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G-PROTEIN COUPLED RECEPTOR KINASES MEDIATE TNF α -INDUCED NF κ B SIGNALING VIA DIRECT INTERACTION WITH AND PHOSPHORYLATION OF I κ B α

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Abstract

Tumor necrosis factor- α (TNF α) is a multifunctional cytokine involved in the pathophysiology of many chronic inflammatory diseases. TNF α activation of the nuclear factor κ B (NF κ B) signaling pathway particularly in macrophages has been implicated in many diseases. We demonstrate here that G-protein coupled receptor kinase-2 and 5 (GRK2 and 5) regulate TNF α -induced NF κ B signaling in Raw264.7 macrophages. RNAi knockdown of GRK2 or 5 in macrophages significantly inhibits TNF α -induced I κ B α phosphorylation and degradation, NF κ B activation, and expression of the NF κ B-regulated gene, macrophage inflammatory protein-1 β . Consistent with these results, over-expression of GRK2 or 5 enhances TNF α -induced NF κ B activity. In addition, we show that GRK2 and 5 interact with I κ B α via the N-terminal domain of I κ B α and that I κ B α is a substrate for GRK2 and 5 *in vitro*. Furthermore, we also find that GRK5 but not GRK2 phosphorylates I κ B α at the same amino acid residues (Ser32/36) as that of IKK β . Interestingly, associated with these results, knockdown of IKK β in Raw264.7 macrophages did not affect TNF α -induced I κ B α phosphorylation. Taken together, these results demonstrate that both GRK2 and 5 are important and novel mediators of a non-traditional I κ B α -NF κ B signaling pathway.

Introduction

Tumor necrosis factor- α (TNF α) is a pleiotropic cytokine secreted by immune cells, in particular by monocytes and macrophages and mediates a number of biological activities ranging from cell proliferation, differentiation and death to inflammation and innate and adaptive immune responses [1]. Importantly, TNF α has been implicated in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis and Crohn's disease and several drugs targeting the TNF α system are already in clinical practice [2]. TNF α mediates its diverse effects through two cell surface receptors: p55TNFR1 and p75TNFR2. TNFR1 is constitutively expressed in most nucleated cells whereas TNFR2 is expressed mainly in immune and endothelial cells. Stimulation of TNFR1 leads to the recruitment of several death domain containing adapter proteins such as TRADD (Tumor necrosis factor receptor 1

associated death domain protein) and RIP1 (Receptor interacting protein-1). This signaling complex interacts with adapter proteins such as TRAF2/5 (TNF receptor associated factor 2/5) and c-IAP1 (Inhibitor of apoptosis 1) which subsequently leads to the activation of signaling pathways that regulate many of the important biological activities of TNF α [3]. Especially important from a physiological as well as pathological perspective is the major role of the “Nuclear factor κ B (NF κ B)” signaling pathway in chronic inflammatory disease [4].

The NF κ B family of transcription factors regulate genes involved in inflammation, innate and adaptive immune responses, cell proliferation, cell adhesion, programmed cell death (apoptosis), and cellular stress response and tissue remodeling (reviewed in [5]). The NF κ B family members include p65 (RelA), p50, RelB, cRel and p52. These NF κ B transcription factors are sequestered in the cytoplasm in the form of homo- or heteromeric complexes with the inhibitory proteins of the I κ B family. The I κ B family members include I κ B α , I κ B β , I κ B ϵ , p105 (NF κ B1), and p100 (NF κ B2). Activation of the NF κ B pathway typically involves the phosphorylation of an I κ B member primarily by the I κ B kinase (IKK) complex. The phosphorylated I κ B then undergoes ubiquitination and subsequent proteolysis leading to the release and translocation of NF κ B into the nucleus where it affects gene transcription (reviewed in [5]). Amongst the various I κ B members, I κ B α plays a critical role in the early activation of NF κ B after ligand stimulation [6]. I κ B α binds to NF κ B subunits p50 and p65 under unstimulated conditions. Upon stimulation I κ B α undergoes rapid phosphorylation by IKK β , followed by ubiquitination and degradation. I κ B α degradation releases the NF κ B subunits p50 and p65, which then translocate into the nucleus to evoke NF κ B-dependent gene transcription (reviewed in [5]). In addition to being physiological substrates for the IKK complex of enzymes, the I κ B family members have also been shown to be substrates for other kinases [7]. In this context, we demonstrated recently that p105 is a substrate for G-protein coupled receptor kinase-5 (GRK5) and that GRK5 phosphorylation of p105 regulates Toll-like receptor-4-induced p105 phosphorylation in macrophages [8].

G-protein coupled receptor kinases (GRKs) are serine/threonine kinases originally discovered for their role in the phosphorylation of G-protein coupled receptors (GPCRs) (reviewed in [9]). The seven mammalian GRKs are divided into three subfamilies based on sequence and functional similarities. The rhodopsin kinase subfamily (GRK1 and GRK7); the GRK2 subfamily (GRK2 and GRK3) and the GRK4 subfamily (GRK4, 5 and 6). All GRKs have a similar structural organization possessing N-terminal, catalytic and C-terminal domains. Interestingly, recent studies have shown that GRKs have functions that go beyond their role in GPCR phosphorylation. For example, GRKs have been shown to phosphorylate a number of cytoplasmic and nuclear proteins as well as additional classes of membrane-localized receptors [10–12]. Moreover, yeast-two hybrid analysis identified NF κ B1 p105 as a GRK2-interacting protein (<http://www.signalinggateway.org/data/Y2H/cgi-bin/y2h.cgi>) while we found that GRK5 but not GRK2 regulates p105 function in macrophages [8].

To better understand the role of GRKs in NF κ B signaling in macrophages and to identify the potential role of these kinases in the regulation of other I κ B members, we tested the role of GRK2 and 5 in the regulation of I κ B α in the context of TNF α signaling in macrophages. Surprisingly, in contrast to the regulation of p105, our studies reveal that both GRK2 and 5 regulate the TNF α -induced I κ B α -NF κ B pathway in Raw264.7 mouse macrophages. We further demonstrate that I κ B α directly interacts with GRK2 and 5 and is differentially phosphorylated by GRK2 and 5. Our observations add new insight into NF κ B signaling as well as demonstrate novel GPCR-independent roles for GRKs.

Materials and Methods

Materials

Mouse TNF α was from PeproTech Inc. (Rocky Hill, NJ). IRDye® 700 labelled NF κ B oligonucleotide probes were from LI-COR Biosciences (Lincoln, NE). Protease inhibitor cocktail tablets were from Roche Diagnostics (Indianapolis, IN). Luciferase assay buffer and substrate were from Promega (Madison, WI). All other reagents were from Sigma unless otherwise noted.

Antibodies—Anti-P50, P65, Lamin B, Protein A/G PLUS-agarose beads, Actin-HRP and polyclonal I κ B α antibody were from Santa Cruz Biotechnology (Santa Cruz, CA), anti-mouse I κ B α and phospho-I κ B α antibodies were from Cell Signaling Technology (Boston, MA), HA monoclonal antibody was from Covance and HA polyclonal antibody from Sigma. GRK2 and 5 monoclonal antibodies were from Upstate Biotechnology.

Plasmids—pcDNAGRK2, pcDNAGRK2-K220R, pcDNAGRK5 and pcDNAGRK5-K215R have been described previously [13,14]. HA-GRK2 was created by excising GRK2 from pRK5-GRK2 construct with EcoRI and SalI and then inserting into pHA-CMV vector. Expression plasmid pCMX-I κ B α (Addgene plasmid 12331) was from Dr. I. M. Verma [15], HA-IKK β (Addgene plasmid # 15470) from Dr. H. Nakano [16], Flag-IKK γ (Addgene plasmid # 11970) from Dr. J. D. Ashwell [17], and Flag-I κ B α from Dr. G. Pei [18]. Adenoviruses expressing GRK2 and GRK5 were kindly provided by Dr. W. J. Koch.

Cell Culture

Raw 264.7 macrophages and HEK293T cells were obtained from ATCC and were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and penicillin (100 units/ml) and streptomycin (100 μ g/ml) at 37° C in 5% CO₂.

RNA interference

Control siRNA pool (against luciferase gene not expressed in macrophages), GRK2 siRNA pool (against mouse GRK2), GRK5 siRNA pool (against mouse GRK5) and IKK β siRNA pool (against mouse IKK β) were purchased from Dharmacon (Dharmacon Research Inc., Lafayette, CO). Raw264.7 macrophages were transfected with siRNA using Amaxa nucleofector (Program D-032) as described previously [8]. The cells were analyzed for knockdown by Western blotting after 48 hours of transfection.

Luciferase assay

HEK293T cells were transfected with an NF κ B promoter luciferase plasmid (pELAM luciferase plasmid [19,20] kindly provided by Dr. E. Latz, University of Massachusetts) and LacZ-expression plasmid [21] (kindly provided by Dr. Philip B. Wedegaertner, Thomas Jefferson University) together with either vector (control) or GRK2/5 wild type or GRK2-K220R/GRK5-K215R kinase deficient expression plasmids. Cells were stimulated with TNF α for 8 hours, lysates prepared and analysed for NF κ B luciferase activity using Promega Luciferase Reporter Assay kit according to manufacturer's instructions. β -Gal activity was determined as described previously [21]. NF κ B luciferase activity was expressed as a ratio of luciferase to beta-galactosidase activity. All experiments were performed in triplicate and repeated at least 3–5 times.

Nuclear Extracts

Nuclear extracts were prepared as described in Nuclear extraction kit (Panomics, CA). Briefly, control or GRK2 knockdown cells in a 60 mm plate were lysed in 500 μ l of the buffer A mix [buffer A (100 mM HEPES, pH 7.9, 100 mM KCl, 100 mM EDTA), 100 mM DTT, 10 μ l protease inhibitor cocktail and 0.38% NP-40]. Sonication was done to disrupt the cell clumps. The lysate was then centrifuged at a maximum speed ($15,000 \times g$) for 3 min at 4°C. Supernatants (cytosolic fraction) were collected and the pellet suspended in 100 μ l of buffer B mix [buffer B (100 mM HEPES, pH 7.9, 2 M NaCl, 5 mM EDTA), protease inhibitor cocktail and 100 mM DTT). The lysates were kept on a rocking platform for ~2 hours, after which the lysates were centrifuged at high speed for 20 min and the supernatants (nuclear fraction) were collected for further analysis.

Electrophoretic mobility shift assay (EMSA)

Electrophoretic mobility shift assays (EMSA) were performed using nuclear extracts [6]. Briefly, ~9 fmol of double stranded oligonucleotide probes corresponding to the human consensus NF κ B sequence end labeled with IRDye® 700 were incubated with 5 μ g of nuclear extracts for 20 min in the dark at room temperature. Samples were then subjected to electrophoresis using 6% non-denaturing polyacrylamide gels and then analysed on LI-COR's odyssey.

Co-immunoprecipitation and Western Blotting

HEK293T cells were transiently transfected using Fugene (Roche) with the indicated expression plasmids in 60-mm cell culture dishes. The cells were washed twice with ice-cold phosphate-buffered saline after 48 hours and were lysed in 400 μ l of cold lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 10% glycerol, 1% NP-40 containing protease and phosphatase inhibitors). Lysates were then incubated with antibody and protein A/G PLUS-agarose beads for 1 hour at 4° C. The beads were washed three times with lysis buffer and the immune complexes eluted with 2X SDS sample buffer. The eluates were then run on SDS-PAGE and transferred to nitrocellulose. Western blotting was then performed as described previously with either fluorescently tagged secondary antibodies using Licor or with HRP-conjugated secondary antibodies using chemiluminescence [22].

Glutathione S-transferase (GST) protein expression and purification

GST (GE HealthCare Biosciences, NJ), GST-I κ B α and GST-I κ B α (Δ 1–75) [23] (kindly provided by Dr. Junan Li, Ohio State University) in pGEX bacterial expression plasmids were expressed and purified as described by the manufacturer (Amersham Biosciences). Briefly, overnight bacterial cultures were diluted 1:100 in 100 ml of fresh LB-ampicillin, grown for 2–3 hours until the OD reached 0.5–0.9. The culture was then induced with IPTG (1 mM) and incubated for another 3 hours after which the cells were pelleted ($6000 \times g$ for 10 min at 4° C) and resuspended in ice cold PBS. Suspended cells were then disrupted using a sonicator and solubilized in 1% Triton-X-100. The cells were then centrifuged at $12,000 \times g$ for 10 min at 4° C and the supernatants collected. These supernatants were incubated with 2 ml of the 50% slurry of Glutathione Sepharose 4B equilibrated with PBS for 30 min and the resin was transferred to a column (Empty Disposable PD-10 column). The column was washed 3 times with PBS after which the fusion proteins were eluted by adding 1 ml of elution buffer (50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0). The purity and integrity of the fusion proteins were analysed and confirmed by SDS-PAGE and Coomassie blue staining.

Overlay assay

Interaction between GST fusion proteins and GRK2 or 5 were determined using an overlay assay [24]. Briefly, GST-fusion proteins (5 µg) were resolved on a 10% SDS-PAGE gel and transferred to nitrocellulose membranes. Membrane was then blocked with 5% w/v fat-free milk in TBS-T and incubated overnight at 4° C in lysates (~400 µg total protein) of HEK293T cells expressing vector, HA-GRK2, or GRK5. Blots were then washed three times with TBST (Tris- Buffered saline-Tween 20) and immuno blotting performed using appropriate antibodies. Blots were then stained with Ponceau to confirm equivalent amount of GST-fusion protein loading.

Phosphorylation assay

In vitro phosphorylation reactions were performed using purified GRK2 and GRK5 with IκBα as a substrate [8]. Purified GST-IκBα or GST (~200 nM each) were incubated with 25 nM GRK2 or GRK5 at 30°C for 15 min in the presence or absence of 2 mg/ml soybean phosphatidylcholine and 60 nM purified Gβγ in 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 5 mM MgCl₂, 0.2 mM ATP, 1–2 µCi of [³²P]ATP in a final volume of 20 µl. Reactions were stopped by the addition of 5 µl of SDS sample buffer and incubation at room temperature for 30 min. Samples were then electrophoresed on a 10% SDS-polyacrylamide gel, the gel was dried, and ³²P-labeled IκBα was visualized by autoradiography and quantified by excising the bands and scintillation counting.

Real time Q-RT-PCR

RNA extraction and real-time Q-RT-PCR were performed as described previously [22]. Briefly, total RNA was extracted using TRIzol reagent (Invitrogen). After sodium acetate–ethanol precipitation and several ethanol washes, the RNA integrity was verified by formaldehyde–agarose gel electrophoresis. Synthesis of cDNA was performed with reverse transcriptase (RT) with the total RNA using the superscript II kit with Oligo-dt (12–18) primers as recommended by the manufacturer (Invitrogen). cDNA was amplified by PCR in a final reaction volume of 25 µl using SYBR Green Supermix (Invitrogen) with 10 pmol of each primer for MIP1β and cyclophilin (for normalization) (primers obtained from IDT DNA Technologies, primer sequence available upon request). Real-time PCR was performed using MX3000P (Stratagene) thermocycler and data analyzed using MX3000P software.

Statistical analysis

All values are represented as mean±SEM. Data were analyzed and statistics performed using GRAPHPAD PRISM software (San Diego, California) using student's t-test (for comparing two groups) and ANOVA (for comparing three or more groups). P value of less than 0.05 was considered significant.

Results

GRK2 mediates TNFα-induced NFκB activation in Raw264.7 macrophages

Previous studies have shown that of the seven GRKs, GRK2 and 5 are expressed at relatively high levels in immune cells including macrophages [25,26]. Furthermore, recent studies have shown that GRKs can regulate GPCR as well as non-GPCR signaling in macrophages [8]. Because TNF receptor-induced NFκB signaling plays a major role in macrophage biology and has been implicated in many chronic inflammatory diseases [4,27], we explored the role of GRKs in TNFα-induced NFκB signaling in macrophages. For this, we first tested the effect of GRK2 knockdown using siRNA pool, on TNFα-induced IκBα phosphorylation and degradation. Treatment of control macrophages with TNFα caused a

time-dependent increase in I κ B α phosphorylation (at serines-32/36) and a subsequent decrease in I κ B α levels (Fig 1). I κ B α phosphorylation was maximal at 5 min after TNF α stimulation, whereas, the consequent decrease in I κ B α levels was maximal at 15 min after stimulation in control cells. However, the TNF α -mediated increase in I κ B α phosphorylation and decrease in I κ B α levels were both significantly blocked in GRK2 knockdown macrophages. Compared to the maximal stimulation of 95 $\pm$ 5% at 5 min in control cells, I κ B α phosphorylation reached only 40 $\pm$ 2% in GRK2 knockdown cells (Fig. 1B). Consistent with the effects on I κ B α phosphorylation, I κ B α degradation was also inhibited in GRK2 knockdown cells compared to control cells. At 15 min after treatment I κ B α levels reached 62 $\pm$ 7% of untreated levels (=100%) in control cells, whereas it reached only 94 $\pm$ 5% in GRK2 knockdown cells (Fig 1C). Importantly, I κ B α levels did not differ between control and GRK2 knockdown cells in the absence of TNF α treatment (0.136 $\pm$ 0.057 in control cells v/s 0.134 $\pm$ 0.057 in GRK2 knockdown cells), suggesting that the effect of GRK2 knockdown on I κ B α levels is specific for stimulated conditions. Furthermore, to rule out the nonspecific effects of the siRNA, we repeated these experiments using individual siRNAs and obtained similar results (Supplementary Figure 1). In addition, siRNA oligos that did not knockdown GRK2 levels did not affect TNF α -induced I κ B α phosphorylation (data not shown). Interestingly, the effect of GRK2 knockdown on I κ B α was specific for TNF α -induced pathway because LPS-induced I κ B α degradation was not inhibited by GRK2 knockdown (Supplementary figure 2).

Because I κ B α phosphorylation and degradation leads to subsequent release and translocation of the NF κ B subunits, primarily p50 and p65, we next examined the nuclear levels of the NF κ B subunits in control and GRK2 knockdown cells before and after TNF α treatment. As with I κ B α phosphorylation/degradation, TNF α -induced p50 and p65 nuclear translocation were significantly inhibited in GRK2 knockdown cells compared to control cells (Fig 1D). These effects of GRK2 are specifically due to a decrease in nuclear translocation and not a decrease in expression levels of these proteins since the cytosolic expression of p50 and p65 did not differ between control and GRK2 knockdown cells (data not shown). Associated with these findings, we also observed that the NF κ B binding activity (assessed by EMSA) was inhibited in GRK2 knockdown macrophages (Fig 1E).

Over-expression of GRK2 causes an increase in TNF α induced I κ B α phosphorylation

To further rule out the possibility of non-specific effects of RNAi, we tested the effects of GRK2 over-expression in TNF α -induced I κ B α phosphorylation in macrophages. Raw264.7 macrophages were transfected with adenoviruses expressing vector or GRK2 and the effect of TNF α -induced I κ B α phosphorylation tested as described earlier. As predicted, over-expression of GRK2 significantly enhanced TNF α -induced I κ B α phosphorylation compared to vector controls (Fig 2).

Role of GRK5 in TNF α -induced I κ B α phosphorylation and degradation

We next tested whether other GRKs can regulate I κ B α phosphorylation/degradation. We especially focused on GRK5 since we previously showed that GRK5 inhibits LPS-induced p105 phosphorylation in macrophages [8]. Interestingly, similar to the effects of GRK2, knockdown of GRK5 using siRNA pool (Fig. 3A) significantly inhibited TNF α -induced I κ B α phosphorylation and degradation (Fig 3B, C & D). TNF α -stimulated I κ B α phosphorylation (Ser32/36) in GRK5 knockdown cells reached only 27 $\pm$ 5% of the maximal response after 5 min compared to 100% in control cells (Fig 3C). Similarly, I κ B α levels after 15 min of TNF α stimulation reached 62 $\pm$ 6% of untreated levels in control cells but only 99 $\pm$ 5% in GRK5 knockdown cells (Fig 3D). Unlike GRK2 knockdown, basal levels of I κ B α were somewhat elevated after GRK5 knockdown (0.361 $\pm$ 0.080 in control vs. 0.563 $\pm$ 0.071 in GRK5 knockdown cells). Similar to GRK2 siRNAs, individual siRNAs

against GRK5 also gave similar results to that of the pool (Supplementary Figure 3). Interestingly, over-expression of GRK5 using adenovirus only modestly enhanced I κ B α phosphorylation (Supplementary Figure 4).

Role of GRKs is specific for I κ B α -NF κ B pathway

In addition to I κ B α phosphorylation, TNF α treatment also induces NF κ B1 p105 (another member of the I κ B family) phosphorylation at Ser932 [28]. Previous studies have shown that GRK2 and 5 can interact with p105 and that GRK5 negatively regulates LPS-induced p105 phosphorylation [8]. To determine whether the observed effects of GRK2/5 knockdown are specific for TNF α -induced I κ B α phosphorylation or whether p105 phosphorylation can also be regulated in a similar manner, we examined TNF α -induced p105 phosphorylation in control and GRK2/5 knockdown macrophages. Interestingly, unlike I κ B α phosphorylation, GRK2/5 knockdown did not affect TNF α -induced p105 phosphorylation (at Ser932) (Supplementary Figure 5 and Supplementary Figure 6). This suggests that the role of GRK2/5 is specific for TNF α -induced I κ B α -NF κ B pathway.

Regulation of NF κ B-dependent gene expression by GRK2 and 5 in macrophages

I κ B α -NF κ B pathway was recently shown to be critical for the expression of MIP1 β (macrophage inflammatory protein 1 β), one of the major chemokines expressed by macrophages [29]. Therefore, we tested whether the role of GRK2 and 5 on the TNF α -induced NF κ B pathway extended downstream of NF κ B activation to MIP1 β mRNA expression. Treatment of control macrophages with TNF α induced MIP1 β mRNA expression by ~4–5-fold. This increase in MIP1 β expression was significantly blocked in both GRK2 and 5 knockdown macrophages (Fig 4A and 4B). These results demonstrate that GRK2 and 5 regulate TNF α -induced I κ B α -NF κ B pathway as well as its physiological gene expression target in macrophages.

Kinase activity of GRK2 and 5 is required for TNF α -induced NF κ B-dependent gene transcription

To further explore the biochemical mechanism by which GRK2 and 5 mediate TNF α -induced NF κ B activity, we next tested whether the kinase activity is essential for the observed effects of the GRKs. For this, we over-expressed vector or wild type or kinase dead GRK2 or GRK5 in HEK293T cells along with an NF κ B reporter plasmid (pELAM luciferase) and LacZ (for transfection normalization). Forty-hours after transfection, cells were serum starved (~3 hours) and were stimulated (or not) with TNF α for 8 hours and luciferase and β -galactosidase activity determined as described in the methods. In vector transfected cells, TNF α stimulation significantly increased NF κ B-luciferase activity (Fig 5). As predicted, over-expression of wild type GRK2 or GRK5 significantly enhanced TNF α -induced NF κ B activity while over-expression of kinase-inactive GRK2 or GRK5 (GRK2-K220R or GRK5-K215R) had no effect (Fig 5A and 5B). This demonstrates that the kinase activities of GRK2 and 5 are required for the observed effects of GRKs in TNF α -induced NF κ B signaling. Interestingly, as observed with I κ B α phosphorylation in macrophages, over-expression of GRK2 but not GRK2-K220R enhanced NF κ B-luciferase activity even in the absence of ligand stimulation (1.0 ± 0.51 activity in vector cells compared to 2.35 ± 1.07 in GRK2 and 0.70 ± 0.32 in GRK2-K220R expressing cells). In contrast, over-expression of GRK5 or GRK5-K215R did not significantly affect basal NF κ B activity (1 ± 0.14 activity in vector cells compared to 1.51 ± 0.17 in GRK5 and 0.86 ± 0.04 in GRK5-K215R expressing cells).

Interaction of GRK2 and 5 with I κ B α

To define the biochemical mechanisms that mediate GRKs' actions, we first tested whether GRKs affect IKK β expression or activity. For this purpose, we knocked down and over-expressed GRK2/5 in Raw264.7 macrophages and HEK293T cells respectively and examined the levels of IKK β . Neither knockdown nor over-expression of GRK2/5 affected IKK β levels in the presence or absence of TNF α (data not shown). To further rule out the effect of GRKs on IKK β activity, we performed an *in vitro* IKK β kinase assay using IKK β immunoprecipitated from cells over-expressing GRK2/5. *In vitro* phosphorylation of GST-I κ B α by immunoprecipitated IKK β was not affected by over-expression of either GRK2 or GRK5 (data not shown). Similarly, the interaction of I κ B α and IKK β was not affected by over-expression of GRKs in HEK293 cells (data not shown). Based on these results we hypothesized that GRKs might mediate their effects via directly interacting with and phosphorylating I κ B α . To first examine if GRK2 or 5 can interact directly with I κ B α , we tested the ability of GST or GST-I κ B α to bind GRK2 and 5 using an overlay blot assay. For this, bacterially expressed and purified GST or GST-I κ B α or GST-I κ B α (76–302) were run on SDS-PAGE, and transferred to nitrocellulose. The membranes were then incubated with HEK293T lysates over-expressing either vector, GRK2 or GRK5 and tested for the ability of GRKs to specifically bind to GST-I κ B α . As hypothesized, both GRK2 (Fig 6A and B) and GRK5 (Fig 7A and B) bound to GST-I κ B α with no significant binding to GST. In addition, neither GRK2 (Fig 6A) nor GRK5 (Fig 7A) interacted appreciably with an N-terminal deletion mutant of I κ B α [I κ B α (76–302)], suggesting that both GRKs primarily interact with I κ B α at the N-terminus. We also tested the ability of GRKs to interact with I κ B α in intact cells and found that immunoprecipitation of I κ B α co-immunoprecipitated both GRK2 and 5 (Fig 6C and Fig 7C in HEK293 cells; Fig 6D in human monocytic cells THP1). Taken together, these results suggest that I κ B α is a direct interaction partner for both GRK2 and GRK5.

Phosphorylation of I κ B α by GRK2 and 5

Experiments using kinase-dead mutants of GRK2 and 5, as well as the experiments described above, suggest that I κ B α may be a substrate for GRKs in TNF α -induced NF κ B signaling. To directly test this, we performed *in vitro* phosphorylation assays using purified GRK2 and 5 with I κ B α as the substrate. GRK5 effectively phosphorylated I κ B α to a stoichiometry of ~0.75 mol/mol (Fig. 8A) and interestingly, phosphorylation was effectively attenuated by the addition of phospholipids, which normally activate GRK5 (Fig. 8B) [30]. In contrast, I κ B α was a relatively poor substrate for GRK2 (Fig 8A), although the phosphorylation was enhanced by the addition of G β γ subunits and phospholipids, known activators of GRK2 (Fig. 8B) [31]. Taken together, these results demonstrate that I κ B α is an *in vitro* substrate for both GRK2 and GRK5.

To identify the GRK phosphorylation sites in I κ B α and to examine if I κ B α is differentially phosphorylated by GRK2 and 5, we first tested whether the known IKK β phosphorylation sites (Ser32/36) are also phosphorylated by GRK2 or GRK5. Indeed, previous studies have shown that these two residues are targeted by additional kinases such as ribosomal S6K and CK II, especially in NF κ B pathways that are largely IKK β -independent [32,33]. To test this, we assessed the ability of GRK2 and GRK5 to phosphorylate a GST-I κ B α S32/36A mutant. Our results show that GRK2 mediated phosphorylation of wild type and mutant GST-I κ B α was comparable, suggesting that these sites are not phosphorylated by GRK2 (Fig 8C). In contrast, GRK5 mediated phosphorylation of the I κ B α mutant was decreased ~60% compared to wild type I κ B α (Fig. 8C), suggesting that GRK5 phosphorylates one or both of these sites. This result was confirmed using an antibody that recognizes phosphoSer32 in I κ B α . For these studies, GST-I κ B α was initially phosphorylated *in vitro* using GRK2, GRK5 or IKK β and the samples were run on SDS PAGE and immunoblotted using anti-I κ B α -

phospho-Ser32. These results show that Ser32 is selectively phosphorylated by GRK5 but not GRK2 and that GRK5 mediated phosphorylation of I κ B α is comparable to that seen with IKK β (Fig 8D). Overall, these results reveal that Ser32 is phosphorylated by GRK5 and that GRK2 and GRK5 phosphorylate distinct residues.

IKK β knockdown does not inhibit TNF α -induced I κ B α phosphorylation in Raw264.7 macrophages

Similar to GRK5, other kinases such as casein Kinase II and ribosomal S6 kinase have been shown to phosphorylate I κ B α at Ser32/36. Interestingly, these kinases were shown to selectively regulate I κ B α -NF κ B pathway in an IKK β -independent manner. Therefore, we hypothesized that because of the role of GRK2 and 5 as “I κ B α kinases”, IKK β may be dispensable in Raw264.7 cells, particularly for TNF α -induced I κ B α phosphorylation. To test this hypothesis, we knocked down IKK β in Raw264.7 macrophages (Fig 9) and tested the effect of TNF α on I κ B α phosphorylation (Ser32/36). As predicted, we found that TNF α -induced I κ B α phosphorylation is not significantly affected by knockdown of IKK β , suggesting that IKK β may be redundant in this system (Fig 9). However, it is possible that the level of knockdown is not sufficient to inhibit I κ B α phosphorylation because of the residual IKK β kinase activity present. To rule out this possibility, we further tested the ability of IKK β knockdown to inhibit LPS-induced I κ B α phosphorylation. Interestingly, our results demonstrate that LPS-induced I κ B α phosphorylation (at Ser32/36) is inhibited by IKK β knockdown and this effect was particularly evident at later time points (Supplementary figure 7). These results suggest that the IKK β plays a crucial role in LPS-induced I κ B α phosphorylation, but not in TNF α -induced I κ B α -NF κ B pathway in Raw264.7 macrophages.

Taken together, our results demonstrate a critical role for GRK2 and 5 in the regulation of TNF α -induced I κ B α -NF κ B pathway in Raw264.7 macrophages and suggest that I κ B α phosphorylation by GRKs might be an essential step in this regulation.

Discussion

G-protein coupled receptor kinases were first discovered for their role in GPCR phosphorylation and desensitization [34,35]. Recent studies, however, have revealed a number of non-GPCR substrates for GRKs [8,10,11,30,36,41]. Although GRKs mediate their cellular effects for the most part through their catalytic activity, recent studies have also proposed a kinase-independent role for GRKs in cellular signaling (via protein-protein interaction with the RH domain). In this regard, GRK2 has been shown to interact with MEK1 and regulate ERK activation in a kinase-independent manner [37]. GRK2 has also been shown to interact with other proteins such as PI3K [38], Akt [39], and GIT [40] and regulate a number of cell biological effects. In addition to the receptor and cytosolic substrates, GRKs, have also been found to phosphorylate nuclear proteins. A physiologically important role for the nuclear localized GRK5 was recently identified by Martini *et al* [41] who showed that the nuclear GRK5 is a HDAC kinase that mediates the epigenetic regulation of gene expression in cardiomyocytes. Taken together these studies suggest that the role of GRKs is much broader than previously appreciated.

Studies have just begun to emerge on the potential role for GRKs in the regulation of various components of the NF κ B pathway. In this regard, we demonstrated that GRK5 stabilizes LPS-stimulated p105 levels in macrophages [8]. More recently, while this manuscript was in preparation, a similar stabilizing role for GRK5 in maintaining I κ B α levels in endothelial cells was shown [42]. Surprisingly in the present study we find that GRK2 and GRK5 are important regulators of I κ B α -NF κ B signaling and mediate TNF α -induced I κ B α phosphorylation. In addition, in contrast to the findings of Sorriento *et al* [42]

in endothelial cells, our results demonstrate that GRK2 and GRK5 mediate TNF α -induced NF κ B-dependent gene transcription in macrophages. Our results further suggests that even with in a given cell type, the role of GRKs is selective for a particular ligand.

Our data further demonstrate that the role of GRK2 and GRK5 on TNF α -induced NF κ B signaling is dependent on the kinase activities because the kinase-deficient GRKs failed to mediate NF κ B activation. Also, *in vitro* kinase assays indicate that I κ B α may be differentially phosphorylated by GRK2 and 5. Our results show that the presence of G $\beta\gamma$ subunits and liposomes significantly enhances the phosphorylation of I κ B α by GRK2. G $\beta\gamma$ subunits have clearly been demonstrated to be important in the translocation of GRK2 to the plasma membrane for GPCR phosphorylation. Whether a similar role for G $\beta\gamma$ subunits in TNF α signaling exists, is presently not known. However, Kawamata *et al* [43] showed recently that TNF α signaling is mediated by activation of G-proteins in adipocytes. In addition, TNF α treatment of THP-1 monocytic cells has been shown to mediate GRK2 translocation to the membrane and affect β -adrenergic receptor desensitization [44], suggesting possible regulation of TNF α -induced GRK2 activity by G-proteins. In contrast to the role of lipids in GRK2 activity, GRK5 phosphorylation of I κ B α appears to be effectively inhibited in the presence of lipids even though previous studies have clearly shown that lipids activate GRK5 [30]. Thus it is possible that GRK5 phosphorylation of I κ B α is regulated by biochemical mechanisms that are distinct from its phosphorylation of other substrates such as synucleins [30] as well as from that of I κ B α phosphorylation by GRK2. Whether these differences in the phosphorylation of I κ B α by GRK2 and 5 translate into regulation of I κ B α in different sub-cellular environments is not known and will be tested in future studies. Other studies have clearly shown that I κ B α -NF κ B complexes can be present in different sub-cellular environments [45,46] and therefore, these complexes could be potentially regulated by GRK2 or 5 depending on the local cellular environment.

IKK β has been identified as the primary kinase that phosphorylates I κ B α . However, there is now extensive evidence that other kinases including IKK α , CK II and ribosomal S6K can phosphorylate I κ B α at the same sites as that of IKK β . This redundancy can in part be explained by the receptor- and cell type-specific regulation of I κ B α -NF κ B pathways [7,32,47]. For example, studies have shown that UV light-induced I κ B α degradation is mediated by phosphorylation of I κ B α by CK II [7]. Also, PMA-induced I κ B α phosphorylation has been shown to involve ribosomal S6K [32]. Similarly, recent studies have shown IKK β -dependent and -independent pathways that regulate I κ B α -NF κ B pathway in human macrophages in response to specific ligands [48]. Our studies clearly suggest that GRKs while necessary for TNF α -induced I κ B α -NF κ B pathway, they are not involved in LPS-induced I κ B α -NF κ B signaling. Interestingly, knockdown of IKK β does not affect TNF α -induced I κ B α phosphorylation (at Ser32/36), but does inhibit LPS-induced I κ B α phosphorylation, suggesting that in Raw264.7 macrophages, GRKs play the role of I κ B α kinases selectively for TNF α signaling. Our *in vitro* kinase reactions further support our findings in macrophages in that, GRK5 is able to phosphorylate some residues in I κ B α that are similar to that of IKK β . Thus it is possible that GRK5 specifically might function in a similar capacity to that of IKK β . If GRK5 can function as an I κ B α kinase, then what is the role of GRK2? It appears from our macrophage experiments that GRK2 is also necessary for TNF α -induced I κ B α phosphorylation (Ser32/36). However, *in vitro* GRK2 does not phosphorylate Ser32/36 and therefore appears to phosphorylate a different set of residues. In cells, GRK2 phosphorylation of these unidentified residues appears to be necessary for phosphorylation of Ser32/36 since knockdown of GRK2 inhibits I κ B α phosphorylation at these two sites. It is also possible the level of IKK β knockdown obtained might not be sufficient to define IKK β -independent regulation because of the residual kinase activity present in the knockdown cells. If this is the case, GRKs might work co-operatively with IKK β in the phosphorylation of I κ B α . In this regard, although Ser32/36 in I κ B α are the well-

characterized IKK phosphorylation sites, there is strong evidence that IKK also phosphorylates less well-characterized sites at the c-terminus [49]. Therefore, in our experiments, we cannot rule out the role of IKK β on phosphorylation of these other sites. Our results, however, indicate that IKK β knockdown can certainly inhibit LPS-induced I κ B α phosphorylation (on Ser32/36) and therefore, suggest that LPS and TNF α signal to NF κ B activation via different mechanisms in Raw264.7 macrophages.

In conclusion, our studies unravel important biochemical roles for GRK2 and 5 in TNF α -induced I κ B α phosphorylation and NF κ B signaling. Because regulation of the NF κ B pathway appears to be receptor-specific as well as cell type-specific, further broad and unbiased proteomic approaches are necessary to identify macrophage-specific and TNFR-specific signaling complexes that mediate NF κ B activation. These studies will undoubtedly identify therapeutic targets for inhibiting TNF α signaling in chronic inflammatory diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations used

TNF α	tumor necrosis factor- α
GRK	G-protein coupled receptor kinase
NF κ B	Nuclear factor κ -B
I κ B	Inhibitory κ -B
TRADD	Tumor necrosis factor receptor 1 associated death domain protein
RIP1	Receptor interacting protein-1
TRAF2/5	TNF receptor associated factor 2/5
c-IAP1	Inhibitor of apoptosis 1
TNFR	TNF α receptor

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Figure 1. GRK2 knockdown in macrophages inhibits TNF α -induced IkB α phosphorylation and degradation

(A, B, C). Raw 264.7 macrophages were transfected with either control siRNA or GRK2 siRNA using Amaxa's nucleofector. Forty-eight hours after transfection, cells were serum starved for ~3 hours, and stimulated or not with TNF α (25 ng/ml) for the indicated time points. Lysates were extracted and run on SDS-PAGE, transferred to nitrocellulose and then immunoblotted with primary antibodies against pIkB α , IkB α and tubulin. Secondary antibodies were fluorescently labeled and the blots were developed using Odyssey's Licor as described before [8]. Representative blots for pIkB α , IkB α , tubulin and GRK2 are shown in

(A). Quantitation is shown in **B** and **C**. *** $p < 0.001$ compared to control; * $p < 0.05$ compared to control. N=7.

D. Raw264.7 macrophages with control or knockdown levels of GRK2 were treated with TNF α as indicated. Nuclear lysates were then extracted and immunoblotted for NF κ B P50 and P65 (Rel A) as shown. Nuclear protein lamin B is shown as a loading control. A representative experiment is shown.

E. Nuclear extracts (from **D** above) were incubated with IR⁷⁰⁰ dye-labeled NF κ B oligonucleotide probes and electrophoretic mobility-shift assay was performed as described in the Materials and Methods. Gels were scanned on Licor Odyssey (Licor Biosciences) to detect binding. A representative experiment is shown.

A.

B.

Figure 2. GRK2 over-expression enhances TNF α -induced IkB α phosphorylation in macrophages Raw264.7 macrophages were infected with adenoviruses encoding empty vector or GRK2 at 50,000 viral particles/cell. Forty-eight hours after infection, cells were serum starved for 3–4 hours, and stimulated with TNF α (25 ng/ml) for indicated times. Lysates were immunoblotted for pIkB α , IkB α and tubulin. A representative blot is shown in (A) and quantitation in (B). * $p < 0.05$ compared to vector. N=3.

Figure 3. GRK5 knockdown inhibits TNF α -induced IkB α phosphorylation and degradation in macrophages

Raw 264.7 macrophages were electroporated with either control or GRK5 siRNA as indicated. Forty-eight hours after electroporation, cells were serum starved for ~3 hours, and stimulated with TNF α (25 ng/ml) for the indicated times. Lysates were immunoblotted for pIkB α , IkB α and tubulin and blots developed using Licor's Odyssey. Representative blots for GRK5 knockdown is shown in (A), pIkB α and IkB α in (B). Quantitation for IkB α phosphorylation and IkB α degradation are shown in (C) and (D) respectively. ***p<0.001 compared to control. N=6.

Figure 4. Expression of Macrophage inflammatory protein-1 β (MIP1 β), an NF κ B-regulated gene, is inhibited by GRK2 or GRK5 knockdown in macrophages

A. Control or GRK2 knockdown Raw264.7 macrophages were treated or not with TNF α for 24 hours and RNA extracted as described in the Methods. Quantitative real-time RT-PCR was performed using MIP1 β specific primers. MIP1 β mRNA levels were normalized to cyclophilin levels. ** $p < 0.01$. N=3.

B. Control or GRK5 knockdown Raw264.7 macrophages were treated with TNF α and mRNA levels of MIP1 β and cyclophilin determined as described in (A) above. *** $p < 0.001$; ** $p < 0.01$. N=3.

Figure 5. Kinase activity of GRK2 and 5 is essential for mediating TNFα-induced NFκB transcriptional activation

A. HEK293T cells were transfected with vector, GRK2 wild type or GRK2-K220R (kinase deficient mutant) expression plasmids along with pELAM-luciferase and LacZ. Forty hours after transfection, cells were stimulated (in triplicates) with different concentrations of TNFα (as indicated) for 8 hours. Lysates were prepared and analysed for luciferase and β-galactosidase activities. Quantitation was performed after normalizing luciferase activity to β-galactosidase activity. Data is expressed as fold basal of the vector transfected cells. Immunoblots showing expression levels of GRK2 wild type and GRK2 kinase deficient mutant are shown in the top panel. *P<0.05. N=4.

B. HEK293T cells were transfected with vector, GRK5 wild type or GRK5-K215R (kinase deficient mutant) expression plasmids along with pELAM-luciferase and LacZ. Cell treatments, activity assays and quantitation were performed as described in (A) above. Immunoblots showing expression levels of GRK5 wild type and GRK5 kinase deficient mutant are shown in the top panel. **P<0.01 N=4.

Figure 6. GRK2 interacts with the N-terminus of IκBα

A. Purified GST-IκBα (full length), GST-IκBα (76–302) and GST alone, (5 μg each) were resolved on a SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated overnight in HEK293T lysates over-expressing HA-GRK2 (**A**) or vector (**B**). Membrane was washed and probed for HA-GRK2 binding to GST-IκBα by immunoblotting using anti-HA antibody. Western blot of the overlay assay is shown in the top panel and a Ponceau stain of the overlay blot is shown in the bottom. Blots are representative of at least four individual experiments. **C.** GRK2 and IκBα were co-expressed in HEK293T cells and the lysates were immunoprecipitated using IκBα polyclonal antibody and the blots were then subjected to immunoblotting as indicated. **D.** Lysates from THP1 monocytic cells were

immunoprecipitated with IkB α polyclonal antibody. Immunoprecipitates were then denatured using sample buffer, boiled and separated on a 10% SDS-PAGE gel. Immunoblotting was performed for GRK2 as shown.

Figure 7. Direct interaction between GRK5 and IκBα

A. Purified GST-IκBα (full length), GST-IκBα (76–302) and GST alone, (5 μg each) were resolved on a SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated overnight in HEK293T lysates over-expressing GRK5 (**A**) or vector (**B**). Membrane was washed and probed for GRK5 binding to GST-IκBα by immunoblotting using anti-GRK5 antibody. Western blot of the overlay assay is shown in the top panel and a Ponceau stain of the overlay blot is shown in the bottom. Blots are representative of at least four individual experiments. **C.** GRK5 and IκBα were co-expressed in HEK293T cells and the lysates were immunoprecipitated using IκBα polyclonal antibody and the blots were then subjected to immunoblotting as indicated.

Figure 8. IκBα is a substrate for GRK2 and GRK5 *in vitro*

Purified GRK2 and GRK5 were prepared as described before [30]. 200 nM purified GST-IκBα or GST alone were incubated with 25 nM GRK2 or GRK5 in buffer containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.1 mM [γ^{32} P] ATP, 5 mM MgCl₂ and the phosphorylation reactions were done for various time points as indicated (Fig 8A). Reaction was stopped with SDS buffer, electrophoresed on a 10% SDS-PAGE, the gel dried and subjected to autoradiography. Phosphorylated IκBα bands were then counted on a liquid scintillation for quantification. In **8B**, the reactions were incubated in the absence or presence of liposomes as indicated and phosphorylation reactions performed for 30 min and stoichiometry quantified as described above. GRK2 phosphorylation samples also contained Gβγ in

addition to liposomes. In Fig 8C, GRK2 and GRK5 were incubated with 200 nM purified GST-I κ B α (wild type or mutant S32A/S36A) in a kinase reaction and phosphorylated I κ B α quantified using liquid scintillation as described in the methods. In Fig 8D, Purified GRK2, GRK5 or IKK β were incubated with GST-I κ B α as a substrate as described above without [γ^{32} P]ATP. Reaction was stopped with SDS buffer, electrophoresed on a 10% SDS-PAGE and transferred to a nitrocellulose membrane. Immunoblotting was performed using an antibody that specifically detects phospho-I κ B α (ser32).

Figure 9. IKK β knockdown does not inhibit TNF α -induced IkB α phosphorylation in Raw264.7 macrophages

Raw 264.7 macrophages were electroporated with either control or IKK β siRNA as indicated. Forty-eight hours after electroporation, cells were serum starved for ~3 hours, and stimulated with TNF α (25 ng/ml) for the indicated times. Lysates were immunoblotted for pIkB α (Ser32/36) and tubulin for normalization. Representative blots are shown in the top and quantitation in the bottom. N=4.