Mol. Cells *30*, 527-532, December 31, 2010
DOI/10.1007/s10059-010-0155-3

Molecules and Cells

Regulation of IκB Kinase by GβL through Recruitment of the Protein Phosphatases

Dong-Joo You[1], You Lim Kim[1], Cho Rong Park[1], Dong-Kyu Kim[1], Jeonghun Yeom[2], Cheolju Lee[2], Curie Ahn[3], Jae Young Seong[1], and Jong-Ik Hwang[1,*]

G protein β-like (GβL) is a member of WD repeat-containing family which are involved in various intracellular signaling events. In our previous report, we demonstrated that GβL regulates TNFα-stimulated NF-κB signaling by interacting with and inhibiting phosphorylation of IκB kinase. However, GβL itself does not seem to regulate IKK directly, because it contains no functional domains except WD domains. Here, using immunoprecipitation and proteomic analyses, we identified protein phosphatase 4 as a new binding partner of GβL. We also found that GβL interacts with PP2A and PP6, other members of the same phosphatase family. By interacting with protein phosphatases, which do not directly bind to IKKβ, GβL mediates the association of phosphatases with IKKβ. Overexpression of protein phosphatases inhibited TNFκ-induced activation of NF-κB signaling, which is an effect similar to that of GβL overexpression. Down-regulation of GβL by small interfering RNA diminished the inhibitory effect of phosphatases, resulting in restoration of NF-κB signaling. Thus, we propose that GβL functions as a negative regulator of NF-κB signaling by recruiting protein phosphatases to the IKK complex.

INTRODUCTION

Nuclear factor kappa B (NF-κB) plays a critical role in a number of cell functions, including immune responses, proliferation, and stress responses, by regulating expression of various genes (Perkins, 2007). Extracellular stimuli, such as microbial and viral infections, proinflammatory cytokines, and some growth factors induce the activation of intracellular signaling molecules that ultimately converge on the IκB kinase (IKK) complex (Hayden and Ghosh, 2004). IκB is phosphorylated by activated IKK at two residues, Ser32 and Ser36, triggering the ubiquitination and subsequent proteasomal degradation of IκB (Karin and Ben-Neriah, 2000). This event results in the release of NF-κB, which translocates to the nucleus and activates transcription of many genes.

The IKK complex, a key regulator of NF-κB signaling, consists of three components: two catalytic subunits, IKKα and IKKβ, and the regulatory subunit, NF-κB essential modulator (NEMO) (Ghosh and Karin, 2002; Li et al., 2000; Yamaoka et al., 1998). Genetic studies have demonstrated that IKKβ is the dominant kinase in the canonical pathway by which proinflammatory cytokines activate NF-κB, whereas IKKα plays an essential role in morphogenic signaling (Pasparakis et al., 2006). Though it lacks catalytic function, NEMO is a key molecule in the activation of the IKK complex. IKKα and IKKβ form homo- and hetero-oligomers through their leucine zipper domains, and a C-terminal helix-loop-helix domain in both proteins mediates recruitment of NEMO to the IKK complex (Ea et al., 2006). Upon stimulation of the canonical pathway, NEMO undergoes Lys63-linked ubiquitination which evokes signaling events by facilitating interactions with proteins that contain ubiquitin-binding domains (Chen et al., 2006). In its ubiquitinated form, NEMO recruits kinases such as transforming growth factor-β-activated kinase-1 (TAK1) to the IKK complex (Burns and Martinon, 2004; Takaesu et al., 2003). Phosphorylation of IKK at serine residues of the N-terminal kinase domain (Ser177 and Ser181 in the case of IKKβ) is a prerequisite for the catalytic activation of IKK and phosphorylation of IκB (Wang et al., 2001).

Recent extensive studies have shown that activated IKK is involved in more than induction of IκB degradation and transcriptional activation of NF-κB. IKKβ phosphorylates and inhibits FOXO3a, which acts as a tumor suppressor by inducing either apoptosis or cell-cycle arrest (Hu et al., 2004). IKKβ also promotes the stability of AU-rich element (ARE)-containing mRNAs, such as cytokines, chemokines, and growth factors by phosphorylating the 14-3-3β protein component of the tristetraprolin/14-3-3β complex and therefore preventing interaction between the complex and AREs (Gringhuis et al., 2005). Insulin resistance and the development of type-2 diabetes caused by pro-inflammatory cytokines such as TNFα and IL-1 might be due to IKKα-dependent phosphorylation of insulin receptor substrate-1 (Gao et al., 2002; He et al., 2006). The increasing number of putative IKK substrates suggests a variety of pathophysiological roles for IKKs. In addition to their substrates, IKK binding partners may exist that regulate enzymatic activity and substrate interactions, providing a means to fine-tune kinase

[1]Graduate School of Medicine, Laboratory of G Protein Coupled Receptors, Korea University, Seoul 136-705, Korea, [2]Life Sciences Division, Korea Institute of Science and Technology, Seoul 136-791, Korea, [3]Transplantation Research Institute, Cancer Research Institute, Seoul National University, Seoul 110-799, Korea
*Correspondence: hjibio@korea.ac.kr

Received June 15, 2010; revised September 13, 2010; accepted September 17, 2010; published online November 23, 2010

Keywords: GβL, IκB kinase, NF-κB, phosphorylation, protein phosphatases

activity and protect cells from the aberrant effects of unconstrained IKK activity.

In a previous report, we demonstrated that G protein β-like (GβL) negatively regulates NF-κB signaling by interacting with IKKβ and blocking its TNFα-stimulated phosphorylation (Kim et al., 2008). GβL consists of six WD-repeat domains that form a circularized β-propeller structure. Many WD-repeat proteins have been identified in eukaryotes and shown to be involved in diverse biological functions, including signal transduction, transcriptional regulation, and apoptosis (Li and Roberts, 2001; Smith et al., 1999). Although GβL has only a simple propeller structure, it might function as an adaptor in intracellular signaling events by tethering various proteins. By analogy to the function of the blades in the G protein β subunit, which contains seven WD domains, each blade in GβL may serve as a protein-binding target. For this reason, GβL is likely to regulate IKK activity by recruiting other functional molecules to IKK or facilitating the interaction between regulatory molecules and IKK.

Here, using immunoprecipitation and subsequent proteomic analyses, we identified protein phosphatase 4 (PP4) as a GβL-interacting protein. Our results further demonstrate that GβL regulates TNFβ-stimulated NF-κB activation through recruitment of phosphatases to IKK.

MATERIALS AND METHODS

Materials

cDNAs of protein phosphatases were purchased from 21C Frontier Human Gene Bank (http://kugi.kribb.re.kr). Nitrocellulose membrane and the enhanced chemiluminascence assay (ECL) kit were from GE Healthcare. Human recombinant TNFα was obtained from R&D systems. All primers used for expression vector construction were purchased from Cosmogentech (Korea). Antibodies including anti-HA antibody and anti-FLAG antibody are products of Sigma. D-luciferin was from Promega. HEK293 cells were purchased from American Type Culture Collection. Cell culture media including DMEM and OptiMEM were obtained from Invitrogen and WELGENE Inc. (Korea). Protease inhibitor cocktail was obtained from Roche. All other chemical reagents were purchased from Sigma.

Immunoprecipitation and silver staining

HEK293 cells maintained in DMEM, supplemented with 10% fetal bovine serum and penicillin/streptomycin, were seed in Poly L-lysine-coated 100 mm dishes at 5×10^6 cells/dish a day before transfection. Using calcium phosphate transfection method, 20 μg of pCMV2-FLAG GβL plasmid were introduced into cells. After 36-48 h, cells were washed with ice-cold PBS and solubilized with 1 ml lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 20 mM NaF, and protease inhibitor cocktail]. The lysates were clarified by centrifugation at 15,000 rpm for 15 min at 4°C and supernatants were incubated with anti-FLAG agarose (Sigma) at 4°C for 2 h. The resin was washed four times with lysis buffer. The bound proteins were eluted by boiling in SDS sample buffer and resolved by SDS-PAGE. Silver staining was performed with the gel as described before (Yan et al., 2000). In brief, gel was fixed with fixing solution containing 45% methanol and 5% acetic acid for 30 min. After 1 h rinsing with distilled water, the gel was sensitized with 0.02% sodium thilsulfate for 1 min. Rinsed gel with distilled water was incubated with 0.1% $AgNO_3$ for 30 min. The signals were developed by incubation with 2% Na_2CO_3 and 0.04% HCOH for about 10 min. After quenching the reaction, the unique protein bands were cut and subjected for mass spectrometric analysis.

In gel digestion

Gel pieces were destained in 100 mM sodium thiosulfate and 30 mM potassium ferricyanide, dehydrated with 100% acetonitrile, and dried at room temperature. Disulfide bonds were reduced by 10 mM dithiothreitol in 25 mM ammonium bicarbonate for 1 h at 56°C. Alkylation was performed via addition of 55 mM iodoacetamide in 25 mM ammonium bicarbonate followed by incubation for 1 h at 25°C in the dark. The gel pieces were washed two times with a 25 mM ammonium bicarbonate, 50% acetonitrile solution, dehydrated with 100% acetonitrile and dried at room temperature. The gel pieces were rehydrated with a solution of sequencing-grade trypsin (12.5 ng/μl; Promega) in 25 mM ammonium bicarbonate and incubated for 16 h at 37°C for protein digestion. Supernatants were transferred to fresh tubes, and the remaining peptides were sequentially extracted by incubating the gel pieces with 50% acetonitrile in 25 mM ammonium bicarbonate, 50% acetonitrile in 0.5% trifluoroacetic acid (TFA) and 70% acetonitrile in 0.5% trifluoroacetic acid (TFA). The extracted peptides were combined and dried in a vacuum evaporator [MIVAC DUO, Genevac (England)].

Mass spectrometric analysis

All nano-HPLC-ESI-MS/MS experiments were performed using the Agilent 1200 nano-flow system (Agilent Technologies, USA) connected to a linear ion trap mass spectrometer (LTQ, Thermo Electron, USA). The reversed phase capillary column was 12 cm in length, 75 μm inner diameter, and in-house packed with 5 μm, 200 Å-pore size Magic C18AQ beads (Michrome BioResources, USA). LC buffers were buffer A (0.1% formic acid in water) and buffer B (0.1% formic acid in acetonitrile). The peptides were eluted in a linear gradient of 10 to 40% acetonitrile over 65 min. The MS survey was scanned from 300 to 2000 m/z, and followed by three data-dependent MS/MS scans with the following options: isolation width, 1.5 m/z; normalized collision energy, 25%; dynamic exclusion duration, 180 s. For all mass spectrometric experiments data was saved in RAW file format (Thermo Scientific, Germany) using the Xcalibur 1.4 with Tune 1.0.

Database searching and validation

The acquired MS/MS spectra were searched using the X1Tendem (open source software, available from http://www.proteome.ca/opensource.html) against the IPI Human database version 3.57. Briefly, precursor mass tolerance was set to -2.0 and + 4.0 Da, fragment ion mass tolerance was set to ± 0.5 Da, cleavage specificity was set to trypsin, allowing for a maximum of two missed cleavages. A fixed modification of carbamidomethylated cysteine (+57.0215 Da) and a variable modification of methionine oxidation (+15.9949 Da) was allowed. Peptide assignment was performed with the Trans Proteomics Pipeline provided by Institute for Systems Biology (TPP, version 4.3, http://www.proteomecenter.org). From The X1Tendem search output, Peptides with probabilities greater than 0.05 were included in the subsequent Protein-Prophet, and proteins having protein probability more than 0.9 were gathered. From each result, the contaminants, e.g. keratin and trypsin, were removed.

Western blotting

HEK293 cells were seeded in 60 mm dishes at 1×10^6 cells/dish 1 day before transfection. The relevant plasmids were introduced into cells with LipofectAMINE 2000 transfection reagent (Invitrogen) following manufacturer's instructions. After immunoprecipitation and SDS-PAGE, the proteins transferred onto a nitrocellulose membrane were probed with the relevant

A

MAEISDLDRQIEQLRRCELIKESEVKALCAKAREILVEESNVQRVDSPVTVCGDIHGQFYDL
KELFRVGGDVPETNYLFMGDFVDRGFYSVETFLLLLALKVRYPDRITLIRGNHESRQITQVY
GFYDECLRKYGSVTVWRYCTEIFDYLSLSAIIDGKIFCVHGGLSPSIQTLDQIRTIDRKQEV
PHDGPMCDLLWSDPEDTTGWGVSPRGAGYLFGSDVVAQFNAANDIDMICRAHQLVMEGYKWH
FNETVLTVWSAPNYCYRCGNVAAILELDEHLQKDFIIFEAAPQETRGIPSKKPVADYFL

B

Swiss prot.	Protein probability	Percent coverage	Unique peptides	Description
P11177	1	15.1	3	PDHB 35 kDa protein
P05388	1	25.5	3	60S acidic ribosomal protein P0
P60510	1	53	9	PPP4C
P02768	1	3.8	2	ALB protein
Q96CX2	0.998	11.1	2	KCTD12

Fig. 1. Identification of the catalytic subunit of PP4 as a GβL-binding partner. After immunoprecipitation of FLAG-GβL, a co-precipitated protein band at 35 kDa in one-dimensional SDS-PAGE was subjected to a proteomic analysis. A tandem mass spectrometry analysis of trypsin-digested protein revealed nine peptide fragments of PP4. (A) Underlined regions indicate peptide fragments detected in mass spectrometry. (B) Mass analysis showed that major protein in the band was catalytic subunit of PP4 among five proteins.

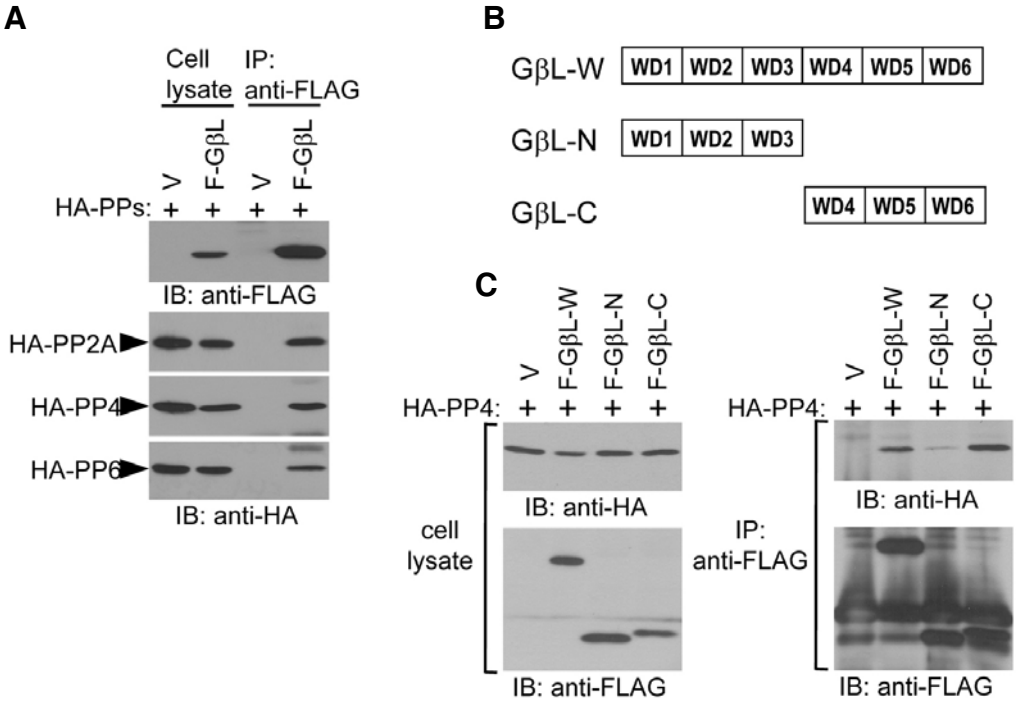

Fig. 2. GβL interacts with protein phosphatases through C-terminal WD domains. (A) Immunoprecipitation of lysates from cells expressing FLAG-GβL and each of the phosphatases showed that GβL interacts with the serine/threonine phosphatase family members PP2A, PP4 and PP6. (B) Schematics of WD domain fragments of GβL. (C) Binding domain-mapping assay. Lysates of HEK293 cells transfected with HA-PP4 and N- or C-terminal domain fragments of FLAG-tagged GβL were co-immunoprecipitated with anti-FLAG antibodies and analyzed by immunoblotting with anti-HA or anti-FLAG antibodies. W, whole; N, N-terminal three WD domains; C, C-terminal three WD domains.

antibodies and then detected using the ECL assay kin. In the experiments with TNFα-treated cells, all cells were cultured in serum-free conditions for 18 h to diminish the serum effects.

Reporter gene assay

To evaluate NF-κB activity, we performed luciferase-based reporter gene assay as described previously (Kim et al., 2008; Lee et al., 2008). In brief, 8×10^4 cells/well HEK293 cells were cultured in 24 well plates. After 15 h, pGL3/NF-κB reporter plasmid (50 ng) and pCMV/β-gal plasmid (20 ng) were transfected into the cells with other relevant plasmids. 48 h later, cells were treated with TNFα (10 ng/ml) for 6 h and harvested with lysis buffer. Luciferase activity of cell extracts was determined using the standard luciferase assay system with a Wallac1420 VICTOR (Perkin-Elmer, USA). Luciferase activities were normalized for transfection efficiency by the β-gal activity. All experiments were repeated at least three times.

RESULTS AND DISCUSSION

The serine/threonine phosphatase PP4 is a GβL-binding protein

GβL has no functional domains other than the unique structure termed a circularized β propeller, which is found in most WD

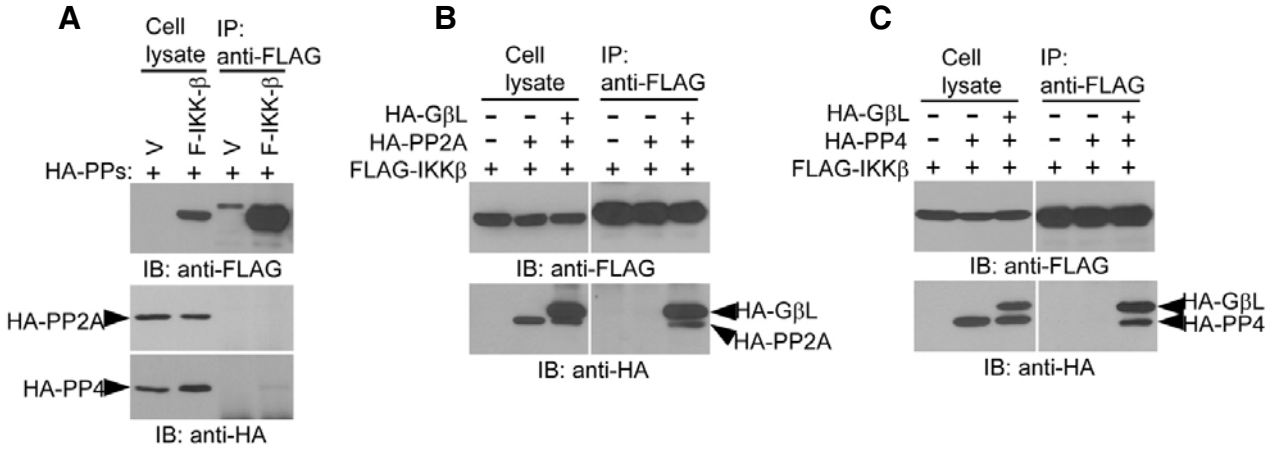

Fig. 3. GβL mediates the association of IKKβ with PP2A or PP4. (A) IKKβ does not directly interact with protein phosphatases. Lysates of HEK293 cells transfected with FLAG-IKKβ and PP2A or PP4 were immunopreciptated with anti-FLAG antibodies and analyzed by immunoblotting with anti-FLAG or anti-HA antibodies. In lane 3, the thin band is nonspecific bands generated from immunoprecipitation, which is also detected in lane 4. (B, C) The protein phosphatases interact with IKKβ in the presence of GβL. Cells expressing IKKβ, protein phosphatases, and GβL were lysed, immunoprecipitated with anti-FLAG antibodies, and analyzed by immunoblotting.

repeat-containing proteins (Kim et al., 2008). Kim et al. (2003) who first shed light on the function of GβL, have demonstrated that GβL regulates nutrient- and growth factor-stimulated signaling by inducing and stabilizing the interaction of raptor with mTOR kinase. The structural and functional properties of GβL imply that it can act as an adaptor by tethering a variety of intracellular molecules to certain complexes to facilitate efficient signaling. To identify GβL-binding molecules, we expressed FLAG-tagged GβL in HEK293 cells and then immunoprecipitated cell lysates with an anti-FLAG antibody followed by proteomic analysis using mass spectrometry. We identified several proteins showing strong binding signals, including mTOR. Interestingly, a protein band at 35 kDa was identified as the catalytic subunit of the serine/threonine phosphatase PP4 based on a mass spectrometry analysis of nine peptide fragments generated by digestion with trypsin (Figs. 1A and 1B). From this result, it is reasonable to speculate that GβL recruits PP4 to its target protein. We previously reported that GβL interacts with IKKβ. Since the catalytic activity of IKKβ is regulated by serine phosphorylation, these results further imply that PP4 likely dephosphorylates, and inactivates, IKKβ by virtue of its association with GβL.

GβL functions as a linker between IKK and protein phosphatases

To confirm the interaction between GβL and PP4, we expressed FLAG-tagged GβL and HA-tagged PP4 in HEK293 cells, and analyzed FLAG-GβL immunoprecipitates by western blotting. As expected, HA-PP4 was detected in FLAG-GβL immunoprecipitates. We also examined whether GβL interacts with PP2A and PP6, which, like PP4, are members of the serine/threonine-specific phosphatase family. As shown in Fig. 2A, both PP2A and PP6 co-immunoprecipitated with GβL, indicating that GβL is capable of associating with this group of protein phosphatases. To further characterize the association of GβL with PP4, we sought to identify the domain of GβL responsible for the interaction. WD domains appear to be binding targets of interacting proteins. In the case of RACK1, a protein kinase C (PKC)-binding protein containing seven WD domains, one or two WD domains are sufficient for interaction with binding partners (McCahill et al., 2002). Because a single WD domain of GβL expressed in HEK293 was not expressed at levels sufficient for detection by Western blotting, we individually expressed FLAG-tagged N-terminal and C-terminal domains of GβL, each of which contains three WD domains. Immunoprecipitation of lysates from cells co-expressing PP4 and GβL N- or C-terminal domain fragments using an anti-FLAG antibody revealed that C-terminal WD domains participate in the interaction with PP4 (Figs. 2B and 2C). The amount of PP4 detected in immunoprecipitates of N-terminal WD domains was negligible compared with the amount of PP4 in C-terminal WD domain immunoprecipitates. Although both PP4 and IKKβ targeted C-terminal WD domains, their binding regions likely do not overlap because each of the three WD domains in the fragment may contribute substantially to binding independent of the others. Fine mapping of the separate binding sites for PP4 and IKKβ await further biochemical studies.

Protein phosphatase regulation of IKK activity has been demonstrated by several groups (Li et al., 2008; Witt et al., 2009). *In vitro* experiments using purified proteins have shown that PP2A inhibits the catalytic activity of IKKs by dephosphorylating serine residues in the kinase domain (DiDonato et al., 1997). The oncoprotein Tax of human T-lymphotropic virus type I constitutively activates IKK by inhibiting NEMO-associated PP2A (Fu et al., 2003). Although PP2A has been identified as a direct binding partner of NEMO, the effect of PP2A on IKK activity is still a matter of debate. Using chemical inhibitors and a NEMO deletion mutant lacking the PP2A binding site, Kray et al. (2005) found that PP2A positively regulates IKK signaling. Our previous studies indicated that GβL did not likely interact with NEMO directly, suggesting that GβL may recruit protein phosphatases to the catalytic subunits of IKK complex. Prior to verifying this hypothesis, we examined direct interactions between IKK and protein phosphatases. As shown in Fig. 3A, neither PP2A nor PP4 was detected in IKKβ immunoprecipitates, excluding the possibility of direct interaction. In contrast, both PP2A and PP4 co-precipitated with IKKβ in the presence of GβL, consolidating the idea that GβL mediates the interaction between protein phophatases and IKK. This result implies that serine/threonine-specific phosphatases including PP2A and PP4 are likely direct modulators of the decreased TNFα-stimulated phosphorylation of IKKβ induced by exogenously

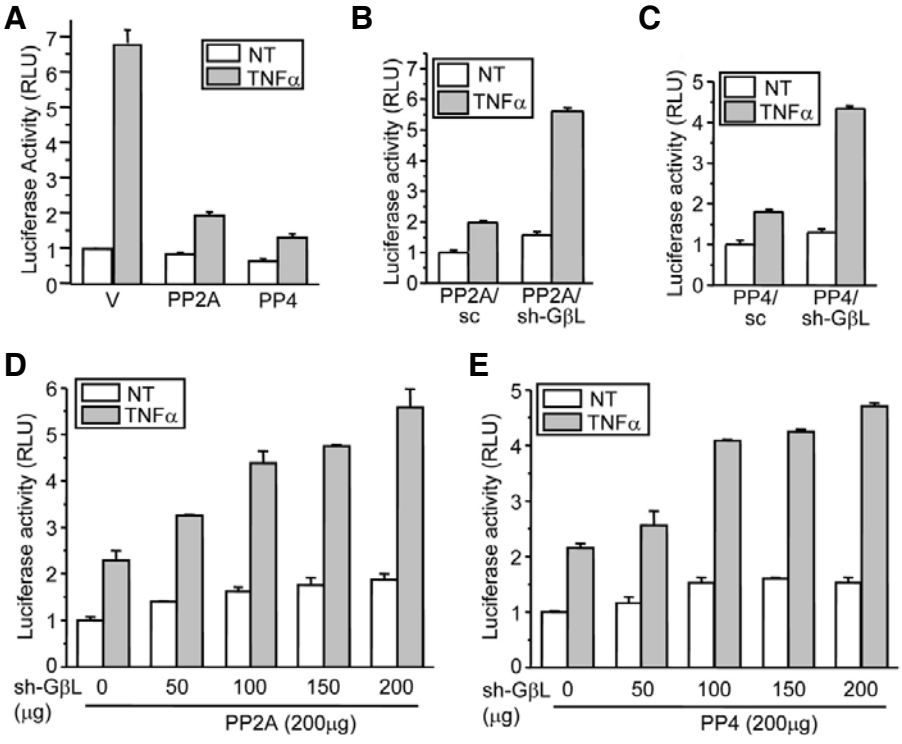

Fig. 4. GβL is responsible for phosphatase inhibition of NF-κB signaling. (A) PP2A and PP4 inhibit TNFα-induced NF-κB activation. HEK293 cells were transfected with an NF-κB-responsive-luciferase reporter plasmid, pCMV/β-gal plasmid, and protein phosphatase expression plasmids. Two days later, cells were treated with TNFα (10 ng/ml) for 6 h, and then luciferase activity was assessed. (B, C) Cells transfected with protein phosphatases and shRNA-GβL were used for luciferase assays. Sc, scrambled RNA; sh, shRNA. (D, E) Cells were transfected with different doses of GβL shRNA and PP2A (D) or PP4 (E), and then used for luciferase assays.

expressed GβL.

The serine/threonine phosphatases PP2A and PP4 negatively regulate TNFα-mediated NF-κB activation through interaction with GβL

TNFα is a proinflammatory cytokine known to stimulate NF-κB signaling. The intracellular signaling cascade that culminates in NF-κB-dependent transcriptional activity comprises multiple cellular events, including subcellular translocation, phosphorylation, and ubiquitination. IKK phosphorylation, in particular, is a pivotal step in the enzymatic activation process that triggers NF-κB release from phosphorylated IκB (Perkins, 2007). To investigate the effect of phosphatases on NF-κB activation, we expressed PP2A or PP4 with an NF-κB responsive promoter-luciferase reporter construct in HEK293 cells. TNFα-stimulated cells were harvested and used for luciferase assays. As shown in Fig. 4A, exogenous expression of either PP2A or PP4 decreased TNFα-stimulated NF-κB activity. Knocking down endogenous GβL expression using a plasmid-based small hairpin inhibitory RNA (shRNA) restored luciferase activity, even in cells expressing exogenous phosphatase (Figs. 4B and 4C). To further confirm the effect of GβL, we co-transfected cells with phosphatase expression plasmids, NF-κB reporter construct, and different amounts of GβL-shRNA. The degree of PP2A- and PP4-mediated down-regulation of luciferase activity decreased with increasing amounts of GβL-shRNA (Figs. 4D and 4E), suggesting that GβL facilitates the association of PP2A and PP4 with IKK, resulting in phosphatase-mediated dephosphorylation of IKK.

Because the WD domains of GβL form six propeller blades, it is reasonable to speculate that GβL serves as an anchor for many intracellular proteins. By verifying the functional role of GβL in promoting the association of protein phosphatases with the IKK complex, the findings of the present study implicate GβL as a negative regulator of NF-κB signaling.

REFERENCES

Burns, K.A., and Martinon, F. (2004). Inflammatory diseases: Is ubiquitinated NEMO at the hub? Curr. Biol. *14*, R1040-R1042.

Chen, F., Bhatia, D., Chang, Q., and Castranova, V. (2006). Finding NEMO by K63-linked polyubiquitin chain. Cell Death Differ. *13*, 1835-1838.

DiDonato, J.A., Hayakawa, M., Rothwarf, D.M., Zandi, E., and Karin, M. (1997). A cytokine-responsive I kappa B kinase that activates the transcription factor NF-kappa B. Nature *388*, 548-554.

Ea, C.K., Deng, L., Xia, Z.P., Pineda, G., and Chen, Z.J.J. (2006). Activation of IKK by TNF alpha requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. Mol. Cell *22*, 245-257.

Fu, D.X., Kuo, Y.L., Liu, B.Y., Jeang, K.T., and Giam, C.Z. (2003). Human T-lymphotropic virus type I Tax activates I-kappa B kinase by inhibiting I-kappa B kinase-associated serine/threonine protein phosphatase 2A. J. Biol. Chem. *278*, 1487-1493.

Gao, Z.G., Hwang, D., Bataille, F., Lefevre, M., York, D., Quon, M., and Ye, J.P. (2002). Serine phosphorylation of insulin receptor substrate 1 by inhibitor kappa B kinase complex. J. Biol. Chem. *277*, 48115-48121.

Ghosh, S., and Karin, M. (2002). Missing pieces in the NF-kappa B puzzle. Cell *109*, S81-S96.

Gringhuis, S.I., Garcia-Vallejo, J.J., Hof, B.V., and van Dijk, W. (2005). Convergent actions of I kappa B kinase p and protein kinase C delta modulate mRNA stability through phosphorylation of 14-3-3 beta complexed with tristetraprolin. Mol. Cell. Biol. *25*, 6454-6463.

Hayden, M.S., and Ghosh, S. (2004). Signaling to NF-kappa B. Genes Dev. *18*, 2195-2224.

He, J.Y., Usui, I., Ishizuka, K., Kanatani, Y., Hiratani, K., Iwata, M., Bukhari, A., Haruta, T., Sasaoka, T., and Kobayashi, M. (2006). Interleukin-1 alpha inhibits insulin signaling with phosphorylating insulin receptor substrate-1 on serine residues in 3T3-L1 adipocytes. Mol. Endocrinol. *20*, 114-124.

Hu, M.C.T., Lee, D.F., Xia, W.Y., Golfman, L.S., Fu, O.Y., Yang, J.Y., Zou, Y.Y., Bao, S.L., Hanada, N., Saso, H., et al. (2004). I kappa B kinase promotes tumorigenesis through inhibition of forkhead FOXO3a. Cell *117*, 225-237.

Karin, M., and Ben-Neriah, Y. (2000). Phosphorylation meets ubiquitination: The control of NF-kappa B activity. Annu. Rev. Immu-

nol. *18*, 621-663.
Kim, D.H., Sarbassov, D.D., Ali, S.M., Latek, R.R., Guntur, K.V.P., Erdjument-Bromage, H., Tempst, P., and Sabatini, D.M. (2003). G beta L, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. Mol. Cell *11*, 895-904.
Kim, Y.L., Kim, J.E., Shin, K.J., Lee, S., Ahn, C., Chung, J., Kim, D.H., Seong, J.Y., and Hwang, J.I. (2008). G beta L regulates TNF alpha-induced NF-kappa B signaling by directly inhibiting the activation of I kappa B kinase. Cell. Signal. *20*, 2127-2133.
Kray, A.E., Carter, R.S., Pennington, K.N., Gomez, R.J., Sanders, L.W., Llanes, J.M., Khan, W.N., Ballard, D.W., and Wadzinski, B.E. (2005). Positive regulation of I kappa B kinase signaling by protein serine/threonine phosphatase 2A. J. Biol. Chem. *280*, 35974-35982.
Lee, S.A., Park, S.H., and Kim, B.C. (2008). Raloxifene, a selective estrogen receptor modulator, inhibits lipopolysaccharide-induced nitric oxide production by inhibiting the phosphatidylinositol 3-kinase/Akt/Nuclear-kappa B pathway in RAW264.7 macrophage cells factor. Mol. Cells *26*, 48-52.
Li, D., and Roberts, R. (2001). WD-repeat proteins: structure characteristics, biological function, and their involvement in human diseases. Cell. Mol. Life Sci. *58*, 2085-2097.
Li, Q.T., Estepa, G., Memet, S., Israel, A., and Verma, I.M. (2000). Complete lack of NF-kappa B activity in IKK1 and IKK2 double-deficient mice: additional defect in neurulation. Genes Dev. *14*, 1729-1733.
Li, H.Y., Liu, H., Wang, C.H., Zhang, J.Y., Man, J.H., Gao, Y.F., Zhang, P.J., Li, W.H., Zhao, J., Pan, X., et al. (2008). Deactivation of the kinase IKK by CUEDC2 through recruitment of the phosphatase PP1. Nat. Immunol. *9*, 533-541.
McCahill, A., Warwicker, J., Bolger, G.B., Houslay, M.D., and Yarwood, S.J. (2002). The RACK1 scaffold protein: A dynamic cog in cell response mechanisms. Mol. Pharmacol. *62*, 1261-1273.
Pasparakis, M., Luedde, T., and Schmidt-Supprian, M. (2006). Dissection of the NF-kappa B signalling cascade in transgenic and knockout mice. Cell Death Differ. *13*, 861-872.
Perkins, N.D. (2007). Integrating cell-signalling pathways with NF-kappa B and IKK function. Nat. Rev. Mol. Cell Biol. *8*, 49-62.
Smith, T.F., Gaitatzes, C., Saxena, K., and Neer, E.J. (1999). The WD repeat: a common architecture for diverse functions. Trends Biochem. Sci. *24*, 181-185.
Takaesu, G., Surabhi, R.M., Park, K.J., Ninomiya-Tsuji, J., Matsumoto, K., and Gaynor, R.B. (2003). TAK1 is critical for I kappa B kinase-mediated activation of the NF-kappa B pathway. J. Mol. Biol. *326*, 105-115.
Wang, C., Deng, L., Hong, M., Akkaraju, G.R., Inoue, J., and Chen, Z.J.J. (2001). TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature *412*, 346-351.
Witt, J., Barisic, S., Schumann, E., Allgower, F., Sawodny, O., Sauter, T., and Kulms, D. (2009). Mechanism of PP2A-mediated IKK beta dephosphorylation: a systems biological approach. BMC Syst. Biol. *3*.
Yamaoka, S., Courtois, G., Bessia, C., Whiteside, S.T., Weil, R., Agou, F., Kirk, H.E., Kay, R.J., and Israel, A. (1998). Complementation cloning of NEMO, a component of the I kappa B kinase complex essential for NF-kappa B activation. Cell *93*, 1231-1240.
Yan, J.X., Wait, R., Berkelman, T., Harry, R.A., Westbrook, J.A., Wheeler, C.H., and Dunn, M.J. (2000). A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization-mass spectrometry. Electrophoresis *21*, 3666-3672.