Phosphorylation-dependent association of the G4-1/G5PR regulatory subunit with IKKβ negatively modulates NF-κB activation through recruitment of protein phosphatase 5

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INTRODUCTION

NF-κB (nuclear factor κB) is a ubiquitous and evolutionarily conserved transcription factor. In non-stimulated cells, NF-κB heterodimers are sequestered in the cytoplasm through interaction with IκB (inhibitor of NF-κB). In response to stimulation with pro-inflammatory cytokines, a multi-subunit protein kinase, the IKK (IκB kinase), is rapidly activated and phosphorylates two critical serine residues in the N-terminal domain of IκB. Phosphorylation of IκB results in ubiquitination of IκB proteins and degradation by the proteasome [1–3]. This process leads to the translocation of NF-κB proteins from the cytoplasm to the nucleus, and turns on the expression of various NF-κB-regulated genes [4–6].

The IKK complex consists of several subunits, including catalytically active IKK1 (IKKα) and IKK2 (IKKβ), and the regulatory subunit NEMO (NF-κB essential modulator, also known as IKKγ), which contains no intrinsic kinase activity. Both IKKα and IKKβ contain the helix-loop-helix and leucine-zipper motifs that are known to interact with essential regulatory subunits [7,8]. Several IKK complex-interacting proteins have been identified as NF-κB-induced negative regulators, including IκBα and A20 [9–12]. We have previously shown that ABIN-2 (A20-binding inhibitor of NF-κB 2) specifically interacts with IKKγ and suppresses TNFα (tumour necrosis factor α)-induced NF-κB activity [13]. ABIN-2 is rapidly induced with similar kinetics as A20 and IκBα during partial hepatectomy-induced liver regeneration [14]. These NF-κB-induced negative regulators may form a negative-feedback control network for controlling NF-κB activation [15].

Several protein phosphatases have been identified as negative regulators of the IKK complex. For example, protein phosphatase PP2Cβ was identified as a negative regulator of NF-κB activation by reducing IKK activity through dephosphorylation of key serine residues in the activation loop of IKKβ [16]. PP2A was also found to positively or negatively regulate IKK activity [17,18]. CUEDC2 (CUE domain-containing protein 2) interacts with IKK to regulate the activity of NF-κB by recruiting PP1 [19]. Moreover, an RNAi (RNA interference) screen has identified 13 phosphatases to be involved in regulating NF-κB signalling [20].

G4-1, also referred to as G5PR, is a member of the PP2A B′ regulatory subunits and has been shown to directly interact with protein phosphatase PP5 and PP2A, and to regulate the phosphorylation status of the GANP/MCM3 (germinal centre-associated nuclear protein/minichromosome maintenance 3) complex during cell-cycle progression [21]. Studies of G4-1 conditional knockout mice demonstrate that loss of the G4-1 in B-cells increases their sensitivity to ACID (activation-induced cell death) triggered by BCR (B-cell receptor) cross-linking [22,23]. G4-1 is also involved in thymocyte development in CD4 and CD8 double-positive survival through INK (c-Jun N-terminal kinase)-mediated apoptosis signalling [24].

Key words: G4-1/G5PR, inhibitor of nuclear factor κB kinase (IKK), nuclear factor κB (NF-κB), protein phosphatase 5.

Abbreviations used: ABIN-2, A20-binding inhibitor of NF-κB 2; AICD, activation-induced cell death; BCR, B-cell receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione transferase; HEK, human embryonic kidney; HEK-293T, HEK-293 cells expressing the large T-antigen of SV40 (simian virus 40); IκB, inhibitor of nuclear factor κB; IKK, IκB kinase; JNK, c-Jun N-terminal kinase; NF-κB, nuclear factor κB; PP, protein phosphatase; O-PCR, quantitative PCR; RIP, receptor-interacting protein; RNAi, RNA interference; RT, reverse transcription; siRNA, small hairpin RNA; siRNA, small interfering RNA; TNFα, tumour necrosis factor α.

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Previous studies have indicated that the autophosphorylated C-terminal serine-rich domain of IKKβ plays an important role in the feedback regulation of IKKβ activity [25]; however, the molecular mechanism underlying the feedback regulation of IKKβ activity by the phosphorylated C-terminus of IKKβ is still unknown. In the present study, we show that G4-1 modulates NF-κB activation by physically associating with active, but not inactive, IKKβ. We further demonstrate that the interaction of G4-1 and IKKβ depends on phosphorylation of the C-terminus of IKKβ. Finally, association of G4-1 to active phosphorylated IKKβ recruits PP5 to the IKK complex and terminates the activation of IKKβ.

EXPERIMENTAL

Plasmid constructs and cell lines

Expression vectors for FLAG-tagged wild-type IKKβ, IKKβ (SS/EE), and the kinase-inactive mutant (K44A), have been described previously [26]. FLAG—GST (glutathione transferase) and FLAG—GST-fused IKKβ expression vectors were constructed by ligating the PCR-amplified cDNA sequence of GST from vector pGEX-4T-3 into a FLAG-tagged vector via HindIII—HindIII sites, followed by verification by DNA sequencing. pCDNA3.1-B-Myc-G4-1 containing the Myc-tagged full-length cDNA sequence of G4-1 and a HindIII/AgeI fragment cloned from HEK (human embryonic kidney)-293 cDNAs was constructed and verified by DNA sequencing. The NF-κB—luciferase reporter, which contains a minimal promoter with five tandem NF-κB-binding sites, and p53—luciferase reporter constructs were obtained from Clontech. Constructs of FLAG—IKKβ (SS/EE) and FLAG—IKKβ-7A were established by oligonucleotide-directed mutagenesis with a QuikChange® kit (Stratagene) and were confirmed by DNA sequencing. GST and GST-fusion proteins were expressed in Escherichia coli under control of the T7 promoter in vector pGEX-4T-3 (Gibco BRL), and the proteins were purified by glutathione-Sepharose beads. Human HepG2 and HEK-293 cDNAs were prepared using standard procedures. Lentiviral constructs, including TRCN0000002803 (#279) and TRCN0000002804 (#1246), which encode shRNAs (small hairpin RNAs) targeting different regions of PP5 mRNA, and TRCN00000055853 (FS), TRCN00000055854 (G8) and TRCN00000055855 (HS), which encode shRNAs targeting different regions of G4-1 mRNA, were obtained from the RNAi Consortium (TRC), National RNAi Core Facility, Academia Sinica, Taiwan, Republic of China. Scrambled siRNA (small interfering RNA) and specific siRNA for PP2A (5'-GC-CAUGACCGGAAUGAAGCAAGAU-3') were purchased from Invitrogen.

Cell transfection and antibodies

The transfection of HEK-293T cells was performed using the standard calcium phosphate precipitation method [27]. The delivery of siRNA was performed using Lipofectamine™ RNAiMAX Transfection Reagent (Invitrogen). Cells were plated in 12-well dishes with 5 × 10^6 cells/well 24 h before transfection. Anti-Myc (9E10) antibody was purchased from Upstate Biotechnology. Monoclonal anti-FLAG (M2) antibody, anti-β-actin (AC-15) antibody and anti-GST (B-14) antibody were purchased from Sigma. Anti-phospho-IKKα (Ser180)/IKKβ (Ser183) antibody was purchased from Cell Signaling Technology. Rabbit polyclonal anti-G5PR (G4-1) antibody was purchased from TransGenic.

Luciferase reporter assay

Cells were co-transfected with expression vectors containing the gene of interest, NF-κB reporter construct and Rous sarcoma virus-β-galactosidase vector to monitor transfection efficiency. At 24 h after transfection, cells were left untreated or treated with 10 ng/ml TNFα for 6 h. Cell extracts were analysed for luciferase and β-galactosidase activities using the dual-light kit (Tropix) according to the manufacturer’s instructions. Luciferase activity was normalized to β-galactosidase activity and expressed as the fold-stimulation relative to vector-transfected cells. The results are means of three separate experiments performed in triplicate. Values are expressed as means ± S.D.

GST pulldown analysis and immunoblotting analysis

Cell lysates were prepared from HEK-293T cells transfected with either pCMV2-FLAG-IKKα, pCMV2-FLAG-IKKβ, pCMV2-FLAG-IKKγ or an empty vector. The cell lysates were pre-cleared with glutathione—agarose beads, and subsequently incubated with soluble GST or GST—G4-1 in the binding buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.1% Nonidet P40 and 1 mM EDTA]. The protein-bound agarose beads were subsequently collected and washed four times with the binding buffer, and the pellet was resuspended in 20 μl of SDS sample buffer [100 mM Tris/HCl, 25% glycerol, 2% SDS and 0.01% Bromophenol Blue (pH 6.8)] containing 5% 2-mercaptoethanol and boiled for 10 min, and then subjected to SDS/PAGE. After transferring to nitrocellulose membranes (PerkinElmer Life Sciences), the membranes were blocked in 2–5% non-fat skimmed milk/TTBS [25 mM Tris/HCl (pH 7.4), 137 mM NaCl, 3 mM KCl and 0.2% Tween 20], followed by incubation with the primary antibodies indicated. Membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies, and levels of proteins of interest were detected by ECL (enhanced chemiluminescence) reagents (Visual Protein Biotechnology).

Preparation for lentiviruses harbouring specific shRNAs, real-time Q-PCR (quantitative PCR) and semi-quantitative RT (reverse transcription)—PCR analysis

HEK-293T cells were transinfected with mixtures of plasmids, including pCMV-Δ R8.91, pMD.G (VSV-G) and pLKO.1 shRNA with the ratio of 0.9:0.1:1. Supernatants containing viruses were collected 48 h after transfection. Cells with stable G4-1 shRNA expression were established by infection with viruses prepared earlier and subsequently selected with puromycin 48 h after infection. Total RNAs were isolated from HepG2 and HeLa stable G4-1-knockdown cells with standard procedures. The following primers were used: G4-1 forward 5′-TTT-TATTTATAGGGCTGCCGCTGCTGAA-3′, and G4-1 reverse 5′-TG-TCTGTTGTGTTGTCACCCGCA-3′; GAPDH (glyceraldehyde-3-phosphate dehydrogenase) forward 5′-GGTGTATTGGGCGCCGGTCGTCACC-3′, and GAPDH reverse 5′-CACACCATGG-AAGAACATGGGGGC-3′; IκBα forward 5′-CTATTCTTCCT-ACCAGCTCAC-3′, and IκBα reverse 5′-CTATAACCTGAGCCGG-3′. B2M forward 5′-AGGAATGCATCCGGCTTCT-ATCCT-3′, and B2M reverse 5′-CTTACCAATCACAATGCGG-3′.
and IκBα reverse 5'-TCATAACGTACAGCTGGCC-3'. An ABI Prism 7500 Fast Real-Time PCR system was used for Q-PCR reactions. The CT and RQ (relative quantification) were calculated by using the ABI 7500 SDS 1.3.1 software. The semi-quantitative RT-PCR analyses were performed and products of the gene-of-interest were resolved using 2% agarose gels and visualized with ethidium bromide staining.

RESULTS

G4-1 negatively regulates the NF-κB signalling pathway

To investigate which survival signalling pathways could be regulated by G4-1, we examined the effect of G4-1 on the p53- and NF-κB-dependent signalling pathway in HEK-293T cells. Overexpression of G4-1 had no effect on p53-induced transcriptional activity (Figure 1A), but suppressed TNFα-induced NF-κB activation (Figure 1B). In order to knockdown expression of G4-1, we examined the efficacy of several lentivirus-based shRNAs, including C3, F8, G8 and H8. The H8 clone showed the best knockdown activity to ectopically expressed Myc-tagged G4-1 protein in HEK-293T cells (Figure 1C). The efficiency of these shRNAs in knocking down endogenous G4-1 mRNA was also confirmed by Q-PCR analysis in human hepatoma HepG2 cells (Figure 1D). Next, we investigated whether silencing endogenous G4-1 expression could enhance expression of NF-κB downstream genes. As expected, knockdown of G4-1 significantly increased the mRNA levels of IκBα, one of NF-κB’s target genes, induced by TNFα treatment in comparison with parental HeLa cells (Figure 1E). In addition, the kinetics of induction of IκBα transcript was modulated due to knockdown of G4-1. When levels of induction of IκBα transcript in parental HeLa cells had already fallen at 60 min after TNFα treatment, levels of induction of IκBα transcript in cells with G4-1 knockdown was still increasing (Figure 1E). Furthermore, using NF-κB activity reporter analysis, when endogenous G4-1 was knocked down by G4-1-specific shRNAs, TNFα-induced NF-κB activity was significantly increased (Figure 1F). Similar effects of these shRNAs on ectopically expressed G4-1 were also observed (Figure 1G). These results suggest that G4-1 may function as a negative regulator of NF-κB activation.

G4-1 selectively inhibits upstream signalling molecules of NF-κB activation

In order to understand how G4-1 inhibits NF-κB activation, we first examined whether the activity of signalling molecules involved in NF-κB activation were inhibited by G4-1. As shown in Figures 2(A) and 2(B), both TNFα- and PMA-mediated NF-κB activation were dose-dependently inhibited by overexpression of G4-1. Furthermore, we found that effects on NF-κB activation mediated by RIP (receptor-interacting protein) (Figure 2C), IKKα (Figure 2D) and IKKβ (Figure 2E) were all significantly suppressed by overexpression of G4-1. However, G4-1 had no effect on p65-mediated NF-κB activation (Figure 2F). These results suggest that G4-1 may target the upstream signalling pathways involved in p65 activation.

G4-1 preferentially interacts with IKK-β, and physical association of G4-1 with IKKβ is kinase-activity-dependent

Since G4-1 inhibited various signalling molecules upon NF-κB activation, we hypothesized that one convergent point of these signalling molecules is the IKKα−IKKβ−IκB complex. To examine whether G4-1 interacts with IKK, we performed in vitro pulldown analysis by incubating recombinant GST or GST−G4-1 protein with cell lysates prepared from cells expressing FLAG−IKKβ, FLAG−IKKγ and FLAG−IKKα. Results of GST pulldown analysis revealed that GST−G4-1 associated with FLAG−IKKβ in the cell lysates, but not FLAG−IKKα or FLAG−IKKγ (Figure 3A). To examine whether the kinase activity of IKKβ is required for its association with G4-1, GST−G4-1 pulldown analysis was performed using cell lysates prepared from HEK-293T cells overexpressing wild-type FLAG−IKKβ, constitutively active FLAG−IKKβ (SS/EE) or kinase-dead FLAG−IKKβ (K44A). As shown in Figure 3(B), catalytically active IKKβ and constitutively active IKKβ were found to interact with GST−G4-1, whereas kinase-dead IKKβ showed very weak interaction with G4-1. To further demonstrate that the kinase activity of IKKβ is essential for its association with G4-1, we examined whether wedelolactone, a specific inhibitor of IKKβ, could block association of IKKβ with G4-1 in vitro. GST−G4-1 pulldown analysis showed that treatment with wedelolactone greatly abolished the interaction of IKKβ with G4-1 in vitro (Figure 3C). As expected, wedelolactone inhibited IKKβ-induced NF-κB activation in a dose-dependent manner (Figure 3D). These results suggest that G4-1 may modulate the NF-κB signalling pathway through associating with kinase-active IKKβ.

G4-1 interacts with the serine-rich C-terminal region of IKKβ

To characterize the interaction between G4-1 and IKKβ, first we mapped the domain of G4-1 for association with IKKβ by using series-deletion mutants of G4-1 (Figure 4A). We co-expressed deletion mutants of G4-1 with either FLAG−GST or FLAG−GST−IKKβ in HEK-293T cells and pulled down FLAG−GST or FLAG−GST−IKKβ to examine the presence of deletion mutants of G4-1. As shown in Figure 4(B), when the N-terminal region encompassing amino acids 1-184 of G4-1 was deleted, G4-1 no longer interacted with IKKβ, indicating that the N-terminal region of G4-1 is required for the IKKβ interaction (Figure 4B). Next, we mapped the domain of IKKβ for association with G4-1 by using series-deletion mutants of IKKβ. GST−G4-1 pulldown analyses were performed by incubating GST−G4-1 with cell lysates prepared from HEK-293T cells overexpressing wild-type FLAG−IKKβ, FLAG−IKKβ (SS/EE), FLAG−IKKβ (K44A), FLAG−IKKβ−733, FLAG−IKKβ−1−645 or FLAG−IKKβ−1−559. As shown in Figure 4(C), GST−G4-1 interacted with wild-type FLAG−IKKβ, FLAG−IKKβ−733, FLAG−IKKβ−1−645 and FLAG−IKKβ−1−559, indicating that the serine-rich C-terminal domain (Figure 4A) of IKKβ is required for G4-1 to interact with IKKβ.

Phosphorylation of the serine-rich C-terminal domain of IKKβ is required for its interaction with G4-1 and for G4-1-mediated down-regulation of IKKβ activity

To investigate whether the autophosphorylated serine residues in the C-terminus of IKKβ are critical for the interaction of IKKβ with G4-1, seven serine residues in the C-terminus of IKKβ were mutated to alanine (Figure 4A), and GST-pulldown analysis was performed to determine the role of phosphorylated serine residues in regulating association with G4-1. As shown in Figure 3(B), G4-1 interacted with the wild-type and constitutively active IKKβ, whereas IKKβ−7A mutants showed reduced association with G4-1. Moreover, IKKβ−7A showed similar activity to activate NF-κB as wild-type IKKβ did (Figure 4D), suggesting that the IKKβ−7A mutant is still functional in activating NF-κB. To determine whether the activity of the IKKβ−7A mutant is
Figure 1  G4-1 modulates the NF-κB activity

(A) HEK-293T cells were transiently transfected with expression constructs of p53, Myc—G4-1, an empty control plasmid or a combination of expression constructs of p53 and Myc—G4-1, and equal amounts of p53-dependent luciferase reporter plasmid. At 24 h after transfection, cells were harvested for luciferase activity analysis. (B) HEK-293T cells were transiently transfected with expression constructs of Myc—G4-1, or an empty control plasmid, and equal amounts of NF-κB-dependent luciferase reporter plasmid. At 24 h after transfection, cells were stimulated with 10 ng/ml TNFα for 4 h prior to harvest for luciferase activity analysis. (C) HEK-293T cells were transiently transfected with vector containing Myc—G4-1 and various vectors harboring sequence encoding distinct G4-1 shRNAs for 48 h prior to harvest of cells for measuring G4-1 protein expression levels by immunoblotting analysis. The knockdown efficiency is shown. (D) q-PCR analysis of G4-1 knockdown by the G4-1 shRNAs indicated stably expressed in HepG2 cells. (E) HeLa cells with vector or specific G4-1 knockdown were analysed for protein and mRNA levels of G4-1 by immunoblotting and RT-PCR respectively. The relative mRNA levels of β2M were analysed by q-PCR in cells carrying vector or stably expressing G4-1 shRNA with or without TNFα treatment for 30 min or the times as indicated. The fold-changes in the expression levels of β2M or B2M (as a control) in control cells with TNFα treatment or G4-1 shRNA-expressing cells with or without TNFα treatment compared with control cells without TNFα treatment (set as 1) are shown. (F) HEK-293T cells were transiently transfected with expression vector for the G4-1 shRNAs indicated, expression vector for Myc—G4-1, an empty control vector, or combinations of vectors for Myc—G4-1 and the G4-1 shRNAs indicated, and equal amounts of NF-κB-dependent luciferase reporter plasmid with or without TNFα stimulation for 4 h prior to harvest for luciferase activity analysis. The results are means of three independent experiments performed in triplicate. The blot of Myc—G4-1 expression level after knockdown with individual shRNAs is shown. * P < 0.05, ** P < 0.01 (measured using a Student’s t test).
Phosphorylation-dependent regulation of NF-κB by G4-1/G5PR

Figure 2  G4-1 inhibits NF-κB activation stimulated by various activators, except p65

HEK-293T cells were transiently transfected with different amounts of Myc−G4-1 (relative amounts are indicated as 0, 0.5×, 1× and 2×; 1× = 300 ng), or an empty vector, and equal amounts of NF-κB-dependent luciferase reporter plasmid. At 24 h after transfection, the cells were either left untreated or stimulated with 10 ng/ml TNFα (A) or 200 ng/ml PMA (B) for 4 h prior to harvest for luciferase activity analysis. HEK-293T cells were transiently transfected with expression vector of RIP (C), IKKα (D), IKKβ (E) or p65 (F), and expression vector of Myc−G4-1or an empty control vector, and equal amounts of NF-κB-dependent luciferase reporter plasmid. Luciferase activity was assessed. The results are means of three independent experiments performed in triplicate.

Figure 3  Association of G4-1 with catalytically active IKK-β, but not inactive IKK-β

(A) Equivalent amounts of GST−vector or GST−G4-1 fusion protein bound to glutathione−Sepharose were incubated with cell lysates prepared from HEK-293T cells transiently transfected with 3 μg of FLAG−IKKα, FLAG−IKKβ or FLAG−IKKγ. After washing, the pulldowns were separated by SDS/PAGE (10 % gels) and analysed by immunoblotting (IB) with specific antibodies as shown.

(B) Equivalent amounts of GST−vector or GST−G4-1 fusion protein bound to glutathione−Sepharose were incubated with the cell lysates prepared from HEK-293T cells transiently transfected with 3 μg of expression vector of FLAG−IKKγ, FLAG−IKKβ (SS/EE), FLAG−IKKβ (K44A) or FLAG−IKKβ (7A). The GST−G4-1 pulldown analyses were performed. (C) The same amounts of GST−vector or GST−G4-1 protein were incubated with lysates of HEK-293T cells 24 h after being transiently transfected with 3 μg of FLAG−IKKβ with or without treatment of the indicated doses of wedelolactone. GST-pulldown analyses were performed. (D) HEK-293T cells were transiently transfected with 0.4 μg of expression vector of FLAG−IKKβ and NF-κB-dependent luciferase reporter vector with or without different doses of wedelolactone treatment. Luciferase activity analyses were performed. The results are means of three independent experiments performed in triplicate.
Figure 4  G4-1 binds to the hyperphosphorylated C-terminal region of IKKβ and regulates NF-κB activity

(A) Schematic diagrams of G4-1 serial-deletion constructs which show different binding ability with IKKβ as indicated (+/−) (upper panel); IKKβ domain structure and serial C-terminal deletion constructs which show different binding ability with G4-1 as indicated (+/−) (middle panel); a schematic diagram of the IKKβ C-terminal serine-rich region sequence which displays the seven serine residues replaced with alanine in the IKKβ-7A mutant (lower panel). (B) GST-pulldown analyses were performed for lysates of HEK-293T cells co-expressing either FLAG—GST or FLAG—GST—IKKβ and wild-type or deletion mutants of G4-1. The pulldowns were separated by SDS/PAGE (10% gels) and analysed by immunoblotting (IB) with specific antibodies as indicated. (C) The GST-pulldown analyses were performed by incubating GST—G4-1 with various cell lysates containing wild-type or mutant forms of FLAG—IKKβ as indicated. (D) Luciferase activity analysis for lysates from HEK-293T cells transiently transfected with 0.4 μg of FLAG—IKKβ, FLAG—IKKβ (SS/EE), FLAG—IKKβ (K44A), FLAG—IKKβ (7A) or an empty control, and equal amounts of NF-κB-dependent luciferase reporter plasmid. The results are means of three independent experiments performed in triplicate. (E) HEK-293T cells were transiently co-transfected with 3 μg of expression vector of FLAG—IKKβ or FLAG—IKKβ (7A) and Myc—G4-1 or empty control vector. At 24 h after transfection, the cells were either left untreated or stimulated with 10 ng/ml TNFα for 30, 60 and 90 min. Whole-cell lysates were analysed by immunoblotting using an antibody against phospho-IKKβ (Ser181), the FLAG tag or the Myc tag. The ratio of induced phospho-IKKβ to the untreated control is shown. (F) HeLa cells with or without G4-1 knockdown which were treated with TNFα for the times indicated were analysed for protein levels of phospho-IκBα, IκBα, G4-1 and actin by immunoblotting. The ratio of levels of phospho-IκBα which was normalized with total IκBα and actin to that of untreated control (set as 1) is shown.
regulated by G4-1, we examined the effect of G4-1 on TNFα-stimulated phosphorylation of IKKβ. We found that the IKKβ-7A mutant showed much higher levels of TNFα-stimulated Ser181 phosphorylation (Figure 4E, lower panel) as compared with that of wild-type (upper panel), and overexpression of G4-1 abolished TNFα-stimulated Ser181 phosphorylation of wild-type IKKβ, but modestly affected the IKKβ-7A mutant. These results strongly support the hypothesis that G4-1 only binds to the phosphorylated C-terminus of IKKβ and inhibits its activity. Since G4-1 down-regulates IKKβ activity, we further examined the effect of knockdown of G4-1 on TNFα-stimulated phosphorylation of IκBα in HeLa cells. HeLa cells with G4-1 knockdown showed much higher levels of TNFα-stimulated IκBα phosphorylation as compared with that of wild-type HeLa cells (Figure 4F). These results demonstrate that G4-1 may function as a negative regulator of the NF-κB signalling pathway via down-regulation of IKKβ activity.

G4-1 suppresses TNFα-induced NF-κB activation through PP5

It has been shown that G4-1 associates with PP2A and PP5 [21]. We wondered which protein phosphatase is involved in G4-1-mediated down-regulation of NF-κB activity. We tested the efficiency of two PP5 shRNAs, shPP5 #279 and shPP5 #1246, to suppress TNFα-induced NF-κB activation in HeLa cells. HeLa cells were transiently transfected with PP5 shRNAs, Myc−G4-1, FLAG−PP5, an empty control vector, combinations of construct of Myc−G4-1 plus the indicated construct of PP5 shRNAs, combinations of FLAG−PP5 plus the indicated construct of PP5 shRNAs, and equal amounts of NF-κB-dependent luciferase reporter plasmid (relative amount to reporter plasmid is indicated as PP5shRNA/Myc−G4-1/FLAG−PP5 = 1.5:1:1). Luciferase activity analysis for lysates from these transfected cells was performed. The expression levels of FLAG−PP5 and Myc−G4-1 of individual cells are shown. The results are means of three independent experiments performed in triplicate. *P < 0.05, measured using a Student’s t-test.
in silencing PP5 expression and found that shPP5 #279 showed much greater efficiency than shPP5 #1246 (Figure 5A, lower panel). As expected (Figure 5A), we found that knockdown of endogenous PP5 by shPP5 #279 not only significantly increased TNFα-stimulated NF-κB activity, but also attenuated the suppressive activity of ectopically expressed G4-1 on TNFα-stimulated NF-κB activity, whereas inefficient knockdown of PP5 by shPP5 #1246 did not have any effect. Furthermore, stable PP5 knockdown in HEK-293T cells overexpressing FLAG-IKKβ were analyzed for the effect of PP5 on TNFα-stimulated phosphorylation of IKKβ (Ser181) and of IκBα (Figure 5B). The levels of TNFα-stimulated phosphorylation of IKKβ and of IκBα were modestly increased by PP5 knockdown. Nevertheless, levels of phosphorylated IKKβ reached a plateau at 60 min after TNFα treatment in control cells (expressing shPP5 #1246), whereas levels of phosphorylated IKKβ were still increasing at 90 min after TNFα treatment in cells with PP5 knockdown. In contrast, PP2A knockdown markedly increased both basal and TNFα-stimulated phosphorylated IKKβ and phosphorylated IκBα (Figure 5C) as compared with that of control cells. In contrast with effects by PP5 knockdown, TNFα-induced phosphorylation of IKKβ reached a plateau at a similar time (30 min) after TNFα treatment in both control cells and cells with PP2A knockdown. Moreover, knockdown of endogenous PP2A did not reverse the suppressive effect of ectopically expressed G4-1 on TNFα-stimulated NF-κB activity (Figures 5D). These results indicate that PP5 is involved in the G4-1-mediated down-regulation of TNFα-stimulated NF-κB activation.

**DISCUSSION**

In the present study, we have shown that the ubiquitously expressed G4-1 protein plays an important role in modulating IKKβ-mediated NF-κB activation. NF-κB is a powerful transcriptional factor which activates hundreds of genes. Therefore the degree of activation of NF-κB should be highly dynamic and tightly controlled. Several functional non-redundant negative-feedback regulators, including IκBα and A20 genes, are rapidly induced by active NF-κB [9–12]. The distinct regulatory role of induced IκBα and A20 on TNFα-induced dynamic activation of IKK and NF-κB has been intensively studied [28]. It was shown that the peak activation of IKK occurred at 10 min after a 1 min pulse of TNFα treatment, and rapidly attenuated to the basal level. However, activation of NF-κB lasts at least 45 min followed by one fast and one slow inactivation dynamic [15]. IκBα and A20 were shown to determine the duration of the first- and second-phase of NF-κB activation respectively. The key question that remains to be answered is how active IKK was rapidly attenuated.

Biochemical analyses have indicated that the rapid inactivation of IKK is dependent on the kinase activity of IKKβ and phosphorylation of its C-terminus [25]. However, the molecular mechanism of how autophosphorylation of the C-terminus of IKKβ inactivates IKKβ is still unknown. The data presented in the present paper indicate that the physical association of G4-1 to wild-type IKKβ (Figure 3C) in vitro. These results support our model that G4-1 only binds to the kinase-active form of IKKβ. Furthermore, data of physical mapping analysis indicate that the C-terminus of IKKβ is essential for G4-1 to bind IKKβ (Figure 4C). The C-terminus of IKKβ has been shown as a multiple autophosphorylation domain of IKKβ in cells [25]. Therefore it is possible that the fully phosphorylated C-terminus of IKKβ is indeed the domain of active IKKβ to interact with G4-1 in cells. This possibility is strongly supported by the results that when multiple autophosphorylated serine residues at the C-terminus of IKK were mutated to alanine, the mutated IKK lost its ability to associate with G4-1 (Figure 3B).

Our results suggest that G4-1 may function as a negative regulator of NF-κB signalling only when the IKK is fully activated. Therefore the initial phase of IKK or NF-κB activation is not subjected to regulation of G4-1, but the kinetics of IKK or NF-κB activation should be modulated by expression levels of G4-1. Consistent with our proposed model, silencing G4-1 resulted in long-lasting induction of IκBα transcript (Figure 1E) and a slower growth in the levels of IκBα protein (Figure 4F) as compared with that of control cells. In addition, higher levels of TNFα-stimulated NF-κB activity were observed in cells with G4-1 knockdown compared with the control cells (Figure 1F). The other prediction of our model is that the IKKβ-7A mutant which cannot bind to G4-1 as much as wild-type should have higher activity than the wild-type IKKβ. This prediction was also confirmed by the results shown in Figure 4(E) that basal and TNFα-stimulated phosphorylation of IKKβ-7A was much higher than that of the wild-type IKKβ. Therefore the results of the present study strongly support the model that the association of G4-1 with the active autophosphorylated IKKβ inactivates IKKβ.

How does G4-1 inactivate IKKβ? Activation of IKKβ is initiated by cytokine-dependent phosphorylation of T loop serine residues, followed by subsequent activation of IKKβ kinase activity. G4-1 has been shown to function as a regulatory subunit of both PP5 and PP2A. The protein phosphatase associated with G4-1 serves as the best candidate to dephosphorylate phosphoserine in the T loop of IKKβ and to inactivate IKKβ. PP2A has been shown to be either a positive or negative regulator of IKK activity [17,18]. The results of the present study showed that knockdown of PP5, but not PP2A, largely abolished the negative regulation of IKKβ activation by G4-1 (Figure 5), which supports our model that G4-1-associated PP5 is responsible for inactivation of IKKβ after TNFα stimulation. Nevertheless, we observed marked increases in overall phosphorylation levels of IKKβ due to knockdown of PP2A (Figure 5). Interestingly, knockdown of PP5 resulted in only modest changes in overall levels of phosphorylation of IKKβ, but it increased the rate of phosphorylation stimulated by TNFα treatment, which was not observed by knockdown of PP2A (Figure 5). These data suggest that PP2A forms a stable complex with IKK, as previously suggested [17], and constantly catalyses dephosphorylation of IKKβ, whereas activated IKKβ recruits the G4-1–PP5 complex to down-regulate IKKβ activity. Analyses on genome-wide knockdown of protein phosphatases and their regulatory subunits have shown that G4-1 acts as a negative regulator for TNFα-induced NF-κB activation [20]. Our observation is consistent with this finding and provides a mechanistic explanation for the regulatory role G4-1 in TNFα-induced NF-κB activation [20]. Our model suggests that once TNFα induces the activation of IKKβ and activated IKKβ autophosphorylates its serine-rich C-terminus, the exposed multiple phosphorylated C-terminus of IKKβ then becomes a target of G4-1. G4-1 associates with the active autophosphorylated C-terminus of IKKβ and, subsequently, the associated PP5 dephosphorylates T loop serine residues of IKKβ to terminate IKKβ activation (Figure 6).

Our findings on NF-κB activation are different from that found in the G4-1 conditional B-cell knockout mice in which no obvious change was observed in NF-κB signalling in B-cells stimulated by BCR cross-linking [21]. The discrepancy between our own findings and others may result from different downstream signalling components between BCR cross-linking in B-cells and TNFα treatment in HeLa cells. In addition, the
functional role of G4-1 depends on the associated phosphatase activity. It is possible that expression levels of G4-1-associated phosphatases are different between lymphocytes and HeLa cells. The physiological significance of our finding is that the G4-1—PP5 complex is a negative regulator of TNFα-induced NF-κB activation. Therefore it is conceivable that G4-1 may negatively regulate TNF-α-stimulated cell proliferation or cell death according to the cell context. It was shown that G5PR (G4-1) knockout rendered B-cells more sensitive to BCR AICD by enhancing the activity of JNK and Bim and impaired BCR-mediated B-cell proliferation [22]. Moreover, BCR cross-linking induced GSPR transcription in AICD-resistant mature splenic IgM+IgD+ B-cells, but not in AICD-susceptible immature IgM+IgDlow B-cells [23]. Thus G4-1 (G5PR) appears to be a positive regulator in survival and cell proliferation of mature B-cells downstream of BCR. Therefore G4-1 and associated phosphatases may differentially regulate cellular activities in a cell-, development- or differentiation-dependent manner.

Our present study showed a distinct mode-of-action of G4-1 to modulate NF-κB activation. There are still many questions that remain to be answered. For example, is G4-1—PP5-mediated feedback control of IKKβ activation a general phenomenon or cell-type specific? Does natural polymorphism of G4-1 and PP5 genes affect this control mechanism of NF-κB activation? Some reports have shown that polymorphic alleles of negative regulators of NF-κB such as A20 are associated with an increased risk of autoimmune disease [29,30]. The inactive mutant of A20 has been shown to associate with human B-cell lymphomas [31,32]. It deserves more detailed scrutiny to know whether deregulation of G4-1 is also involved in particular diseases such as autoimmune diseases or cancer.

ACKNOWLEDGEMENTS

We thank Dr Hiroyasu Nakano (Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan) for wild-type IKKα and kinase-inactive IKKβ constructs, and the National RNAi Core Facility for providing the RNAi reagents. We thank Mr Larry Paris for editorial assistance prior to submission.

FUNDING

This work was supported, in part, by the National Science Council [grant numbers NSC 95-2311-B-182-002, NSC 95-2323-B-182-001, NSC 96-2311-B-182-005-MY3] and Chang Gung University [grant numbers CMRPD140213, CMRPD140103, CMRPD160473], to C.-K.C.

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AUTHOR CONTRIBUTION

Chao-Wei Chiang and Wei-Kuang Liu contributed equally in performing the experiments and writing part of the paper. Chen-Kung Chou and Chi-Wu Chiang designed experiments, analysed the data and wrote the paper. All authors read and approved the final manuscript.


