation of GPCRs has yet to be determined.

Recent studies on antigen receptor signaling pathways have demonstrated that a scaffold protein, CARMA1, is required for antigen receptor-induced NF-κB activation and functions downstream of PKC (11–13). After the stimulation of T cell receptor, it induces activation of PKCζ, a novel isoform of PKC family members. The activated PKCζ phosphorylates CARMA1 (14, 15), which, in turn, recruits Bcl10 and MAL13 to form a CARMA1–Bcl10–MAL13 (CBM) complex. The formation of the CBM complex has been shown to activate IKK through an ubiquitination-dependent pathway (16–19), leading to activation of NF-κB. Because the CBM complex functions downstream of PKCs in antigen receptor signaling pathways, we postulated that the CBM complex may also play an important role downstream of PKCs in GPCR-induced signaling pathways. In this study, we have tested this hypothesis and demonstrated that Bcl10 is required for GPCR-induced NF-κB activation after stimulation by LPA and ET-1.

Results

To examine whether Bcl10 is required for NF-κB activation induced by GPCRs, we first expressed the constitutively active mutants of Gq, G12, and Gq12/13, leading to the activation of various transcription factors, including NF-κB (8).

It has been shown that NF-κB is the key transcription factor mediating LPA-induced expression of cytokines and prosurvival factors (9). Although the molecular mechanism by which LPA induces NF-κB activation remains to be determined, earlier studies on signaling pathways induced by LPA receptors and other GPCRs suggest that PKC mediates GPCR-induced NF-κB activation (9, 10). However, how PKC activates the IKK complex after the stimulation of GPCRs has yet to be determined.

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To examine whether Bcl10 is required for NF-κB activation induced by GPCRs, we first expressed the constitutively active mutants of Gq and Gα12, which have been shown to mediate GPCR-induced NF-κB activation (20), in human embryo kidney 293T cells together with or without siRNA for Bcl10 (Fig. 1A). We found that expression of Gq and Gα12 effectively activated NF-κB, whereas the transfection of Bcl10 siRNA reduced the expression of Bcl10 (Fig. 1B) and inhibited Gqα and Gα12-induced NF-κB activation (Fig. 1A). Together, these results

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The authors declare no conflict of interest.

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Abbreviations: GPCR, G protein-coupled receptor; IKK, IκB kinase; LPA, lysophosphatidic acid; ET-1, endothelin-1; MEF, mouse embryonic fibroblast; AP-1, activator protein-1; IRES, internal ribosome entry site; PI3K, phosphatidylinositol 3-kinase; PMA, phorbol-12-myristate-13-acetate; MIP-2, macrophage inflammatory protein-2.

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suggest that Bcl10 may be involved in G protein-mediated NF-κB activation.

To further investigate whether Bcl10 is required for GPCR-induced NF-κB activation after a physiological stimulation, we made mouse embryonic fibroblasts (MEFs) from Bcl10−/− and Bcl10+/− mice. These cells were stimulated, and the nuclear extracts were prepared. LPA-induced NF-κB activation was not defective in Bcl10+/− MEF cells (Fig. 2A). Importantly, the expression of Bcl10 restored LPA-induced NF-κB activation in Bcl10−/− MEF cells (Fig. 2B). These results confirmed that Bcl10 is indeed a critical signaling molecule for LPA-induced NF-κB activation.

Previous studies also suggest that activation of phosphatidylinositol 3-kinase (PI3K) contributes to the activation of NF-κB induced by GPCRs (20). To investigate whether PI3K is involved in LPA-induced NF-κB activation, we pretreated MEF cells with or without a potent PI3K inhibitor, Wortmannin, which inhibits PI3K activation. LPA-induced activation of NF-κB and AP-1 was examined by EMSA. Interestingly, we found that NF-κB activation induced by both LPA and TNF-α was not inhibited with this inhibitor (Fig. 4A) in Bcl10−/− MEF cells, although this inhibitor significantly reduced the basal level of Akt phosphorylation (Fig. 4B). In contrast, LPA-induced NF-κB activation was significantly depleted in Bcl10−/− MEF cells (Fig. 4A).

Together these data indicate that the Bcl10-mediated, but not PI3K-Akt-mediated, pathway is required for LPA-induced NF-κB activation.

To investigate whether the requirement of Bcl10 in LPA-induced signaling pathways is specific for NF-κB activation, we...
determined MAP kinase activation after the stimulation of Bcl10+/+ and Bcl10−/− MEF cells with LPA. The activation of JNK and ERK in these cells was examined by Western blotting analyses using antibodies against phospho-JNK or phospho-ERK. We found that LPA and phorbol-12-myristate-13-acetate (PMA)/ionomycin stimulation induced the phosphorylation of JNK and ERK in both Bcl10+/+ and Bcl10−/− MEF cells (Fig. 4C). Therefore, these results indicate that the deficiency of Bcl10 does not affect the activation of MAP kinases induced by LPA.

To examine whether Bcl10 is required for other GPCR-induced NF-κB activation, we stimulated Bcl10+/+ and Bcl10−/− MEF cells with ET-1, another GPCR ligand, which also induces NF-κB activation. Similar to LPA, ET-1 effectively induced NF-κB activation in Bcl10+/+ MEF cells, but it failed to do so in Bcl10−/− MEF cells (Fig. 5A). Consistent with the requirement of PKC isoforms in GPCR-induced NF-κB activation, although stimulation of Bcl10+/+ MEF cells with PMA plus ionomycin, which activates PKCs, effectively activated NF-κB (Fig. 5A), this stimulation failed to induce NF-κB activation in Bcl10−/− MEF cells (Fig. 5A), suggesting that PKC-induced NF-κB activation depends on Bcl10. Because earlier studies suggest that PKC is also involved in integrin-induced NF-κB activation (23), we hypothesized that integrin-induced NF-κB also depends on Bcl10. Surprisingly, we found that osteopontin and fibronectin, which activate αvβ3 and αvβ1 integrin, respectively, effectively induced NF-κB activation in both Bcl10+/+ and Bcl10−/− MEF cells (Fig. 5B). In addition, we examined whether Toll-like receptor-induced NF-κB depends on Bcl10 and found that LPS induced comparable levels of NF-κB activation in both Bcl10+/+ and Bcl10−/− MEF cells (Fig. 5B). Together, these results indicate that Bcl10 is selectively involved in GPCR-induced NF-κB activation.

IL-8 is the major chemoattractant and activator for neutrophils and is a key component of innate immunity. Recently, it was reported that LPA-induced IL-8 production depends on NF-κB in human bronchial epithelial cells (10). Because Bcl10 mediates LPA-induced NF-κB activation, we examined whether LPA-induced IL-8 production would be defective in Bcl10−/− MEF cells. To test this hypothesis, supernatants from LPA-stimulated Bcl10+/+ and Bcl10−/− MEF cells were analyzed for the production of macrophage inflammatory protein-2 (MIP-2), the
murine homolog of human IL-8. Although LPA stimulation induced a significant increase of MIP-2 production in Bcl10/H11001/H11002 MEF cells, this induction was significantly reduced in Bcl10/H11002/H11002 MEF cells (Fig. 6A). Thus, the defect in LPA-induced NF-κB activation resulted in impaired production of MIP-2 in Bcl10/H11002/H11002 MEF cells after LPA stimulation.

Discussion

GPCRs play pivotal roles in cell proliferation, differentiation, and survival. Although many studies indicate that stimulation of GPCRs leads to NF-κB activation, the molecular mechanism by which GPCRs induce NF-κB activation remains largely unknown. Bcl10 is an adaptor protein that mediates antigen receptor-induced NF-κB in lymphocytes. However, the physiological role of Bcl10 in nonlymphoid cells is completely unknown. In this study, we demonstrate that Bcl10 is required for GPCR-induced NF-κB activation. We have found that NF-κB activation induced by LPA or ET-1 is significantly defective in Bcl10/H11002/H11002 MEF cells (Figs. 2–5). This defect of NF-κB activation is very specific, because Bcl10 deficiency neither affects TNF-α-, LPS-, or integrin-induced NF-κB activation, nor GPCR-induced MAP kinase activation (Figs. 4 and 5). Although we only investigated NF-κB activation induced by two GPCRs, we were able to show that NF-κB activation induced by constitutively active mutants of Gα subunits was defective when endogenous Bcl10 expression was inhibited by Bcl10 siRNA (Fig. 1), suggesting that other GPCRs may also use the Bcl10-dependent pathway to activate NF-κB. Together, our results provide genetic evidence that GPCR-induced NF-κB activation depends on Bcl10.

Because Bcl10 forms a complex with MALT1 to activate the IKK complex through ubiquitination of the IKKα/NEMO subunit of the IKK complex in antigen receptor signaling pathways, it is likely that Bcl10 also regulates IKK activation through
forming a complex with MALTI in GPCR-induced signaling pathways. Consistent with this hypothesis, Bcl10 has been shown to associate with MALTI in nonlymphoid cells (24). When the expression level of MALTI was partially inhibited by expressing MALTI siRNA, we found that NF-κB activation induced by the constitutively active mutant of Goα was also partially defective [supporting information (SI) Fig. 7], suggesting that MALTI is also involved in GPCR-induced NF-κB activation. Nevertheless, additional studies, using cells deficient in MALTI, will be needed to definitely demonstrate whether MALTI is required for GPCR-induced NF-κB activation.

Recent studies indicate that TAK1 is required for NF-κB activation induced by TNF receptor and Toll-like receptor family members (25, 26). Interestingly, it has been shown that TAK1 is inducibly associated with Bcl10 in B cells after the stimulation of BCR. Therefore, it is possible that TAK1 is also involved in GPCR-induced NF-κB activation through forming a complex with Bcl10. Consistent with this hypothesis, we found that suppressing the expression of TAK1 using TAK1 siRNA inhibited Goα-induced NF-κB activation (data not shown), suggesting that TAK1 is required for GPCR-induced NF-κB activation. However, we will need to use TAK1−/− cells to confirm this finding.

Previous studies suggest that PI3K may play a role in GPCR-induced NF-κB activation. However, our data show that inhibition of the PI3K pathway has no effects on LPA-induced nuclear localization of NF-κB (Fig. 4), suggesting that the PI3K pathway is not involved in LPA-induced IKK activation. However, it remains to be determined whether PI3K signaling is involved in the regulation of NF-κB transcriptional activity inside nuclei after LPA stimulation. In addition, it will be important to investigate whether NF-κB activation induced by other GPCRs depends on the PI3K pathway.

Although it remains to be determined how GPCRs regulate Bcl10, previous studies in antigen receptor-induced signaling pathways have provided a potential mechanism by which Bcl10 is regulated by upstream signaling components. In lymphocytes, the stimulation of antigen receptors activates PKCs, which phosphorylate CARMA1. The phosphorylated CARMA1 further recruits Bcl10 into the antigen receptor complexes. The recruitment of Bcl10 activates MALTI and TRAF6, leading to activation of IKK. Previous studies have shown that PKC is also involved in LPA-and other GPCR-induced NF-κB activation. Therefore, it is likely that Bcl10 may also be regulated in a similar manner in the GPCR signaling pathway. Because CARMA1 only expresses in lymphocytes, CARMA3, the CARMA1 homolog in nonlymphoid cells, may function downstream of PKC in GPCR signaling pathways. Therefore, the PKC–CARMA–Bcl10 module may constitute a common axis to transduce signals from PKC to IKK downstream of antigen receptors and GPCRs (Fig. 6B). Consistent with this model, we recently generated CARMA3-knockout mice and found that CARMA3 is required for GPCR-induced NF-κB activation (B. Grabiner and X.L., unpublished work). Together, these results provide genetic evidence that the PKC–CARMA–Bcl10–IKK signaling axis exists in multiple tissues and functions in a similar mechanism.

Materials and Methods

Reagents. L-α-LPA (C18:1cis-9) was from Sigma–Aldrich (St. Louis, MO), PMA was from Calbiochem (San Diego, CA). TNF-α was from Upstate Biotechnology (Lake Placid, NY). Antibodies against IκBα was a kind gift from Shao-Cong Sun (Pennsylvania State University, State College, PA). Antibodies against JNK, Erk, Akt, and their phosphorylated forms were from Cell Signaling (Beverly, MA). Antibodies specific for Bcl10, Goα, and Actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Bcl10 siRNA and relative transfection reagents were obtained from Dharmaco (Lafayette, CO). The target sequences for Bcl10 gene silencing are: GAAAUUUUCUGUCGAAACAUUU (sense sequence) and 5′-PAUGUUUCGACAGAUAUCU (antisense sequence). The plasmids encoding Goα(Q209L) and Gα12(Q229L) were kindly provided by Richard D. Ye (University of Illinois, Chicago, IL).

Cell Culture and Transfection. Mouse Bcl10−/− and Bcl10+/− MEF cells were cultured in DMEM supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 units/ml). Cells were grown in 8% CO2 at 37°C. For all experiments, 1–2 × 106 cells were plated into 10-cm tissue culture plates. After 24–48 h, cells were starved in DMEM with 1% FBS and antibiotics for an additional 12–18 h. LPA (prepared as a stock of 1 mg/ml) in PBS containing 10 mg/ml essentially fatty acid-free BSA (Sigma) and other reagents or vehicles were added to achieve the concentrations specified. Wortmannin treatment was carried out by adding Wortmannin (Biosource, Inc., Camarillo, CA) to a final concentration of 8 μM for 1 h before LPA stimulation.

Human embryo kidney 293T cells were maintained in DMEM supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin (100 units/ml). Cells were grown in 5% CO2 at 37°C and passed every 3 days. Cells (0.1 × 106 per well) were plated in 12-well plates the day before transfection. Bcl10 siRNA transfection was performed according to the manufacturer’s instructions (Dharmacon, Inc.). Forty-eight hours after siRNA transfection, cells were transfected with reporter plasmid encoding 5× NF-κB-luc (60 ng) and pEF-REnilla-luc (10 ng) together with active mutants of Gaα, Goα(Q209L), and Gα12(Q229L), or pCMV5 vector DNA (80 ng per sample) by using the calcium phosphate precipitation method. Six hours later, cells were serum-starved for 16–18 h. Cell lysates prepared for luciferase activities were measured with dual-luciferase assay kits (Promega, Madison, WI). NF-κB activities were determined by normalization of NF-κB-dependent Firefly luciferase to Renilla luciferase activity.

EMSA. Nuclear extracts were prepared from stimulated MEF cells. Nuclear extracts (10 μg) were mixed with [32P]labeled NF-κB, AP-1, or Oct-1 probes (Promega) and incubated at room temperature for 15 min. The mixtures were then subjected to electrophoresis on a 6% polyacrylamide gel and autoradiography.

Integrin-Induced NF-κB Activation. Six-well dishes were coated with recombinant mouse osteopontin (2 μg/ml), fibronectin (10 μg/ml), or poly-L-Lys (5 μg/ml) in PBS overnight at 4°C. The dishes were washed with PBS. A total of 2 × 106 serum-starved MEF cells were plated onto the dishes for 2 h. For TNF-α and LPS stimulation, 10 ng/ml TNF-α or 10 ng/ml LPS was added to poly-L-Lys-plated cells 30 min before harvest. After stimulation, the cells were collected and lysed, and nuclear extracts were prepared. Nuclear extracts were then subjected to EMSA for the detection of NF-κB activation.

Western Blot. After different stimulations, MEF cells were rinsed with PBS and then lysed in ELB buffer (10 mM Hepes, pH 7.4/250 mM NaCl/1 mM EDTA/1% Nonidet P-40) and supplemented with the mixture of protease inhibitors (Roche, Indianapolis, IN). Cell lysates were then subjected to SDS/PAGE, transferred onto nitrocellulose, and probed with individual antibodies.

Retroviral Infection. Cell lines, transfected with a retroviral vector that encodes Bcl10 or the empty retroviral vector, were provided by Demin Wang (The Blood Center of Southeastern Wisconsin, Milwaukee, WI). These virus-producing cells were kept in DMEM supplemented with 10% FCS and antibiotics. After 90% confluence, cultured media were collected every 24 h. For
infections of MEF cells, $1 \times 10^6$ MEF cells were resuspended in freshly collected supernatant from retrovirus-generating cell lines and kept in culture, and culture medium was replaced with freshly collected retroviral supernatant every 24 h. At 48 h, MEF cells were starved in DMEM/1% FCS for 12–18 h, and LPA stimulation was carried out as described above.

**ELISA.** A total of $1 \times 10^6$ MEF cells were plated into 10-cm dishes for 24 h in DMEM supplemented with 10% FCS and antibiotics. The medium was then replaced with DMEM/0.1% FCS and antibiotics for 18 h. LPA was then added to a final concentration of $10^{-6}$ M. The cells were then cultured for another 24 h. Culture supernatants were collected and cleared by centrifugation and stored at $-80^\circ$C for ELISA analysis. The MIP-2 level was determined by using the MIP-2 Quantikine kit from R&D Systems (Minneapolis, MN) according to the manufacturer’s instruction manual.

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