Biochemical and Biophysical Research Communications 461 (2015) 307–313

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

GRK6 phosphorylates IκBα at Ser^{32}/Ser^{36} and enhances TNF-α-induced inflammation

Yuki Ohba, Michio Nakaya*, Kenji Watari, Akiomi Nagasaka, Hitoshi Kurose

Department of Pharmacology and Toxicology, Graduate School of Pharmaceutical Sciences, Kyushu University, Fukuoka 812-8582, Japan

ARTICLE INFO

Article history:
Received 19 March 2015
Available online 13 April 2015

Keywords:
GRK
NF-κB
IκBα
TNF-α
BRET
Inflammation

ABSTRACT

G protein-coupled receptor kinases (GRKs) comprise a family of seven serine/threonine kinases that phosphorylate agonist-activated G protein-coupled receptors (GPCRs). It has recently been reported that GRKs regulate GPCR-independent signaling through the phosphorylation of intracellular proteins. To date, several intracellular substrates for GRK2 and GRK5 have been reported. However, those for GRK6 are poorly understood. Here we identified IκBα, a negative regulator of NF-κB signaling, as a substrate for GRK6. GRK6 directly phosphorylated IκBα at Ser^{32}/Ser^{36}, and the kinase activity of GRK6 was required for the promotion of NF-κB signaling after TNF-α stimulation. Knockout of GRK6 in peritoneal macrophages remarkably attenuated the transcription of inflammatory genes after TNF-α stimulation. In addition, we developed a bioluminescence resonance energy transfer (BRET) probe to monitor GRK6 activity. Using this probe, we revealed that the conformational change of GRK6 was induced by TNF-α. In summary, our study demonstrates that TNF-α induces GRK6 activation, and GRK6 promotes inflammatory responses through the phosphorylation of IκBα.

1. Introduction

G protein-coupled receptor kinases (GRKs), which are composed of seven subfamilies (GRK1–7), are known as kinases that phosphorylate G protein-coupled receptors (GPCRs) [1]. When GPCRs are activated by binding to their agonists, GRKs phosphorylate them. Subsequently, β-arrestins bind to the receptor, inhibiting further G protein activation by blocking receptor-G protein coupling [2]. In addition to the regulation of GPCR desensitization, it has recently been revealed that GRKs phosphorylate not only GPCRs but also non-GPCRs [3]. Furthermore, this phosphorylation leads to the regulation of GPCR-independent intracellular signaling pathways [4]. For instance, GRK2 is reported to phosphorylate p38 and impair MKK6-induced p38 activation [5]. Among 7 GRK subfamilies, the intracellular substrates for GRK2 and GRK5 have been well elucidated [3,4]. On the other hand, those for other GRKs are poorly understood.

Nuclear factor-κB (NF-κB) is a transcription factor that regulates the expression of genes involved in various responses, including inflammation, anti-apoptosis, and immunity [6]. The NF-κB family is composed of the five related proteins RelA (p65), RelB, c-Rel, NF-κB1 (p50/p105), and NF-κB2 (p52/p100), forming various homo- and heterodimers [7]. In the resting state, NF-κB dimers are inactive in the cytoplasm because of the association with the inhibitor of NF-κB (IκB). When IκB kinase (IKK) is activated by extracellular stimuli, IKK phosphorylates IκB. Then, IκB undergoes ubiquitination followed by proteasome-induced degradation. Following this, NF-κB dimers are released, and they migrate to the nucleus. These dimers bind to specific DNA sequences and activate a variety of NF-κB target genes [8]. Among IκB isoforms, IκBα is known to be involved in inflammation. IκBα degradation leads to the nuclear translocation of the p65:p50 heterodimer that promotes the transcription of inflammatory genes, including IL-1β, IL-6, and inducible nitric oxide synthase (iNOS) [9,10]. Although IKK is well known to phosphorylate IκBα at Ser^{32}/Ser^{36}, a recent study demonstrated that GRK5 also phosphorylated IκBα at Ser^{32}/Ser^{36} and promoted

Abbreviations: GRK, G protein-coupled receptor kinase; GPCR, G protein-coupled receptor; MKK6, mitogen-activated protein kinase kinase 6; NF-κB, nuclear factor-κB; IκB, inhibitor of NF-κB; IKK, IκB kinase; IL, interleukin; GIT, GRK interactor; TNF-α, tumor necrosis factor α; PM, peritoneal macrophages; TEPM, thioglycollate-elicited peritoneal macrophages; SM, splenic macrophages; BMDM, bone marrow-derived macrophages; M-CSF, macrophage colony-stimulating factor; siRNA, small interfering RNA; BRET, bioluminescence resonance energy transfer; Ctrl, control; WT, wild-type; KO, knockout; iNOS, inducible nitric oxide synthase.

* Corresponding author. Department of Pharmacology and Toxicology, Graduate School of Pharmaceutical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka, 812-8582, Japan. Fax: +81 92 642 6878.
E-mail address: nakaya@phar.kyushu-u.ac.jp (M. Nakaya).

http://dx.doi.org/10.1016/j.bbrc.2015.04.027
0006-291X/

NF-κB signaling [11]. Therefore, we expected that other kinases could phosphorylate IκBα and regulate inflammatory responses through the activation of NF-κB signaling.

We recently revealed that GRK6 directly phosphorylated GIT1, which is known as a scaffold protein, and promoted efficient apoptotic cell engulfment [12]. This suggests that GRK6 also phosphorylates non-GPCR substrates and regulates various cellular signaling, as done by GRK2 and GRK5. In this study, we attempt to demonstrate the possibility that GRK6 is involved in TNF-α-induced inflammation through the activation of NF-κB signaling.

2. Materials and methods

2.1. Mice

We purchased C57BL/6J mice from Charles River Japan. GRK6-deficient mice were obtained from Dr. R. J. Lefkowitz and Dr. R. T. Premont (Duke University). Mice were kept under pathogen-free conditions in accordance with the regulations of Institutional Animal Care and Use Committee. All experimental procedures were performed under the guidelines by Kyushu University.

2.2. Preparation of peritoneal macrophages, thioglycollate-elicited peritoneal macrophages, and splenic macrophages

Peritoneal macrophages (PM) were collected from intact mice and thioglycollate-elicited peritoneal macrophages (TEPM) were collected from mice four days after intraperitoneal injection with sterile 4% thioglycollate (BD). They were euthanized and their peritoneal cavities were exposed. We injected 10 mL of Dulbecco's Modified Eagle Medium (DMEM) (nacalai tesque) into the abdominal cavities and harvested these fluids. Then, PM and TEPM were filtrated through 40 μm nylon meshes (Kyoshin Rikoh) and plated onto non-treated 6-well plates. Splenic macrophages (SM) were collected from wild-type mice. Their spleens were extracted and minced. After that, the minced spleens were filtrated through 40 μm nylon meshes and plated onto non-treated 6 well plates.

2.3. Cell culture and transfection

NIH3T3 cells, HEK293 cells, PM, TEPM, and Plat-E cells, a retrovirus-packaging cell line [13], were cultured in DMEM with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin solution (nacalai tesque). Bone marrow-derived macrophages (BMDM) were prepared, as previously described [14]. BMDM and SM were maintained in MEM-α (Life Technologies) containing 10% FBS and macrophage colony-stimulating factor (M-CSF) prepared from the conditioned medium of L929 cells transformed with a human M-CSF expression plasmid [15]. HEK293 cells and Plat-E cells were transfected with X-tremeGENE 9 (Roche Diagnostics). NIH3T3 cells were transfected with Lipofectamine 2000 (Life Technologies). Lipofectamine RNAiMAX (Life Technologies) was used for siRNA transfection. NIH3T3 cells were infected with retrovirus using the supernatants of Plat-E cells, as previously described [16].

2.4. Plasmid constructs and siRNA

The cDNAs encoding mouse GRK5, GRK6, and IκBα were amplified from a mouse thioglycollate-elicited peritoneal macrophage cDNA library. The cDNAs, fused with Flag or HA at the N-termini (Flag-GRK5, Flag-GRK6, and HA-IκBα) were subcloned into pMXs-puro or pcDNA3. Kinase-inactive forms of each Flag-tagged GRK [GRK5 (K215R) and GRK6 (K215R)] and a dominant-negative mutant of HA-tagged IκBα [IκBα (S32A/S36A)] were generated by site-directed mutagenesis using a QuickChange mutagenesis kit (Stratagene). GRK6 siRNA was purchased from Life Technologies. The target sequence for mouse GRK6 was 5′-AAUAA-CAGGCGCCCAAUGGGCUGGC-3′. As a control, Stealth RNAi siRNA negative control hi GC (Life Technologies) was used.

2.5. Real time reverse transcription polymerase chain reaction

Total RNA was purified using a RNeasy Mini Kit (QIAGEN), according to the protocol of the manufacturer. Expression of mRNA was quantified by real time reverse transcription polymerase chain reaction (RT-PCR) using TaqMan™ probes after cDNA synthesis. In absolute quantification, we created the standard curves of known concentration plasmids (pcDNA3-Flag-GRK5 and pcDNA3-Flag-GRK6). Then, we compared mRNA expression with the standard curves and extrapolated these values. TaqMan™ probes for mouse *Grk5* (Assay ID: Mm00517039_m1), mouse *Grk6* (Assay ID: Mm00442425_m1), mouse *Il1b* (Assay ID: Mm00434228_m1), mouse *Il6* (Assay ID: Mm00446190_m1), mouse *Nos2* (Assay ID: Mm00440502_m1), and mouse *Gapdh* (Assay ID: Mm99999915_g1) were purchased from Applied Biosystems. The sequences of TaqMan™ primer pairs and probe purchased from Sigma Aldrich are described below.

Mouse *Il1b*: 5′-TGCCACCTTTTGACAGTGATGA-3′ (forward), 5′-GCCCAGGTCAAAGGTTTGGAA-3′ (reverse), 5′-FAM-ACCTGTTCTTT GAAGTTGACGGACCCCA-TAMRA-3′ (probe).

Data were normalized to GAPDH.

2.6. Western blot analysis

Samples were loaded onto SDS-PAGE and subjected to western blot with the following antibodies: anti-phospho-NF-κB p65 (Ser^{536}) (1:2,000, CST), anti-NF-κB p65 (1:2,000, CST), anti-phospho-IκBα (Ser^{32}/Ser^{36}) (1:2,000, CST), anti-IκBα (1:2,000, CST), anti-GRK6 (1:4,000, Santa Cruz), anti-GAPDH (1:20,000, Santa Cruz), and anti-Flag (1:20,000, Sigma Aldrich). Anti-rabbit IgG or anti-mouse IgG secondary antibodies conjugated to horseradish peroxidase were purchased from CST and Santa Cruz. Densitometric analysis was conducted using ImageJ software.

2.7. Immunoprecipitation

Immunoprecipitation was performed, as previously reported [12]. In brief, NIH3T3 cells infected with the retrovirus carrying Flag-GRK6 were washed and scraped with lysis buffer (50 mM Tris-Cl, pH 7.5, 300 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.5% sodium deoxycholate) that contained a protease inhibitor cocktail (nacalai tesque). After centrifugation, the supernatants of scraped cells were collected and gently rotated overnight at 4 °C after adding 20 μL of Anti-Flag M2 Affinity Gel (Sigma Aldrich). The beads were washed three times and eluted with 2 × SDS sample buffer. After centrifugation, the supernatants were collected and subjected to SDS-PAGE, followed by western blot with the previously mentioned antibodies.

2.8. In vitro kinase assay

An *in vitro* kinase assay was performed, as previously described [12]. Recombinant proteins [GST-IκBα (WT) and GST-IκBα (S32A/S36A)] were purified from BL21 (DE3) (Novagen). Baculovirus-expressed recombinant full-length GRK6 (200 ng; Carna Biosciences) was incubated with GST-IκBα (WT; 5 μg) or GST-IκBα (S32A/S36A; 5 μg) in 20 μL kinase buffer [20 mM Tris-Cl, pH 7.5, 5 mM $MgCl_2$, and 1 mM dithiothreitol (Sigma Aldrich)] containing 100 μM Adenosine 5′-triphosphate (Sigma Aldrich) for 30 min at 30 °C. The reaction was stopped by adding 4 × SDS sample buffer

and boiling. The samples were then loaded onto SDS-PAGE and subjected to western blot.

2.9. Luciferase assay

NIH3T3 cells were transfected with empty vector, pcDNA3-Flag-GRK6, or pcDNA3-Flag-GRK6 (K215R) using Lipofectamine 2000 (Life Technologies). pGL4.32 [luc2P/NF-κB-RE/Hygro] (Promega) and pRL-TK (Promega) vector were simultaneously transfected. Thirty-six hours after transfection, cells were serum-starved with 0.1% FBS for 12 h and then stimulated with mouse TNF-α (10 ng/mL; PeproTech) for 6 h. Lysates were prepared and analyzed for firefly and *Renilla* luciferase (*R*luc) activities using the Dual-Luciferase Reporter Assay System (Promega). The intensity of each luciferase was measured using a Multilabel Reader Mithras LB 940 (Berthold Technologies).

2.10. Bioluminescence resonance energy transfer assay

To evaluate the conformational change of GRK6, a bioluminescence resonance energy transfer (BRET) experiment was performed, as previously described [17]. The plasmid of *R*luc(h)-GRK6-GFP^2 was constructed and transfected into HEK293 cells. Forty-eight hours after transfection, the cells were collected and washed with DMEM/F12 (Life Technologies). The cells were suspended in BRET assay buffer (1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 1 g/L D-glucose, and 2 mg/L aprotinin in PBS). After that, 25 μL of the cell suspension was dispensed into a 96-well white microplate, and 25 μL of mouse TNF-α (10 ng/mL final concentration) was stimulated for the indicated time. After stimulation, DeepBlueC™ (Biotium) was added at a final concentration of 5 μM, and the 400 nm (*R*luc) and 515 nm (GFP^2) emissions were immediately measured using a Multilabel Reader Mithras LB 940 (Berthold Technologies).

2.11. Statistical analysis

The results were presented as the means ± SEM of at least three independent experiments. Statistical significance of differences was determined using the Student's *t* test, one-way analysis of variance (ANOVA) with Tukey's multiple comparison test, or two-way ANOVA with *post hoc* Bonferroni test.

3. Results

3.1. GRK6 directly phosphorylates IκBα at Ser^{32}/Ser^{36}

During our investigation of the role of GRK6 in macrophages, we found that the expression level of GRK6 was much higher than that of GRK5, which belongs to the same subfamily as GRK6, in macrophages collected from the mouse peritonea, bone

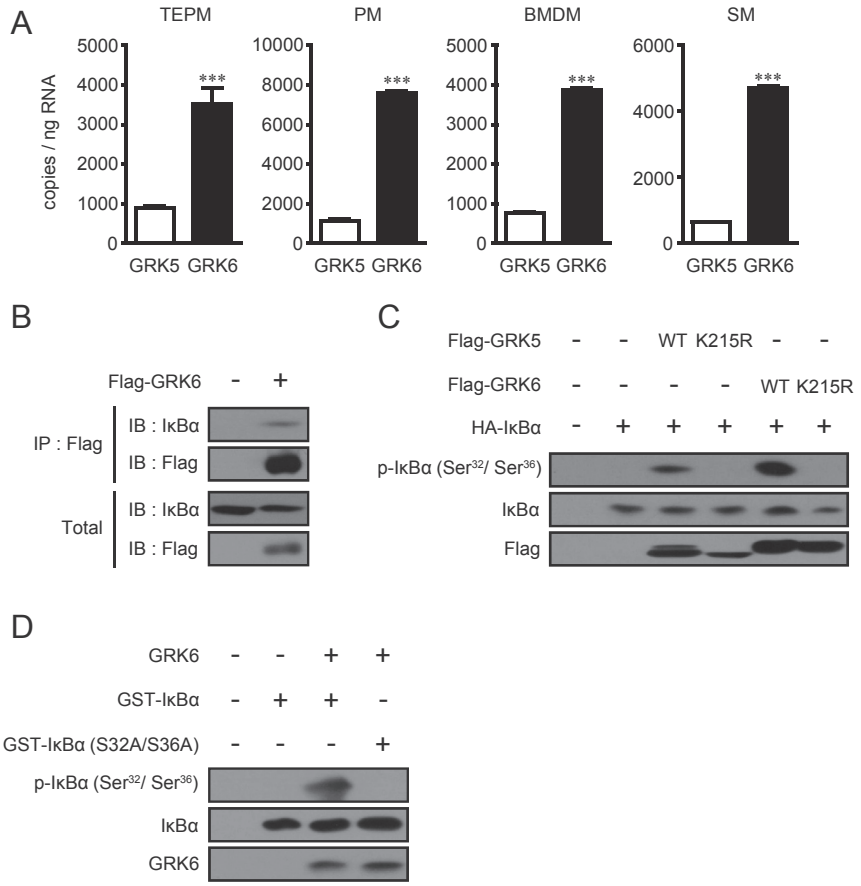

Fig. 1. GRK6 directly phosphorylates IκBα at Ser^{32}/Ser^{36}. (A) The absolute mRNA expression of GRK5 and GRK6 in macrophages. Total RNA extracted from these macrophages was subjected to real time RT-PCR using an absolute quantification method. TEPM, thioglycollate-elicited peritoneal macrophages; PM, peritoneal macrophages; BMDM, bone marrow-derived macrophages; SM, splenic macrophages. ***$P < 0.001$. n = 3–4. (B) GRK6 interacted with IκBα. NIH3T3 cells infected with the empty retrovirus vector or the virus carrying the cDNA for Flag-tagged GRK6 were lysed and then immunoprecipitated with anti-Flag antibody. The immunoprecipitated and total samples were subjected to western blot with antibodies against Flag and IκBα. (C) Overexpression of GRK6 leads to IκBα phosphorylation at Ser^{32}/Ser^{36}. HEK293 cells transiently transfected with the indicated plasmids were lysed and subjected to western blot with anti-phospho-IκBα (p-IκBα) antibody, anti-IκBα antibody, and anti-Flag antibody. (D) GRK6 directly phosphorylated IκBα at Ser^{32}/Ser^{36}. *In vitro* kinase assays were performed and samples were subjected to western blot with antibodies against phospho-IκBα (p-IκBα), IκBα, and GRK6.

marrow, and spleen (Fig. 1A). Because GRK5 is demonstrated to phosphorylate IκBα at Ser^{32}/Ser^{36} and promote NF-κB signaling in the macrophage cell line RAW 264.7 [11], we postulated that GRK6 modulates IκBα phosphorylation and regulates inflammatory responses mediated by NF-κB signaling in macrophages. To test this idea, we first examined the interaction between GRK6 and IκBα. NIH3T3 cells-overexpressing Flag-GRK6 were lysed and immunoprecipitated with an antibody against Flag tag. Western blot analysis using anti-IκBα antibody revealed that GRK6 interacted with IκBα (Fig. 1B). We next investigated whether GRK6 was involved in the phosphorylation of IκBα at Ser^{32}/Ser^{36}. In HEK293 cells, the overexpression of GRK5 (WT) induced IκBα phosphorylation, as previously reported [11], whereas that of kinase-inactive mutant of GRK5, GRK5 (K215R), did not (Fig. 1C). Similarly, GRK6 (WT) but not GRK6 (K215R) strongly induced IκBα phosphorylation in HEK293 cells (Fig. 1C). We further examined if GRK6 directly phosphorylated IκBα. We purified GST-tagged IκBα and GST-IκBα (S32A/S36A), which was a dominant-negative mutant of IκBα, and performed an *in vitro* kinase assay. Consequently, phosphorylated IκBα at Ser^{32}/Ser^{36} was greatly increased in the presence of GRK6 (Fig. 1D). These results indicated that IκBα was a substrate for GRK6.

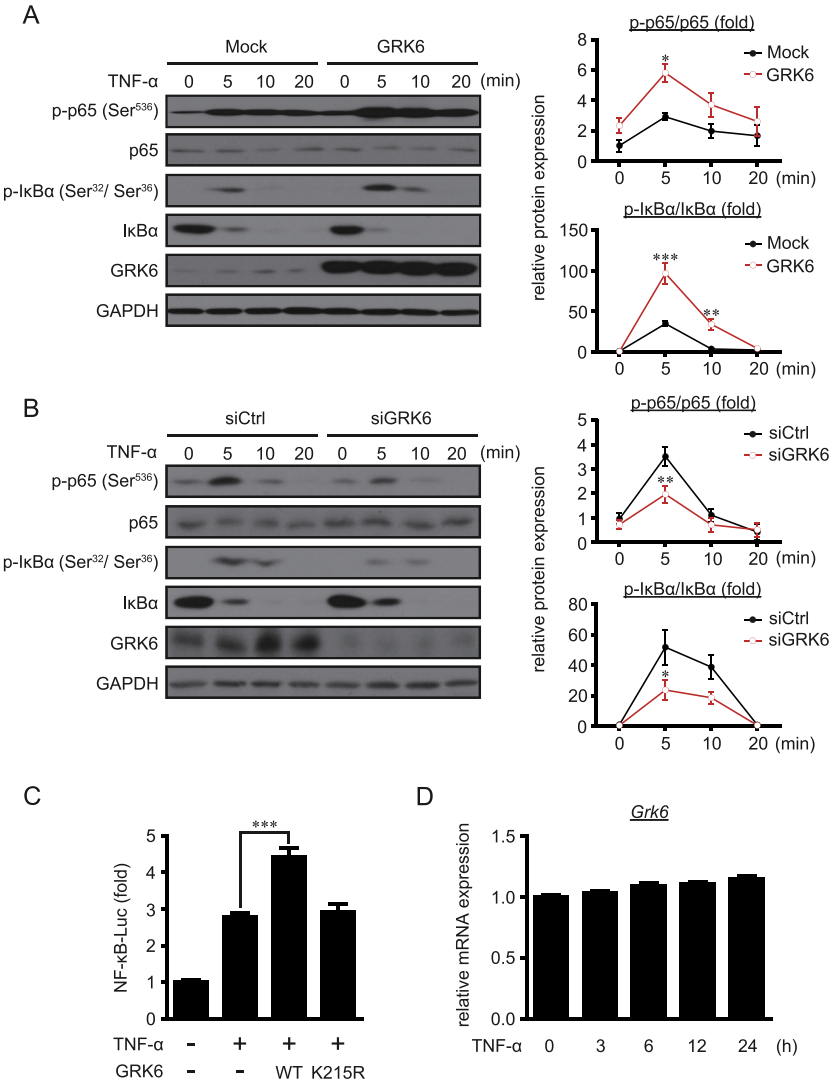

Fig. 2. GRK6 enhances TNF-α-induced NF-κB signaling in a kinase activity-dependent manner. (A) GRK6 overexpression enhanced TNF-α-induced NF-κB signaling. NIH3T3 cells were infected with the empty retrovirus vector or the virus carrying the cDNA for Flag-tagged GRK6 at the N-terminus. Thirty-six hours after infection, cells were serum-starved for 12 h and then stimulated with mouse TNF-α (10 ng/mL) for the indicated times. Samples were subjected to western blot with the indicated antibodies. Phospho-p65 (p-p65) and phospho-IκBα (p-IκBα) were normalized with total-p65 and total-IκBα, respectively. Black and red lines are mock and overexpression of GRK6, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. n = 3. (B) Knockdown of GRK6 inhibited TNF-α-induced NF-κB signaling. NIH3T3 cells were transfected with control (siCtrl) or GRK6 (siGRK6) siRNA. Thirty-six hours after transfection, cells were serum-starved for 12 h and then stimulated with mouse TNF-α (10 ng/mL) for the indicated times. Samples were subjected to western blot using the indicated antibodies. Phospho-p65 (p-p65) and phospho-IκBα (p-IκBα) were normalized with total-p65 and total-IκBα, respectively. Black and red lines are siCtrl and siGRK6, respectively. $^{*}P < 0.05$ and $^{**}P < 0.01$. n = 3. (C) The kinase activity of GRK6 contributes to the promotion of TNF-α-induced NF-κB transcriptional activation. NIH3T3 cells were transfected with empty vector, pcDNA3-Flag-GRK6, or pcDNA3-Flag-GRK6 (K215R). Luciferase reporter vectors were concomitantly transfected with each gene. Thirty-six hours after transfection, cells were serum-starved for 12 h and then stimulated with mouse TNF-α (10 ng/mL) for 6 h. Lysates were prepared and then firefly and *Renilla* luciferase (*R*luc) activities were analyzed. $^{***}P < 0.001$. n = 4. (D) The mRNA expression of GRK6 in NIH3T3 cells after TNF-α stimulation. NIH3T3 cells were stimulated with mouse TNF-α (10 ng/mL) for the indicated times. Total RNA extracted from NIH3T3 cells was subjected to real time RT-PCR. n = 4. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.2. GRK6 enhances TNF-α-induced NF-κB signaling in a kinase activity-dependent manner

Phosphorylation of IκBα at Ser^{32}/Ser^{36} induces IκBα degradation via the ubiquitin and proteasome pathway, leading to NF-κB activation [18]. Because TNF-α is a major cytokine to elicit IκBα phosphorylation, we treated NIH3T3 cells-overexpressing GRK6 with TNF-α to examine whether GRK6 regulates NF-κB signaling. TNF-α promotes the phosphorylation of IκBα at Ser^{32}/Ser^{36} and subsequently its degradation, resulting in the phosphorylation of p65 at Ser^{536}, one of the components of NF-κB. Phosphorylation of IκBα and p65 were further increased by the overexpression of GRK6 (Fig. 2A). Conversely, GRK6 knockdown by siRNA significantly decreased the phosphorylation level of these proteins after TNF-α stimulation (Fig. 2B). We then tried to determine whether the kinase activity of GRK6 is required for the promotion of TNF-α-induced NF-κB signaling. We performed luciferase assay using luciferase reporter plasmids containing an NF-κB response element (NF-κB-Luc) in NIH3T3 cells. When GRK6 (WT) was overexpressed, TNF-α-induced NF-κB activity was significantly enhanced. On the other hand, the overexpression of GRK6 (K215R) did not affect the activity (Fig. 2C). The mRNA expression of GRK6 was mostly unchanged in NIH3T3 cells after TNF-α stimulation (Fig. 2D). These results indicated that GRK6 enhanced TNF-α-induced NF-κB signaling in a kinase activity-dependent manner.

3.3. The conformational change of GRK6 is induced by TNF-α stimulation

Because GRK6 regulated TNF-α-mediated NF-κB signaling, we speculated that TNF-α induced the conformational change that affects GRK6 activity. To monitor the structural change of GRK6, we developed an intramolecular BRET probe that detects this change. A similar intramolecular BRET probe has been used to analyze the conformational change of β-arrestins [17,19,20]. As shown in Fig. 3A, we fused *R*luc(h) to the N terminus of GRK6 and GFP^2 to its C terminus. We overexpressed this *R*luc(h)-GRK6-GFP^2 construct in HEK293 cells and found that the BRET ratios were gradually increased after TNF-α stimulation (Fig. 3B). The time course of increase in BRET ratio after TNF-α stimulation was similar to that in TNF-α-induced IκBα phosphorylation in HEK293 cells (Fig. 3C). This data indicated that the conformation of GRK6 was changed by TNF-α stimulation.

3.4. Knockout or knockdown of GRK6 attenuates the transcription of inflammatory genes after TNF-α stimulation in TEPM

GRK6 enhanced NF-κB activity through the direct phosphorylation of IκBα. Thus, we finally examined whether GRK6 could promote the expression of inflammatory genes after TNF-α stimulation in TEPM. It has been well known that the expression levels of IL-1β, IL-6, and iNOS are increased by TNF-α stimulation depending on NF-κB activity [9,10]. Therefore, we measured the mRNA expression of these genes in TEPM treated with the siRNA targeting GRK6. As shown in Fig. 4A, the knockdown efficiency of GRK6 mRNA was approximately 50%. Knockdown of GRK6 significantly suppressed the transcription of IL-1β, IL-6, and iNOS after TNF-α stimulation (Fig. 4B). Furthermore, TEPM derived from GRK6-deficient mice also showed a significant decrease in the expression level of these genes after TNF-α stimulation (Fig. 4C). These results demonstrated that GRK6 enhanced inflammatory responses by promoting the transcription of NF-κB-regulated inflammatory genes in macrophages.

4. Discussion

GRK6 is one of the GRK subfamilies that were originally identified as kinases for GPCRs. In this report, we disclosed that GRK6 promoted inflammatory responses by phosphorylating IκBα at Ser^{32}/Ser^{36}. The direct phosphorylation of IκBα by GRK6 is required for the promotion of TNF-α-induced NF-κB activation that enhances the transcription of inflammatory genes in macrophages. Using a

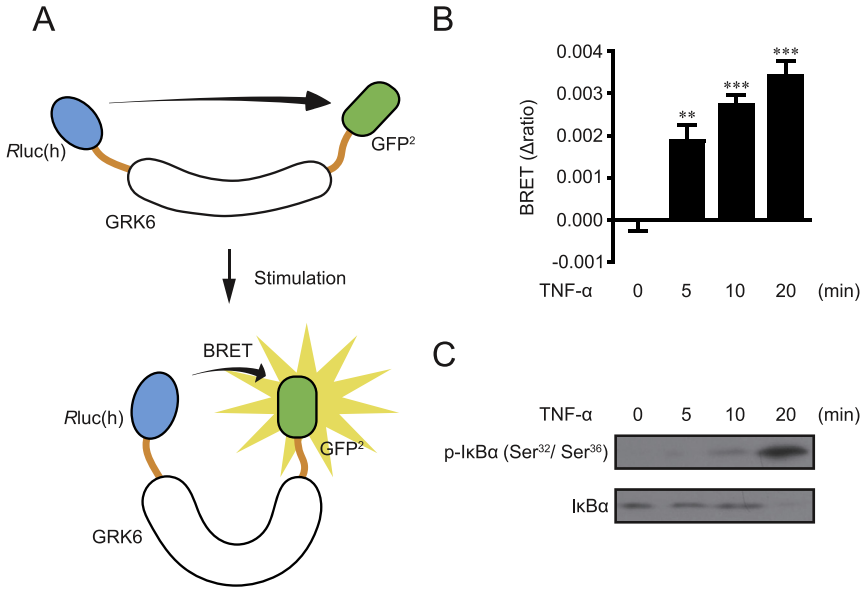

Fig. 3. The conformational change of GRK6 is induced by TNF-α stimulation. (A) Schematic image of the intramolecular BRET probe, *R*luc(h)-GRK6-GFP^2. The distance between *R*luc(h) and GFP^2 depends on the conformational changes of GRK6. BRET ratio increases when *R*luc(h) comes close to GFP^2. (B) The conformational change of GRK6 was induced by TNF-α stimulation. HEK293 cells were transfected with *R*luc(h)-GRK6-GFP^2. Forty-eight hours after the transfection, cells were harvested and split into a 96-well plate. Then, they were stimulated with mouse TNF-α (10 ng/mL) for the indicated times. After the stimulation, the *R*luc(h) substrate, DeepBlueC™ was added to a final concentration of 5 μM. Analysis was performed by calculating the ratio of the intensity of GFP^2 to that of *R*Luc(h). $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. TNF-α 0 min group. n = 6. (C) Time course of IκBα phosphorylation in HEK293 cells after TNF-α stimulation. HEK293 cells were stimulated with mouse TNF-α (10 ng/mL) for the indicated times. Samples were subjected to western blot using the indicated antibodies.

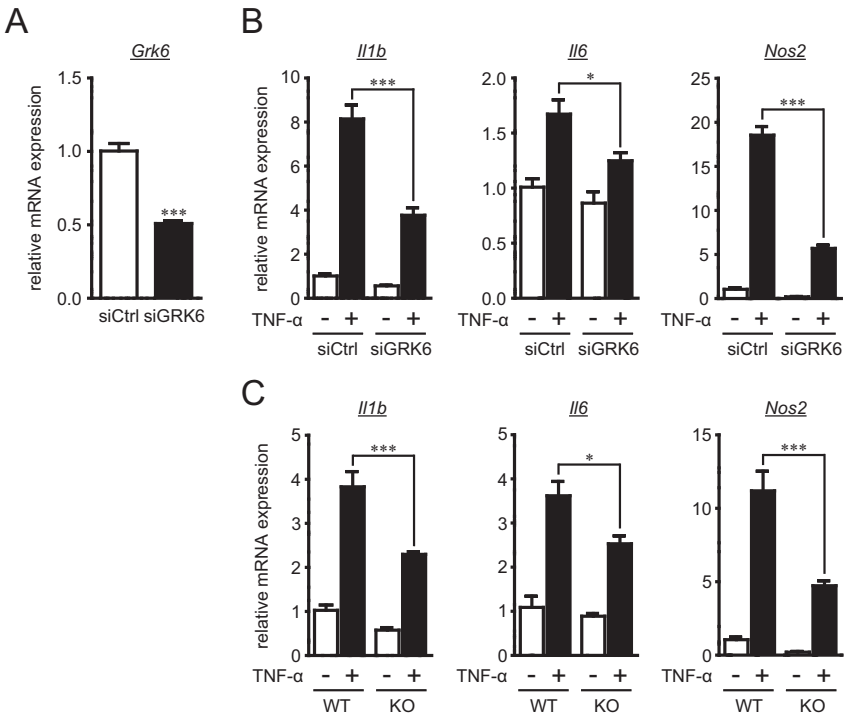

Fig. 4. Knockout or knockdown of GRK6 attenuates the transcription of inflammatory genes after TNF-α stimulation in TEPM. (A) The knockdown efficiency of GRK6 mRNA by siRNA in TEPM. Forty-eight hours after the transfection of siRNA, cells were harvested and subjected to real time RT-PCR. $^{***}P < 0.001$. n = 4. (B) The mRNA expression of inflammatory genes in control or GRK6-knockdown TEPM after TNF-α stimulation. Thirty-six hours after the transfection of siRNA, cells were serum-starved for 12 h and then stimulated with mouse TNF-α (10 ng/mL) for 3 h. The mRNA expression of inflammatory genes encoding IL-1β, IL-6, and iNOS was analyzed by real time RT-PCR. $^{*}P < 0.05$ and $^{***}P < 0.001$. n = 4. (C) The mRNA expression of inflammatory genes in WT or GRK6-deficient TEPM after TNF-α stimulation. Cells were serum-starved for 12 h and then stimulated with mouse TNF-α (10 ng/mL) for 3 h. The mRNA expression of inflammatory genes encoding IL-1β, IL-6, and iNOS was analyzed by real time RT-PCR. $^{*}P < 0.05$ and $^{***}P < 0.001$. n = 4–6.

BRET probe, *R*luc(h)-GRK6-GFP^2, our experiment also demonstrated that the conformational change of GRK6 may reflect GRK6 activation.

In this study, we revealed that GRK6 directly phosphorylated IκBα at Ser^{32}/Ser^{36} and regulated NF-κB signaling. On the other hand, GRK5, which belongs to the same subfamily as GRK6, was also reported to function as a kinase for IκBα [11]. As shown in Fig. 1C, when the expression levels of GRK5 and GRK6 were nearly the same, the amounts of phosphorylated IκBα induced by GRK5 and GRK6 were also almost the same. This result suggests that in macrophages, where the expression level of GRK6 was much higher than that of GRK5 (Fig. 1A), GRK6 rather than GRK5 contributes to NF-κB-regulated responses.

To date, it has long been obscure whether GRK6 undergoes conformational change when it is activated, though the structural analysis of GRK6 [21] suggested that GRK6 forms a different structure from that in the steady state when its kinase activity was changed. GRK6 was originally identified as a kinase that recognizes and phosphorylates activated GPCR, leading to its desensitization. Thus, it would be interesting to examine the conformational change of GRK6 during GPCR desensitization by the intramolecular BRET probe (Fig. 3A). This examination may reveal that the conformational change of GRK6 upon GPCR desensitization is different from that upon TNF-α stimulation (Fig. 3B).

GRK6 has been reportedly involved in the progression of various diseases, including Parkinson's disease [22], colitis [23], lung cancer [24], and autoimmune diseases [12]. Therefore, GRK6 is a potential therapeutic target for these diseases. Although antagonists for GRK2 have been discovered, such as paroxetine and balanol [25], those for other GRKs, including GRK6, have not been well developed. GRK2 antagonists, namely paroxetine and balanol, are reported to directly interact with GRK2 and induce conformational change at the kinase domain of GRK2 [25]. Therefore, it is expected that agonists or antagonists for GRK6 also change its structure. Our BRET assay system may be useful in the context of exploring therapeutic compounds that modulate the activity of GRK6 because it detects GRK6 conformational change.

In summary, we demonstrated for the first time that GRK6 directly phosphorylated IκBα at Ser^{32}/Ser^{36} and promoted TNF-α-induced inflammation. Although we newly identified IκBα as a substrate for GRK6, the intracellular substrates for GRK6 are largely unknown. Because GRK2 and GRK5 phosphorylate various intracellular proteins, such as single transmembrane proteins, cytosolic proteins, and nuclear proteins [4], we expect that GRK6 also phosphorylates these proteins and elicits its own functions. Further studies are required for the identification of GRK6 intracellular substrates and the specific roles of GRK6 that differ from GRK2 and GRK5.

Conflict of interest

All authors have no conflict of interest.

Acknowledgments

We thank Dr. R.T. Premont (Duke University) for GRK6-deficient mice. This study was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan [to M.N. (25713007) and H.K. (25253011)]; from Grant-in-Aid for JSPS Fellows (K.W.) (13J03105); The Takeda Science Foundation and The Naito Foundation, (M.N); from Platform for Drug Discovery, Informatics, and Structural Life Science from the Ministry of Education, Culture, Sports, Science and Technology, Japan. (M.N., H.K.)

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.027.

References

[1] C. Ribas, P. Penela, C. Murga, et al., The G protein-coupled receptor kinase (GRK) interactome: role of GRKs in GPCR regulation and signaling, Biochim. Biophys. Acta 1768 (2007) 913–922.

[2] E. Reiter, R.J. Lefkowitz, GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling, Trends Endocrinol. Metab. 17 (2006) 159–165.

[3] K. Watari, M. Nakaya, H. Kurose, Multiple functions of G protein-coupled receptor kinases, J. Mol. Signal 9 (2014) 1.

[4] E.V. Gurevich, J.J. Tesmer, A. Mushegian, et al., G protein-coupled receptor kinases: more than just kinases and not only for GPCRs, Pharmacol. Ther. 133 (2012) 40–69.

[5] S. Peregrin, M. Jurado-Pueyo, P.M. Campos, et al., Phosphorylation of p38 by GRK2 at the docking groove unveils a novel mechanism for inactivating p38MAPK, Curr. Biol. 16 (2006) 2042–2047.

[6] M. Hinz, C. Scheidereit, The IkappaB kinase complex in NF-kappaB regulation and beyond, EMBO Rep. 15 (2014) 46–61.

[7] V.F. Shih, R. Tsui, A. Caldwell, et al., A single NFkappaB system for both canonical and non-canonical signaling, Cell. Res. 21 (2011) 86–102.

[8] D. Bhatt, S. Ghosh, Regulation of the NF-kappaB-mediated transcription of inflammatory genes, Front. Immunol. 5 (2014) 71.

[9] P. Viatour, M.P. Merville, V. Bours, et al., Phosphorylation of NF-kappaB and IkappaB proteins: implications in cancer and inflammation, Trends Biochem. Sci. 30 (2005) 43–52.

[10] D. Fukumura, S. Kashiwagi, R.K. Jain, The role of nitric oxide in tumour progression, Nat. Rev. Cancer 6 (2006) 521–534.

[11] S. Patial, J. Luo, K.J. Porter, et al., G-protein-coupled-receptor kinases mediate TNFalpha-induced NFkappaB signalling via direct interaction with and phosphorylation of IkappaBalpha, Biochem. J. 425 (2010) 169–178.

[12] M. Nakaya, M. Tajima, H. Kosako, et al., GRK6 deficiency in mice causes autoimmune disease due to impaired apoptotic cell clearance, Nat. Commun. 4 (2013) 1532.

[13] S. Morita, T. Kojima, T. Kitamura, Plat-E: an efficient and stable system for transient packaging of retroviruses, Gene Ther. 7 (2000) 1063–1066.

[14] K. Watari, M. Nakaya, M. Nishida, et al., beta-arrestin2 in infiltrated macrophages inhibits excessive inflammation after myocardial infarction, PLoS One 8 (2013) e68351.

[15] S. Takeshita, K. Kaji, A. Kudo, Identification and characterization of the new osteoclast progenitor with macrophage phenotypes being able to differentiate into mature osteoclasts, J. Bone Min. Res. 15 (2000) 1477–1488.

[16] M. Nakaya, M. Tanaka, Y. Okabe, et al., Opposite effects of rho family GTPases on engulfment of apoptotic cells by macrophages, J. Biol. Chem. 281 (2006) 8836–8842.

[17] M. Nakaya, S. Chikura, K. Watari, et al., Induction of cardiac fibrosis by beta-blocker in G protein-independent and G protein-coupled receptor kinase 5/beta-arrestin2-dependent signaling pathways, J. Biol. Chem. 287 (2012) 35669–35677.

[18] M.S. Hayden, S. Ghosh, Regulation of NF-kappaB by TNF family cytokines, Semin. Immunol. 26 (2014) 253–266.

[19] P.G. Charest, S. Terrillon, M. Bouvier, Monitoring agonist-promoted conformational changes of beta-arrestin in living cells by intramolecular BRET, EMBO Rep. 6 (2005) 334–340.

[20] A.K. Shukla, J.D. Violin, E.J. Whalen, et al., Distinct conformational changes in beta-arrestin report biased agonism at seven-transmembrane receptors, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 9988–9993.

[21] D.T. Lodowski, V.M. Tesmer, J.L. Benovic, et al., The structure of G protein-coupled receptor kinase (GRK)-6 defines a second lineage of GRKs, J. Biol. Chem. 281 (2006) 16785–16793.

[22] R.R. Gainetdinov, L.M. Bohn, T.D. Sotnikova, et al., Dopaminergic supersensitivity in G protein-coupled receptor kinase 6-deficient mice, Neuron 38 (2003) 291–303.

[23] N. Eijkelkamp, C.J. Heijnen, A. Lucas, et al., G protein-coupled receptor kinase 6 controls chronicity and severity of dextran sodium sulphate-induced colitis in mice, Gut 56 (2007) 847–854.

[24] S.K. Raghuwanshi, N. Smith, E.J. Rivers, et al., G protein-coupled receptor kinase 6 deficiency promotes angiogenesis, tumor progression, and metastasis, J. Immunol. 190 (2013) 5329–5336.

[25] D.M. Thal, K.T. Homan, J. Chen, et al., Paroxetine is a direct inhibitor of g protein-coupled receptor kinase 2 and increases myocardial contractility, ACS Chem. Biol. 7 (2012) 1830–1839.