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GβL regulates TNFα-induced NF-κB signaling by directly inhibiting the activation of IκB kinase

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ABSTRACT

The transcriptional activation of NF-κB, a critical player in both physiological and pathological cellular responses to diverse cytokines, is dependent on IKK activation. Although molecular mechanisms underlying IKK activation have been well elucidated, the processes that negatively regulate IKK activity are still largely unknown. Using yeast two-hybrid screening, we have identified GβL as an interacting partner of IKKβ. In this study, we demonstrate that GβL interacts with IKKα and IKKβ in vitro and in vivo. The C-terminal WD domains of GβL are required for the interaction with both the kinase domain and leucine zipper domain of IKKβ. Overexpression of GβL inhibits TNFα-induced activation of NF-κB signaling, while down-regulation of GβL expression by small interfering RNA enhances NF-κB activity. GβL constitutively interacts with IKKβ, and this interaction is enhanced by TNFα treatment. GβL also inhibits TNFα-induced phosphorylation of IKKs. Taken together, these data suggest that GβL is involved in the negative regulation of TNFα-stimulated NF-κB signaling through a direct interaction with IKK.

1. Introduction

Nuclear factor kappa B (NF-κB) is activated in response to various stimuli, including microbial and viral infections, proinflammatory cytokines, and some growth factors [1,2] As a transcription factor, NF-κB regulates the expression of numerous genes controlling programmed cell death, immune responses, inflammation, proliferation, cancer, and tissue remodeling [3,4]. There are five members of the NF-κB family expressed in mammals, which interact to form homo- or heterodimers [5]. In quiescent cells, NF-κB complexes are retained in the cytosol through binding to the inhibitory protein, IκB. Upon stimulation of cytokines and other reagents, IκB is phosphorylated by the IκB kinase (IKK) complex, which results in ubiquitination and proteasome-mediated degradation of IκB. Relieved of IκB inhibition, NF-κB then translocates to the nucleus [1,2].

The cytoplasmic signaling pathways that lead to NF-κB activation in response to various stimuli converge on a complex of two protein kinases with high sequence similarity, IKKα and IKKβ, and a regulatory subunit, NEMO (NF-κB essential modulator, also called as IKKγ) [6,7]. NEMO is a key molecule in the activation of the IKK complex in the canonical pathway of NF-κB activation [8,9].

To date, several IKK activators have been identified through extensive investigations into signaling networks. RIP is recruited to the TNF receptor I, which interacts with TNFα and is polyubiquitinated in a Lys63-dependent manner [9,10]. TAB2 binds to and recruits TAK1 (transforming growth factor-β-activated kinase 1) to polyubiquitinated RIP [11]. Ubiquitination of NEMO on Lys63 allows RIP-TAB2-TAK1 to phosphorylate and activate IKKα (Ser176, Ser180) and IKKβ (Ser177 and Ser181) [12,13]. In alternative NF-κB activation pathways, NIK (NF-κB inducing kinase), which itself is regulated by TRAF3, phosphorylates IKKα and stabilizes the interaction of IKKα with p100 (the precursor of NF-κB member p52), which is likely to lead to p100 processing and the activation of p52/RelB [14,15]. Atypical isoforms of protein kinase C (ζ and λ/ι), which are activated by TNFα and interleukin 1, also play critical roles during NF-κB activation by phosphorylating IKKβ at Ser177 and Ser181 of the catalytic domain [16]. Trans-autophosphorylation and conformational changes through multimerization are also pivotal steps in IKK activation [17,18].

In addition to the phosphorylation of IκB proteins, activated IKK phosphorylates several other proteins, including NF-κB subunits, which might result in stimulation of anti-apoptotic, proinflammatory, proliferative, and tumor-promoting pathways [2]. The transcription factor FOXO3a, which plays a role in the induction of apoptosis or cell-

Abbreviations: IKK, IκB kinase; NF-κB, Nuclear factor kappa B; TNF, Tumor necrosis factor; NEMO, NF-κB essential modulator; RIP, Receptor-interacting protein; TAK1, TGF-β-activated kinase 1; TAB2, TAK1-binding protein 2; TRAF3, TNF receptor-associated factor 3; NIK, NF-κB inducing kinase; ARE, AU-rich element; shRNA, Small hairpin RNA; GST, Glutathione *S*-transferase; HA, Hemagglutinin.

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cycle arrest, is phosphorylated and inactivated by IKKβ [19]. IKKβ phosphorylation of 14-3-3β inhibits the function of the tritetraprolin (TPP)/14-3-3β complex, which regulates the stability of mRNA by binding AU-rich element (ARE), possibly stabilizing cytokine and growth factor mRNAs that contain ARE sequences [20]. Insulin receptor substrate 1 (IRS1) is also phosphorylated by IKKβ, which is likely to be a pivotal event in TNFα-mediated inhibition of insulin signaling [21,22]. Although the number of known targets has increased, it is still unclear how particular cellular contexts dictate IKK's interaction with specific substrates. The signalosome complex, which includes IKK, might also contain unknown IKK binding partners that regulate its enzymatic activity and mediate IKK–substrate interactions. Recently, it has been reported that CUE domain-containing protein 2 (CUEDC2), which contains a ubiquitin-binding domain, interacts with IKK and acts as an adaptor protein to target IKK for dephosphorylation [23].

In this study, we present the identification of novel IKK interacting proteins uncovered in a yeast two-hybrid screening. GβL, one of the novel IKK binding partners, directly interacts with both the kinase domain and leucine zipper domain of IKKβ through three C-terminal WD domains. GβL negatively regulates TNFα-induced NF-κB activation by inhibiting IKK phosphorylation and IκB degradation, dependent on TNFα stimulation. These results demonstrate a role for GβL as a negative regulator in the NF-κB pathways that respond to inflammatory stimulation, through the regulation of IKKβ activity.

2. Materials and methods

2.1. Materials

Nitrocellulose membrane and the enhanced chemiluminascence assay (ECL) kit were purchased from Amersham Pharmacia biotech. Human recombinant TNFα was purchased from R&D systems. All primers used for expression vector construction were purchased from Cosmogentech (Seoul, Korea). Anti-IKKβ antibody, anti-phosphoIKKα/β (Ser180/ser181) antibody, anti-GβL, and anti-tubulin antibody were purchased from Cell Signaling (Beverly, MA). Protease inhibitor cocktail was purchased from Roche. All other chemical reagents, including anti-HA antibody, anti-FLAG antibody, and anti-FLAG M2 agarose affinity gel were products of Sigma.

2.2. Yeast two-hybrid screening

Yeast two-hybrid screening was performed in the L40 yeast strain with His3 and β-galactosidase reporter systems, as described previously [24,25]. Mouse IKKβ DNA fragments encoding the region covering the RelB-transactivation domain containing a putative leucine zipper motif (aa301–540) or helix-loop-helix (HLH) and NEMO-binding domain (NBD) (aa541–757) were inserted into pBHA (LexA fusion vector) and used as baits to screen a human lymphocyte cDNA library constructed in the pACT plasmid (Clontech, Palo Alto, CA). A total of 2×10^6 clones were screened for interacting proteins. Positive clones were verified by His growth and β-galactosidase assay. Plasmid DNAs from positive colonies were isolated and sequenced. The interacting proteins were identified by data bank search using the National Center for Biotechnology Information (NCBI) BLAST with sequencing results.

2.3. Plasmid constructs

In order to express GST-fusion proteins in *E. coli*, the gene fragments of IKKβ (aa1–300, aa301–540, and aa541–756) and GβL (aa1–326, aa1–158, and aa159–326) were amplified by PCR from the original cDNA clones and inserted into EcoRI and XhoI sites of pGEX4T-1 plasmid (Amersham Pharmacia Biotech) or pCDNA3HA (HA-inserted form of pCDNA3, Invitrogen). Wild type and site-directed mutants in conserved region of WD repeats of GβL were previously constructed in pRK5-HA [26]. All IKKβ constructs (full length, aa1–300, aa301–757, aa1–540, and aa301–540-deleted forms), IKKα, NEMO, and IκB were inserted into EcoRI and SalI sites of pCMV2-FLAG plasmids.

2.4. Immunoprecipitation and western blotting

HEK293 cells maintained in DMEM, supplemented with 10% fetal bovine serum and penicillin/streptomycin, were plated in 60 mm dishes at 1×10^6 cells/dish 1 day before transfection. The relevant plasmids were transfected with LipofectAMINE 2000 transfection reagent (Invitrogen) following the manufacturer's instructions. After 36–48 h, cells were washed with ice-cold PBS and solublized with 1 ml lysis buffer (50 mM Tri–HCl (pH7.5), 150 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail). The lysates were clarified by centrifugation at 15,000 rpm fro 15 min at 4 °C and supernatants were incubated with anti-FLAG antibody-conjugated beads (Sigma) at 4 °C for 2 h. The beads were washed four times with lysis buffer, and the bound proteins were eluted by boiling in SDS sample buffer and resolved by SDS-PAGE. The proteins transferred onto a nitrocellulose membrane were probed with the relevant antibodies and then detected using the ECL assay kit. In the experiments with TNFα-treated cells, all cells were cultured in serum-free conditions for 18 h to diminish the serum effects.

2.5. Interaction between endogenous IKKβ and GβL

HEK293 cells grown on 100 mm dish were extracted in lysis buffer and then clarified by centrifugation at 15,000 rpm for 15 min at 4 °C. The supernatants were incubated with anti-IKKβ antibodies (Cell Signaling) and protein G-agarose beads at 4 °C for 4 h. The beads were washed four times with lysis buffer. The bound proteins were eluted by boiling in SDS sample buffer, resolved on SDS-PAGE gel, and then subjected to immunoblot analysis with anti-GβL antibodies.

2.6. In vitro binding assay

pGEX4T-1 plasmids containing the gene for each fragments of IKKβ and GβL were transformed into *E. coli* BL21 strain, and expression of the GST-fusion proteins was induced by 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 25 °C for 4 h. HEK293 cells transfected with HA-GβL gene or FLAG-IKKβ gene were harvested with lysis buffer. Cell lysates were clarified by centrifugation at 15,000 rpm for 15 min at 4 °C and supernatants were incubated with GST-fusion proteins purified with glutathione sepharose 4B for 3 h at 4 °C. The beads were washed four times with lysis buffer, and the bound proteins were analyzed by SDS-PAGE and western blotting with appropriate antibodies.

2.7. Reporter gene assay

8×10^4 cells/well HEK293 cells were cultured in 24 well plates. After 15 h, pGL3/NF-κB reporter plasmid (50 ng), pCMV/β-gal plasmid (20 ng), and other relevant plasmids (400 ng) were transfected into the cells. Approximately 48 h later, cells were treated with TNFα (10 ng/ml) (R&D systems) 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, Wellesely, MA). Luciferase activities were normalized for transfection efficiency by the β-gal activity. All experiments were repeated at least three times.

2.8. RNA interference

shRNA target sequence for human GβL was 5′-CAGTTGCTTATCCA-GATGTGAC-3′ in the 3′ untranslated region. Synthesized shRNA

cassette was inserted between the BamHI and XhoI sites immediately downstream of the H1 promoter in pBluescript II SK plasmid (Stratagene).

3. Results

3.1. Identification of GβL as an IKKβ interacting protein

To identify novel binding partners in IKK signaling networks, we employed the yeast two-hybrid system to screen a human lymphocyte cDNA library with domains of the IKKβ protein. When we screened for two-hybrid interactions with the RelB-transactivation domain containing a putative leucine zipper motif (aa301–540), we identified 30 positive clones from a total of 2×10^6 transformants. Sequence analysis showed that three of these clones encoded the fragment of human GβL (aa180–326) that contains part of the fourth WD domain and two C-terminal WD domains. In addition, several novel clones were identified in the two-hybrid screening. To confirm the interaction between IKKβ and GβL, HA-tagged GβL and FLAG-tagged IKKβ were transiently co-transfected into HEK293 cells. In western blot analysis, HA-GβL was detected in FLAG-IKKβ precipitates (Fig. 1A). Then, to determine the specific interaction of endogenous GβL and IKKβ, an immunoprecipitation assay with anti-IKKβ antibodies was performed. Both proteins were expressed in HEK293 cells, and GβL was co-precipitated with IKKβ (Fig. 1B), suggesting that the GβL–IKKβ interaction is physiologically relevant.

3.2. Mapping of domains responsible for the GβL–IKKβ interaction

To further understand the association between GβL and IKKβ, we endeavored to determine the interaction domains of each binding partner. First, we constructed expression plasmids for several GST-fusion fragments of IKKβ, as shown in Fig. 2A. In in vitro pull-down assays using GSH agarose, GβL was precipitated only with the leucine zipper fragment of IKKβ, consistent with the results of the yeast two-hybrid assay (Fig. 2B). However, in the immunoprecipitation assays performed with mammalian cells expressing GβL and individual fragments of IKKβ, GβL interacted with the kinase domain fragment and leucine zipper fragment of IKKβ, but not with the fragment including the helix-loop-helix and the NEMO-binding domain (Fig. 2C). The apparent discrepancy of these results might be due to the presence of additional members of the protein complex in mammalian cells or to conformational differences in IKKβ expressed in mammalian cells and prokaryotic cells, such as post-translational modification of the kinase domain fragment.

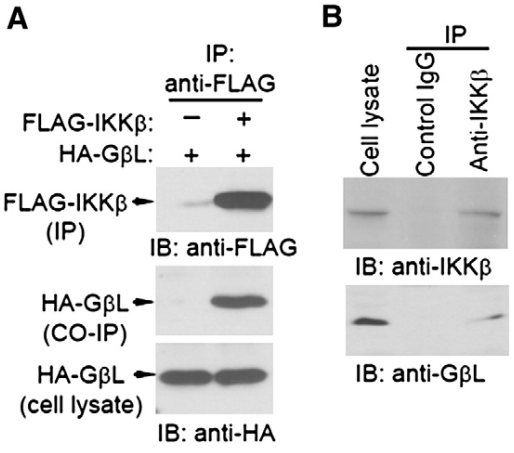

Fig. 1. GβL interacts with IKKβ (A) Lysates of HEK293 cells co-transfected with HA-GβL and FLAG-IKKβ were immunoprecipitated with anti-FLAG antibodies and analyzed by western blotting with anti-FLAG and anti-HA antibodies. GβL was precipitated in the presence of FLAG-IKKβ. (B) Lysates of HEK293 cells were immunoprecipitated with anti-IKKβ antibodies and analyzed by western blotting with anti-IKKβ and anti-GβL antibodies. GβL was precipitated in the presence of anti-IKKβ antibodies, but not in the presence of control IgG.

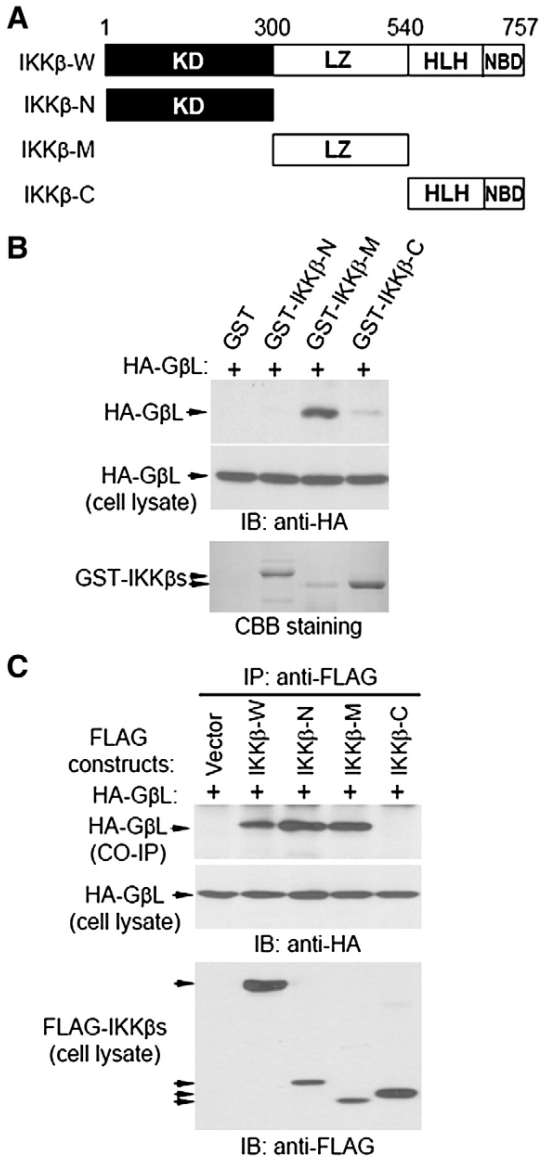

Fig. 2. IKKβ interacts with GβL through its kinase domain and leucine zipper domain. (A) Fragment forms of IKKβ used in domain-mapping experiments. KD, kinase domain; LZ, leucine zipper; HLH, helix-loop-helix; NBD, NEMO-binding domain. (B) In vitro binding assay. Lysates of HEK293 cells expressing HA-GβL were precipitated with GST-tagged forms of IKKβ fragments purified with GSH agarose and analyzed by immunoblotting with anti-HA antibodies. (C) Co-immunoprecipitation assay. Lysates of HEK293 cells transfected with HA-GβL and each fragment of IKKβ (FLAG-tagging form) were immunoprecipitated with anti-FLAG antibodies, and analyzed by immunoblotting with anti-HA or anti-FLAG antibodies.

GβL, which is a member of the WD repeat protein family, contains six WD domains and no other known functional domains [27]. In case of Rack1, which is a PKC-interacting protein containing seven WD domains, one or two WD domains are sufficient for interactions with binding partners [28]. To examine whether GβL interacts with IKKβ by a similar mechanism, we constructed separate WD domain fragments from GβL. Because the expression levels of single WD domain fragments in HEK293 cells were too low to be detected in immunoblotting assays, we roughly divided GβL into the three N-terminal WD domains and the three C-terminal WD domains (Fig. 3A), then

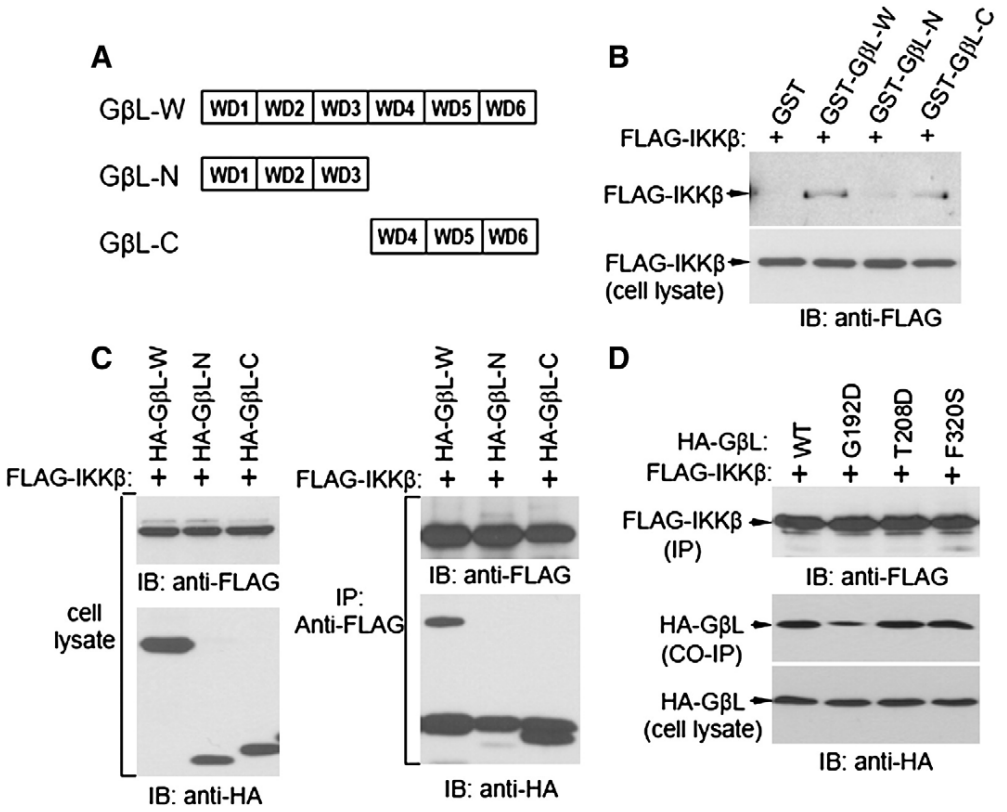

Fig. 3. The three C-terminal WD domains of GβL participate in the interaction with IKKβ. (A) Schematics of WD domain fragments of GβL. (B) In vitro binding assays: Lysates of HEK293 cells expressing FLAG-IKKβ were precipitated with GST-tagged forms of GβL fragments purified with GSH agarose and analyzed by immunoblotting with anti-FLAG antibodies. (C) Lysates of HEK293 cells transfected with FLAG-IKKβ and each fragment of GβL (HA-tagged form) were used for immunoprecipitation with anti-FLAG antibodies and analyzed by immunoblotting with anti-FLAG or anti-HA antibodies. (D) G192 of GβL is required for the interaction with IKKβ. Lysates of HEK293 cells transfected with FLAG-IKKβ and each single point mutant of GβL (HA-tagged form) were immunoprecipitated with anti-FLAG antibodies and analyzed by immunoblotting.

expressed each as HA- or GST-fusion proteins. In vitro pull-down assays using GSH agarose and immunoprecipitation assays with anti-FLAG antibody revealed that the C-terminal WD domains participated in an interaction with IKKβ, consistent with the result from the yeast two-hybrid assay (Fig. 3B, C). There are several conserved residues in the WD domains which might confer a functional interaction with other molecules [26]. We generated GβL mutants in which we substituted these residues with other amino acids and examined the affinity of each mutant protein for IKKβ by immunoprecipitation analysis. As shown in Fig. 3D, interaction of GβL mutants G192D with IKKβ was substantially decreased, indicating that this residue is likely to be structurally important for IKKβ interaction.

3.3. GβL specifically interacts with IKKα and IKKβ in the IKK signaling complex

There are three main components of the IKK complex, as described previously. IKKα and IKKβ are very similar in structure, showing 70% amino acid homology, and containing N-terminal kinase domains, leucine zipper, helix-loop-helix, and NEMO-binding domains. NEMO, which is considered to be a regulatory protein, contains two coiled-coils, a leucine zipper, and a zinc-finger domain in its 419 amino acid sequence [2]. To determine the specificity of the interaction of GβL and components of the IKK complex, we performed immunoprecipitation assays with HEK293 cells expressing GβL and each of the IKK proteins. GβL interacted strongly with IKKα and IKKβ, but very weakly with NEMO, although NEMO expression levels were higher than the other IKK proteins (Fig. 4). This suggests that IKKα and IKKβ, but not NEMO, are likely to be direct binding partners of GβL.

3.4. GβL negatively regulates TNFα-stimulated activation of NF-κB signaling pathways

WD repeat-containing proteins have been implicated as adaptors or regulators for a variety of functions, including signal transduction, transcription regulation, and apoptosis [29]. GβL has also been reported

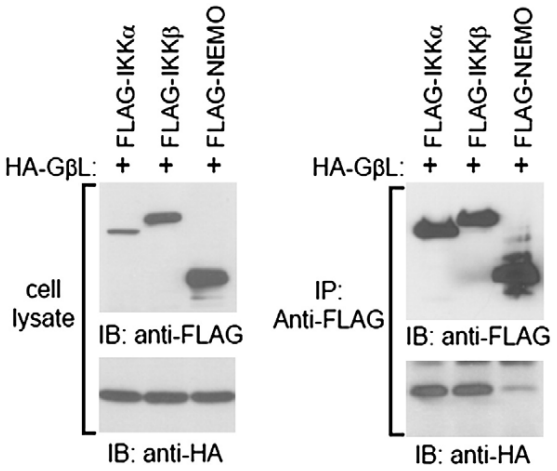

Fig. 4. GβL interacts with the IKK complex. Lysates of HEK293 cells transfected with HA-GβL and each FLAG-tagged form of IKKα, IKKβ, and NEMO (IKKγ) were immunoprecipitated with anti-FLAG antibodies and analyzed by western blotting with anti-FLAG and anti-HA antibodies. Substantial amounts of GβL were precipitated in the presence of IKKα and IKKβ, but GβL was barely detected in the NEMO precipitates.

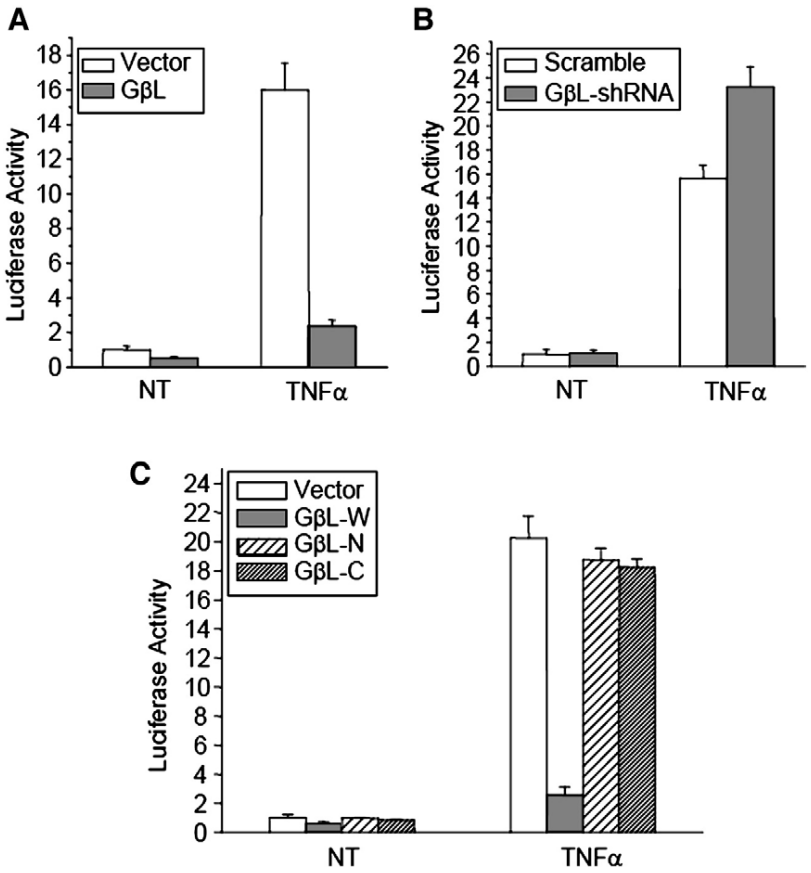

Fig. 5. GβL negatively regulates NF-κB activation. (A) GβL inhibits TNFα-induced NF-κB activation. HEK293 cells (8×10^4) were transfected with a NF-κB luciferase reporter plasmid (50 ng), pCMV/β-gal plasmid (20 ng), and an expression plasmid for HA-GβL (400 ng, grey bars) or an empty plasmid (400 ng, white bars). Two days later, cells were treated with TNFα (10 ng/ml) or left untreated for 6 h, and then processed for reporter assays. (B) NF-κB activity was enhanced in the GβL-knockdown cells. HEK293 cells were transfected with reporter plasmid and GβL–shRNA plasmid (grey bars) or scrambled plasmid (white bars). At 48 h after transfection, cells were treated with TNFα for 6 h. Reporter assays were performed the same as in A. (C) Expression of GβL fragments does not affect TNFα-induced NF-κB activation. The same experiments were performed with cells transfected with reporter plasmid and the plasmid containing full length GβL, N-terminal GβL, or C-terminal GβL. The data are representative of three independent experiments performed in triplicate. Mean±SD is shown.

to be a regulator of the rapamycin-sensitive pathways through an interaction with mTOR [26]. To investigate the physiological function of the GβL–IKKβ interaction in the NF-κB signaling pathway, we introduced the GβL gene along with a NF-κB reporter gene into HEK293 cells. GβL overexpression slightly lowered basal NF-κB activity in untreated cells and, moreover, GβL dramatically suppressed TNFα-induced NF-κB transcriptional activity (Fig. 5A). To determine whether endogenous GβL modulates NF-κB activation, we knocked down GβL expression using a plasmid-based shRNA, as described in Materials and methods. TNFα-induced luciferase activity was potently enhanced in GβL-knockdown cells, compared with the control (Fig. 5B). These data demonstrate that GβL is likely to be a negative regulator of TNFα-induced NF-κB activation pathways. According to the results of co-immunoprecipitation experiments, the three C-terminal WD domains of GβL participate in an interaction with IKKβ (Fig. 3D), implying that this fragment would somehow influence NF-κB activation. However, TNFα-stimulated NF-κB activation was not affected by expression of either the N-terminal or C-terminal fragment of GβL (Fig. 5C). From this result, it is speculated that GβL is not likely to regulate IKK-mediated NF-κB pathways through a direct interaction, but rather by recruiting other regulators to the IKK complex.

3.5. GβL blocks IκB degradation by inhibiting phosphorylation-dependent activation of IKKs

Phosphorylation and proteasome-mediated degradation of IκB by IKKβ are a prerequisite for TNFα-stimulated NF-κB activation. To determine the effect of GβL on TNFα-dependant IκB degradation, cells expressing HA-GβL and FLAG-IκB were stimulated with TNFα. The amount of IκB in the cell lysates was decreased by TNFα treatment, whereas it was not changed in the presence of exogenous GβL, implying that GβL is likely to block TNFα-induced IκB degradation (Fig. 6A). This was later confirmed in GβL-knockdown cells. We stimulated the cells expressing FLAG-IκB and GβL shRNA with TNFα and measured the expression level of FLAG-IκB by western blotting. Basal levels of FLAG-IκB in GβL shRNA expressing cells were lower than that in control shRNA containing cells. Furthermore, upon TNFα treatment, FLAG-IκB levels were considerably decreased with GβL-knockdown compared to the shRNA control cells (Fig. 6B).

To examine the possibility that the stability of IκB is regulated by a direct interaction of GβL and IκB, we attempted immunoprecipitation from cells expressing exogenous HA-GβL and FLAG-IκB. However, HA-GβL was not detected in FLAG immune complexes, suggesting that there was no direct interaction between GβL and IκB (Fig. 6C). Taking previous results into consideration, we then speculated that the interaction of GβL and IKK was likely to affect phosphorylation and degradation of IκB by IKK.

Activation of IKK is dependent on phosphorylation on Ser181 of IKK-β and Ser180 of IKKα [12,13]. To investigate the effect of GβL on IKK phosphorylation, we used cells expressing exogenous GβL. Phosphorylation of IKKs was substantially increased in response to TNFα treatment in HEK293 cells transfected with a control vector; in contrast, phosphorylation of IKKs was barely detected in the cells transfected with the HA-GβL expression plasmid (Fig. 6D). We next

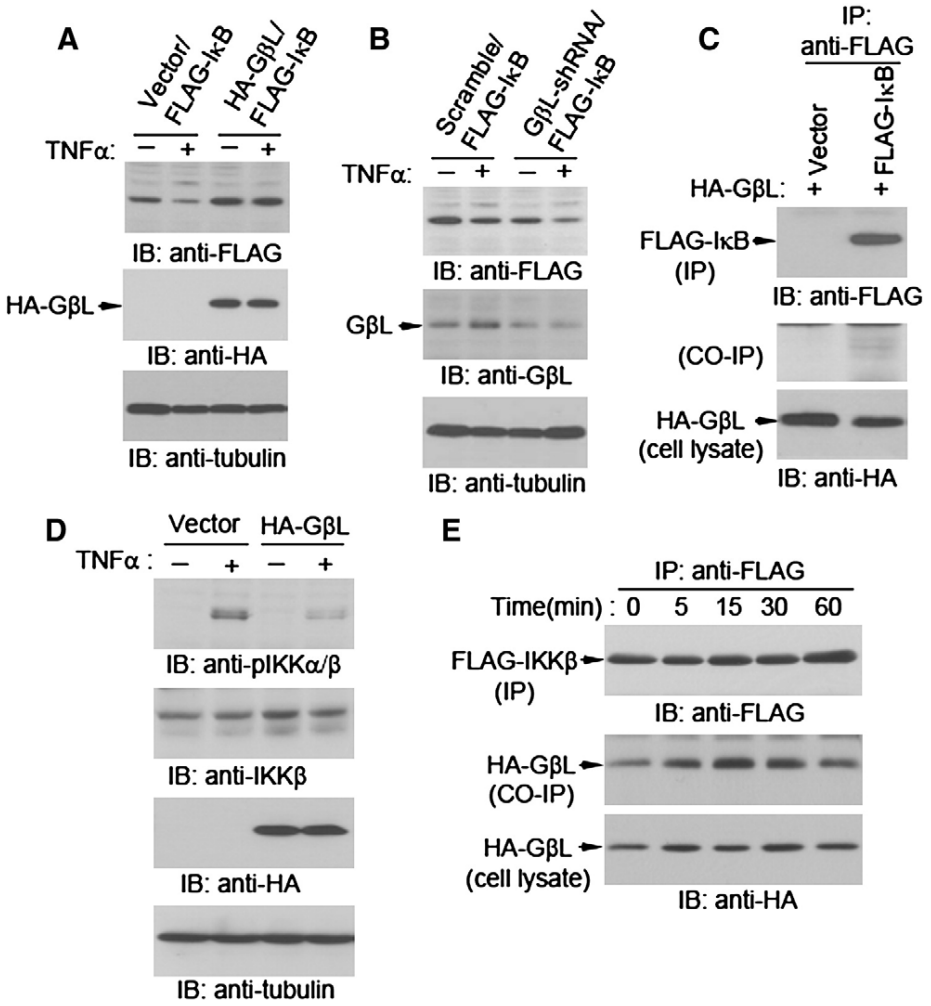

Fig. 6. GβL blocks IκB degradation by inhibiting phosphorylation-dependent activation of IKKs. (A–B) HEK293 cells were transfected with the expression plasmids for HA-GβL and FLAG-IκB (A), or GβL–shRNA plasmid and expression plasmid for FLAG-IκB (B). After 48 h, cells were treated with TNFα (10 ng/ml) for 30 min and harvested with lysis buffer. Lysate (30 μg) was loaded in each well and used for western blotting with appropriate antibodies. (C) IκB does not interact with GβL. Lysates of HEK293 cells transfected with the expression plasmids of HA-GβL and FALG-IκB were used for immunoprecipitation using anti-FLAG antibodies. Precipitates were analyzed by western blotting with anti-HA or anti-FLAG antibodies. (D) GβL inhibits IKK phosphorylation. HEK293 cells transfected with HA-GβL plasmid were cultured for 18 h in serum-free condition and treated with TNFα for 15 min. The cell lysates (20 μg/lane) were used for western blotting with anti-pIKKα/β antibodies. (E) Interaction between IKKβ and GbL is enhanced by TNFα. HEK293 cells transfected with HA-GβL and FLAG-IKKβ plasmids were cultured for 18h in serum-free condition and treated with TNFα. Cell lysates were used for immunoprecipitation using anti-FLAG antibodies. Precipitates were analyzed by western blotting with appropriate antibodies.

performed immunoprecipitation assay from cells expressing FLAG-IKKβ and HA-GβL to test whether there is a dynamic interaction between GβL and IKKβ in an extracellular stimulation-dependent manner. In unstimulated states, GβL is constitutively bound to IKKβ, and this interaction is enhanced within 5 min of TNFα treatment, peaking at 15 min (Fig. 6E). Taken together, we conclude that GβL negatively regulates IKKβ activity by interacting with IKKβ in TNFα-mediated NF-κB signaling pathways.

4. Discussion

Activation of IKK is a pivotal step in transducing the signals triggered by various extracellular stimuli to the activation of NF-κB, culminating in the expression of various genes. As most kinases go through activation and deactivation by other signaling molecules in response to certain stimuli, IKK activity is thought to be regulated by kinases and other interaction partners [1,30]. Although a large body of results has been reported on the activation mechanisms of IKK and NF-κB signaling, models describing the negative regulation of IKK, such as its inhibition and deactivation, are still underdeveloped. The present study demonstrates that GβL, a member of the WD repeat proteins, interacts with IKKβ and regulates its activation in response to inflammatory stimulation.

The WD domain is comprised of four small strands of antiparallel β sheets with 40–60 residues that contain the Gly-His (GH) dipeptide 11–24 residues from the N-terminus and the Trp-Asp (WD) dipeptide at the C-terminus [29,31]. Most WD repeat proteins contain a cluster of 4–16 copies of the WD domain, which are speculated to form a circularized β propeller structure by virtue of crystal structure analysis of the G protein β subunit [32]. A large number of WD repeat proteins have been identified in eukaryotes and biological functions of some members have been defined, ranging from signal transduction, transcriptional regulation, and cell cycle to apoptosis, while the functions of many proteins are still unknown [33]. GβL, containing 6 WD repeats, was first defined by Kim et al. as a regulator of the mTOR pathway that participates in nutrient and growth factor-mediated signaling to S6 kinase 1 [26]. Furthermore, its expression is positively regulated by insulin in fully differentiated adipocytes [27]. Nevertheless, the ubiquitous expression pattern of GβL, with different levels in various tissues, and the simple propeller structure lacking any other functional domains enables us to speculate that GβL has functional roles in multiple signal pathways.

Because it was the bait for the yeast two-hybrid screening, we postulated that a region of IKKβ containing the leucine zipper participates in an interaction with GβL. The hypothesis was found to be correct in in vitro and in vivo binding assays with IKKβ fragments and recombinant GβL protein. However, the kinase domain of IKKβ also interacts with GβL with a relatively strong affinity. Based on these results, it is reasonable to assume that binding targets of GβL likely exist in both the kinase domain and leucine zipper region of IKKβ. With respect to GβL, the C-terminal WD repeats were found to be responsible for the interaction with IKKβ. Moreover, the results of site-directed mutagenesis of the conserved residues of these WD domains indicate that the fourth WD domain is crucial for the interaction with IKKβ, because a residue in this domain, Gly192, is required for the interaction. This residue is also important for the interaction between GβL and mTOR [26], suggesting that Gly192 may be an essential residue for WD domain integrity and for forming an architecture fit for molecular interaction. In immunoprecipitation assays for subtype-specific binding, GβL interacted strongly with IKKα and IKKβ, but only barely with NEMO. Although the IKK complex involved in classical NF-κB pathways contains IKKα-IKKβ dimers and a regulatory subunit, NEMO, both IKKα and IKKβ influence widespread signaling pathways, independent of NF-κB signaling, and NEMO is dispensable for IKK activation in non-canonical NF-κB pathways [34]. Above all, NEMO lacks a kinase domain, which is a major binding target of GβL. Based on these facts, it is conceivable that NEMO is not a direct binding partner of GβL.

Recent intensive investigations have revealed that several intracellular molecules, such as SMAD7, RBCK1, and NUMBL, negatively regulate NF-κB activation through different signaling pathways [35–37]. In addition, CUEDC2, whose function has yet to be well defined, is the first protein found to directly inhibit IKK activity [23]. In this work, we identify GβL as a new negative regulator of the IKK/IκB/NF-κB signaling axis. Overexpression of GβL inhibits TNFα-induced NF-κB activation and down-regulation of GβL expression enhances NF-κB activity. However, fragment proteins containing C-terminal WD domains of GβL do not inhibit TNF-induced NF-κB activation, in spite of its interaction with IKKβ. These findings suggest that GβL acts as an adaptor protein to mediate NF-κB inhibition by interacting with other proteins and affecting the behavior of IKK and its neighbors.

Upon TNFα stimulation, IκB is phosphorylated by IKKα/β and goes through proteosomal degradation [2]. In our experiments, the IκB level was not changed on TNFα treatment in cells expressing exogenous GβL; however, IκB degradation by TNFα was accelerated when GβL expression was down-regulated. In the case of βTrCP/Slimb, which contains two structural motifs, an F-box at the N-terminus and seven WD repeats at the C-terminus, interaction with IκB occurs through the WD repeats, recruiting IκB to the SCF complex allowing ubiquitin-conjugating enzymes to ubiquitinate IκB [38,39]. Together with the results of this study, one could hypothesize that the WD repeats of GβL interact with IκB and inhibit its interaction with βTrCP and subsequent proteosomal degradation. However, results from immunoprecipitation assays revealed that GβL was not a part of IκB immune complexes, implying that GβL is not a direct regulator of IκB degradation. When we examined the direct effect of GβL on IKK, TNFα-stimulated IKK phosphorylation was significantly down-regulated in the presence of exogenous GβL. Since phosphorylation by upstream kinases is a prerequisite for IKK activation [12,13], GβL seems to regulate IKK activity by inhibiting IKK phosphorylation. Because GβL seems to act as an adaptor, new GβL-binding proteins, which may directly affect IKK phosphorylation, should be identified in future studies. GβL is likely to regulate proinflammatory cytokine-stimulated cellular responses by inhibiting the action of IKK through direct interaction.

5. Conclusion

In summary, GβL is identified as a binding partner of IKKβ using the yeast two-hybrid method. The kinase domain and leucine zipper region of IKKβ are required for interaction with the C-terminal WD repeats of GβL. Overexpression and down-regulation of gene expression by shRNA revealed that GβL inhibits TNFα-induced NF-κB activation. GβL might be a negative regulator of NF-κB signaling through inhibition of phosphorylation-dependent IKK activation.

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