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Diacylglycerol kinase α enhances protein kinase Cζ-dependent phosphorylation at Ser311 of p65/RelA subunit of nuclear factor-κB

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ABSTRACT

We recently reported that diacylglycerol kinase (DGK) α enhanced tumor necrosis factor-α (TNF-α)-induced activation of nuclear factor-κB (NF-κB). However, the signaling pathway between DGKα and NF-κB remains unclear. Here, we found that small interfering RNA-mediated knockdown of DGKα strongly attenuated protein kinase C (PKC) ζ-dependent phosphorylation of a large subunit of NF-κB, p65/RelA, at Ser311 but not PKCζ-independent phosphorylation at Ser468 or Ser536. Moreover, knockdown and overexpression of PKCζ suppressed and synergistically enhanced DGKα-mediated NF-κB activation, respectively. These results strongly suggest that DGKα positively regulates TNF-α-dependent NF-κB activation via the PKCζ-mediated Ser311 phosphorylation of p65/RelA.

1. Introduction

It is well recognized that a variety of lipid second messengers that are present in low abundance perform specific tasks during a wide range of biological processes in eukaryotic cells. It is essential that the cellular concentrations of such signaling lipids are strictly regulated by the action of metabolic enzymes. Diacylglycerol kinase (DGK) phosphorylates diacylglycerol to yield phosphatidic acid [1–4]. Diacylglycerol is an established activator of conventional and novel protein kinase Cs (PKCs), Unc-13, and Ras guanyl nucleotide-releasing protein [5,6]. Phosphatidic acid has also been reported to regulate a number of signaling proteins, such as phosphatidylinositol-4-phosphate 5-kinase, Ras GTPase-activating protein, Raf-1 kinase, and atypical PKC [1–4,7].

Mammalian DGK is known to exist as a large protein family consisting of ten isozymes classified into five subtypes according to their structural features [1–4]. The type I DGKs (α-, β-, and γ-isozymes) contain two sets of Ca^{2+}-binding EF-hand motifs at their N-termini. We recently demonstrated that DGKα is a novel positive regulator of nuclear factor-κB (NF-κB), which suppresses tumor necrosis factor-α (TNF-α)-induced melanoma cell apoptosis [8]. However, it is not yet known how DGKα activates NF-κB.

NF-κB is a heterodimeric transcription factor that is predominantly composed of the 65 and 50 kDa subunits of the Rel family [9,10]. In resting cells, NF-κB is mainly retained in the cytoplasm by the inhibitor of NF-κB (IκB) family of proteins, which mask the nuclear translocation signal of the transcription factor. Upon cell stimulation, two defined serine residues in the N-terminus of IκB proteins are phosphorylated by IκB kinase (IKK), thus triggering their ubiquitination and subsequent degradation by the 26S proteasome. In this manner, the NF-κB proteins are released for translocation to the nucleus and subsequent induction of a variety of κB-dependent genes. Conformational changes by phosphorylation of p65 (also called RelA) are also important for the activation of NF-κB [9,10]. For example, in addition to IκB, IKKs phosphorylate p65 at Ser468 and Ser536 [11,12]. PKCζ was reported to directly phosphorylate p65 at Ser311 in a TNF-α-dependent manner [13]. In this case, however, factor(s) upstream of PKCζ remain unclear.

Here we present several lines of evidence indicating that DGKα positively regulates the PKCζ-mediated Ser311 phosphorylation of p65 in response to TNF-α. We propose that DGKα is a novel key regulator in the TNF-α–PKCζ–NF-κB pathway.

Abbreviations: DGK, diacylglycerol kinase; GFP, green fluorescent protein; IκB, inhibitor of NF-κB; IKK, IκB kinase; NF-κB, nuclear factor-κB; PKC, protein kinase C; siRNA, small interfering RNA; TNF-α, tumor necrosis factor-α

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2. Materials and methods

2.1. Cell culture

AKI cells were maintained as described previously [8].

2.2. Antibodies

Anti-pig DGKα polyclonal antibody (cross-reactive with the human enzyme) was prepared as described previously [14]. Anti-NF-κB p65 mouse monoclonal (F-6), anti-phospho-IκBα mouse monoclonal (B-9), anti-PKCζ rabbit polyclonal (C-20), anti-actin goat polyclonal (C-11), and anti-green fluorescent protein (GFP) mouse monoclonal (B-2) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-IκBα and anti-phospho-NF-κB p65 Ser468 and Ser536 antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-NF-κB p65 phospho-Ser311 rabbit polyclonal antibody was from Abcam (Abcam, Tokyo, Japan).

2.3. Plasmids

Human PKCζ cDNA was amplified by PCR using human whole brain cDNA (Clontech-Takara Bio, Tokyo, Japan) as template and then subcloned into the pEGFP-C3 expression vector (Clontech-Takara Bio). The authenticity of the cDNA construct was confirmed by DNA sequencing. The expression plasmid of GFP-tagged porcine DGKα was prepared as described previously [8]. Cells were transiently transfected with cDNAs using Effectene transfection reagent (Qiagen, Tokyo, Japan) according to the manufacturer's instructions.

2.4. RNA interference

The following small interfering RNA (siRNA) oligonucleotides were used to knockdown the expression of endogenous DGKα or PKCζ: DGKα Stealth Select RNAi™ (Invitrogen, Tokyo, Japan, catalog numbers HSS102626 and HSS102627, here designated DGKα#1 and DKGα#2, respectively); PKCζ Validated Stealth RNAi™ (Invitrogen; catalog number, VHS41533); and Stealth RNAi™ Negative Control Med GC Duplex (Invitrogen; catalog number, 12935-300). AKI cells were transfected with these siRNAs using Lipofectamine™ RNAiMAX Reagent (Invitrogen) according to the manufacturer's instructions.

2.5. Western blot analysis

Western blotting was performed as described previously [8].

2.6. Luciferase reporter assay

The luciferase reporter assay was performed as described previously [8].

3. Results

3.1. DGKα does not affect phosphorylation of IκB

It is well known that IκB proteins are phosphorylated by IKKs in response to TNF-α. Thus, to investigate how DGKα activates NF-κB, we first examined TNF-α-induced phosphorylation levels of IκB in DGKα-silenced AKI cells. In control cells, TNF-α strongly enhanced the phosphorylation of IκBα (Fig. 1A and B). When DGKα was knocked down (Fig. 1C), the phosphorylation level was not significantly changed (Fig. 1A and B). Moreover, we confirmed that protein levels of IκBα were not markedly altered by the DGKα knockdown (Supplementary Fig. 1). These results indicate that DGKα is not involved in the phosphorylation of IκBα.

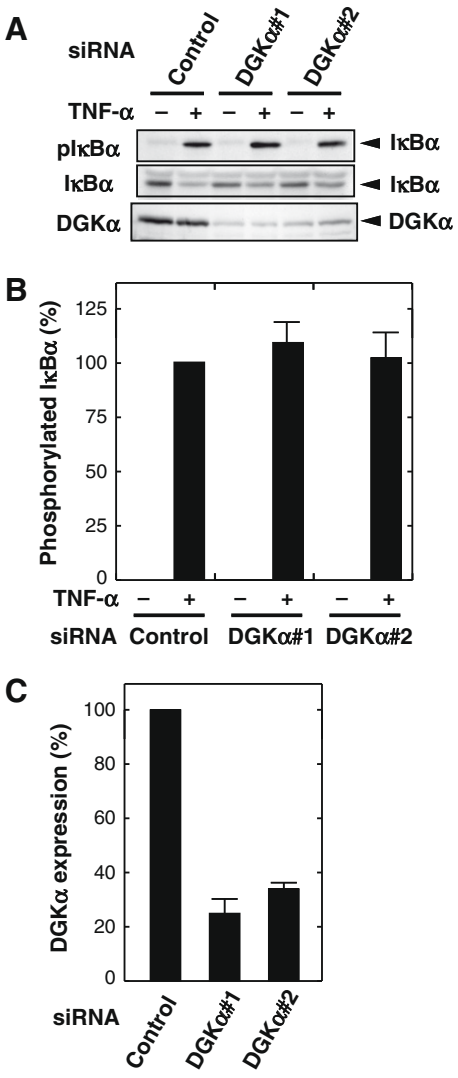

Fig. 1. Effects of DGKα knockdown on TNF-α-induced phosphorylation of IκBα in AKI melanoma cells. (A) AKI melanoma cells were transfected with control siRNA or siRNA (10 nM) targeting DGKα as indicated. After 48 h, the cells were further incubated with or without 50 ng/ml TNF-α for 5 min and harvested. Cell lysates (10 μg protein) were analyzed by Western blotting with antibodies against phospho-IκBα (pIκBα), IκBα, and DGKα as indicated. The relative intensities of bands of phospho-IκBα (B) and DGKα (C) are shown. The results are presented as the percentage of the value for control siRNA (TNF-α (+)) and represent the mean ± S.D. of the values obtained in three separate experiments.

3.2. DGKα enhances phosphorylation of p65 at Ser311 but not at Ser468 or Ser536

Because phosphorylation of a large subunit of NF-κB, p65 (also called RelA), is also essential for NF-κB activation [9,10], we next investigated phosphorylation levels of p65. Ser468 and Ser536 in p65 were reported to be phosphorylated by IKKs [11,12] (Fig. 2A), which also phosphorylate IκB. However, silencing of DGKα failed to affect the phosphorylation levels of Ser468 and Ser536 (Fig. 2B). Phosphorylation of Ser311 in p65 is also known to be required for NF-κB activation [13] (Fig. 2A). Intriguingly, knockdown of DGKα markedly decreased the phosphorylation levels of Ser311 (Fig. 2B and C). We confirmed that expression levels

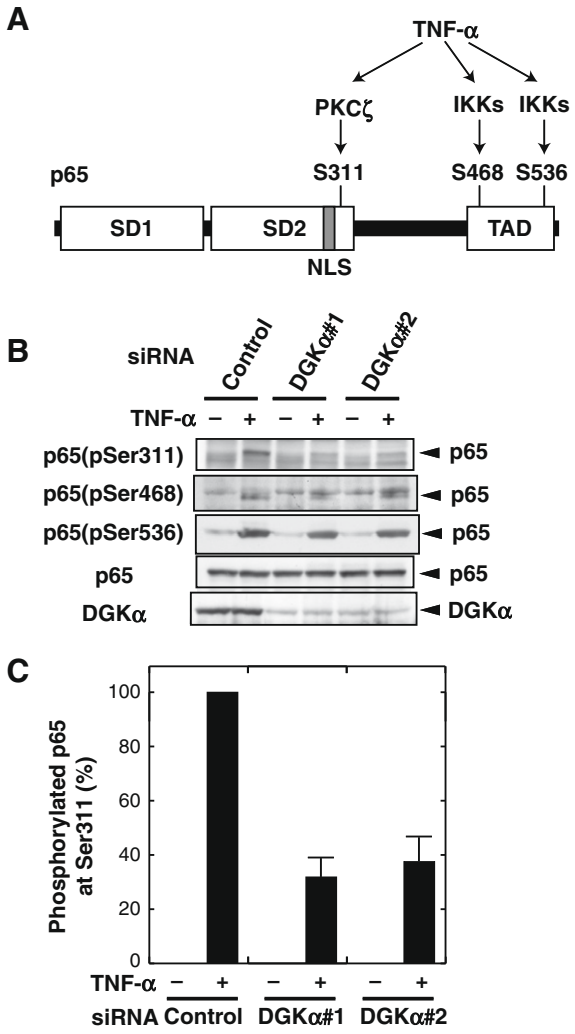

Fig. 2. Effects of DGKα knockdown on TNF-α-induced phosphorylation of NF-κB p65 subunit in AKI melanoma cells. (A) Schematic diagram of the NF-κB p65 subunit. Several serine residues phosphorylated by TNF-α stimulation are shown. NLS, nuclear localization sequence; SD, subdomain; TAD, transactivation domain. (B) DGKα-knockdown AKI melanoma cells were treated as described in Fig. 1. TNF-α-induced phosphorylation of p65 was analyzed by Western blotting with antibodies against phosphoserines of p65 (pSer311, pSer468, and pSer536), p65, and DGKα as indicated. (C) Relative intensities of bands of phospho-Ser311. Results are presented as the percentage of the value for control siRNA (TNF-α (+)) and represent the mean ± S.D. of the values obtained in three separate experiments.

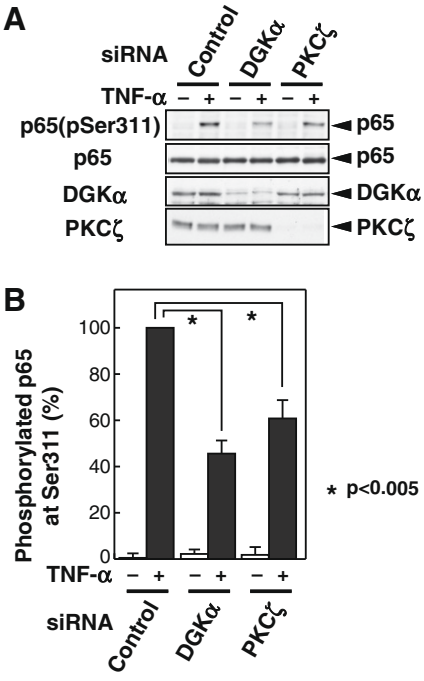

Fig. 3. Effects of PKCζ knockdown on TNF-α-induced phosphorylation of the NF-κB p65 subunit in AKI melanoma cells. (A) AKI melanoma cells were transfected with control siRNA, DGKα siRNA (#1), or PKCζ siRNA (10 nM) as indicated. After 48 h, cells were further incubated with or without 50 ng/ml TNF-α for 5 min and harvested. Cell lysates (10 μg protein) were analyzed by Western blotting with antibodies against phospho-Ser311-p65, p65, DGKα, and PKCζ as indicated. (B) Relative intensities of bands of phospho-Ser311-p65. Results are presented as the percentage of the value for control siRNA (TNF-α (+)) and represent the mean ± S.D. of the values obtained in three separate experiments. Statistical significance was determined using Student's *t*-test.

of p65 were not altered by the silencing of DGKα (Fig. 2B). Moreover, there was no difference between siRNAs #1 and #2, which recognize different sequences in the DGKα gene, arguing against an off-target effect of the siRNAs.

3.3. DGKα-dependent phosphorylation of p65 at Ser311 is mediated by PKCζ

Because PKCζ was reported to phosphorylate Ser311 of p65 [13], we verified that this protein kinase indeed participates in the TNF-α-dependent Ser311 phosphorylation of p65 in AKI cells. As shown in Fig. 3, siRNA-mediated depletion of PKCζ significantly inhibited the Ser311 phosphorylation of p65. The result indicates that PKCζ, at least in part, mediates the Ser311 phosphorylation of p65 induced by TNF-α in AKI cells.

We next examined whether PKCζ mediated the DGKα-regulated Ser311 phosphorylation of p65 observed in Fig. 2. As previously reported [8], overexpression of DGKα markedly enhanced NF-κB activity (Fig. 4A). Knockdown of PKCζ (Fig. 4B) significantly impaired the DGKα-mediated enhancement of NF-κB activity (Fig. 4A). We next checked the effects of overexpression of PKCζ on NF-κB activity enhanced by DGKα overexpression. Although overexpression of DGKα or PKCζ slightly enhanced NF-κB activity, simultaneous overexpression of these proteins synergistically augmented NF-κB activity (Fig. 4C). These results strongly suggest that PKCζ is involved in DGKα-mediated NF-κB activation.

4. Discussion

We recently demonstrated that DGKα positively regulated TNF-α-induced activation of NF-κB [8], which suppressed melanoma cell apoptosis. However, it was not clear how DGKα activated NF-κB. In this study, we showed that the PKCζ-mediated Ser311 phosphorylation of p65 is involved in the positive regulation of TNF-α-dependent NF-κB activation by DGKα.

siRNA-mediated knockdown of DGKα failed to show detectable effects on IκB phosphorylation and its degradation (Fig. 1 and Supplementary Fig. 1). Moreover, the phosphorylation levels of Ser468 and Ser536 in NF-κB were not affected by DGKα depletion (Fig. 2). Thus, it is likely that DGKα selectively regulates the PKCζ-mediated Ser311 phosphorylation of p65. p65 is known to have several other phosphorylation sites, such as Ser276 and Ser529 [9,10]. Because the PKCζ-mediated phosphorylation at Ser311 was reported to be critical for TNF-α-induced activation of NF-κB [13], Ser311 phosphorylation is, at least in part, essential for DGKα-regulated NF-κB activation.

Our results did not show whether PKCζ acted upstream or downstream of DGKα. However, because it was reported that PKCζ

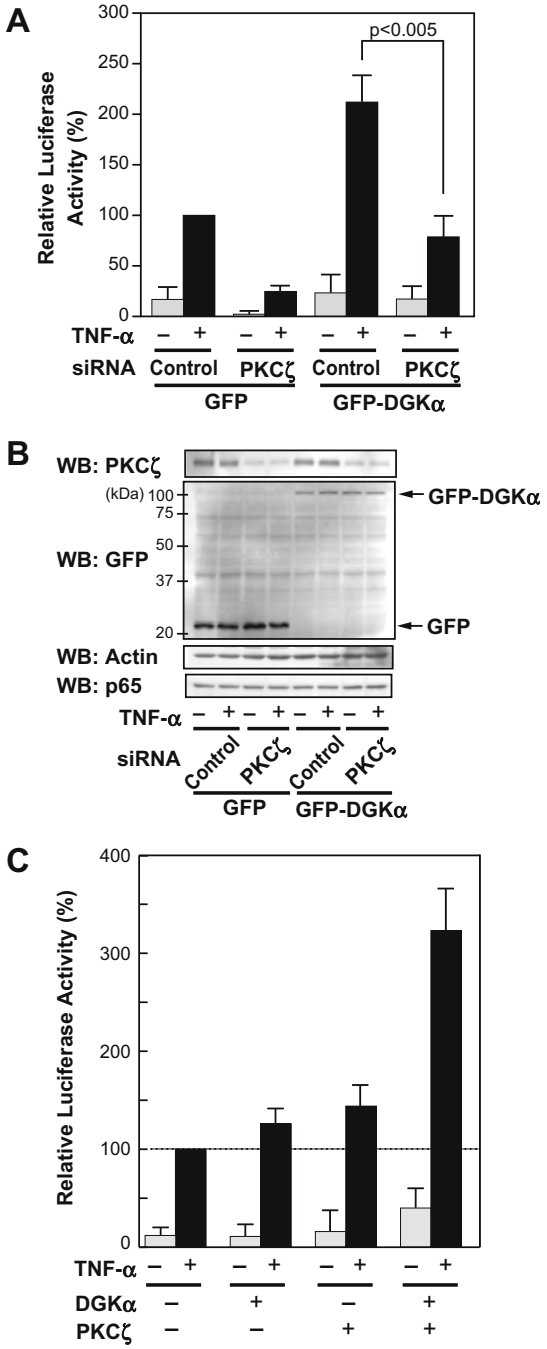

Fig. 4. Effects of overexpression and knockdown of PKCζ on NF-κB activity enhanced by DGKα overexpression in AKI melanoma cells. (A and B) AKI melanoma cells were transfected with either control or PKCζ siRNA as indicated. After 24 h, cells were cotransfected with pNF-κB-Luc vector (Clontech-Takara Bio, 40 ng/ml), pSV-β-galactosidase control vector (Clontech-Takara Bio, 40 ng/ml), and with either GFP or GFP-DGKα expression plasmids (120 ng/ml) as indicated. After 24 h, cells were further incubated with or without 50 ng/ml of TNF-α for 12 h. (A) Luciferase activity was measured and normalized to β-galactosidase activity. The results are presented as the percentage of the value of control siRNA plus GFP (TNF-α (+)) and represent the mean ± S.D. of the values obtained in three separate experiments. Statistical significance was determined using Student's *t*-test. (B) Cell lysates (10 μg of protein) were analyzed by Western blotting with antibodies against PKCζ, GFP, actin, and p65 as indicated. (C) AKI melanoma cells were cotransfected with pNF-κB-Luc vector (40 ng/ml), pSV-β-galactosidase control vector (40 ng/ml), and the indicated combinations of expression plasmids (60 ng/ml each): GFP (−), GFP-DGKα (DGKα (+)), and GFP-PKCζ (PKCζ (+)). After 24 h, cells were further incubated with or without 50 ng/ml of TNF-α for 12 h. Luciferase activity was measured and was normalized to β-galactosidase activity. The results are presented as the percentage of the value of control transfection (TNF-α (+)) and represent the means ± S.D. of the values obtained in three separate experiments.

directly phosphorylated p65 [13], DGKα is probably an upstream regulator of PKCζ. Interestingly, phosphatidic acid was reported to activate PKCζ directly in vitro [15]. However, it is not clear at present whether phosphatidic acid produced by DGKα directly activates PKCζ. We cannot rule out the possibility of other factors bridging between DGKα and PKCζ.

The transcription factor NF-κB is a crucial regulator of many physiological and pathological processes, including control of the adaptive and innate immune responses, inflammation, proliferation, tumorigenesis, and apoptosis. Thus, the tight regulation of NF-κB activity within a cell is extremely important. However, the regulatory mechanisms are not yet fully elucidated. For example, upstream regulators of PKCζ-dependent NF-κB activation [13] have not been identified. The results of the present study strongly suggest that DGKα is an upstream regulator. Further study on the mechanism underlying the DGKα–PKCζ signaling pathway is needed to better understand the complex regulation of the important signaling hub NF-κB.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.09.017.

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