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RIOK3 interacts with caspase-10 and negatively regulates the NF-κB signaling pathway

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Abstract RIOK3 was initially characterized as a homolog of *Aspergillus nidulans* sudD and showed down-regulation at the invasive front of malignant melanomas, but the molecular mechanism remains elusive. Here, we report that overexpression of RIOK3 inhibits TNFα-induced NF-κB activation, but down-regulation of endogenous RIOK3 expression by siRNA potentiates it. A yeast two-hybrid experiment revealed that RIOK3 interacted with caspase-10, and further, a GST pull-down assay and endogenous coimmunoprecipitation validated the interaction. We subsequently showed that the interaction was mediated by the RIO domain of RIOK3 and each death effector domain of caspase-10. Interestingly, our data demonstrated that RIOK3 suppressed caspase-10-mediated NF-κB activation by competing RIP1 and NIK to bind to caspase-10. Importantly, the kinase activity of RIOK3 was confirmed to be relevant to NF-κB signaling. Taken together, our findings strongly suggest that RIOK3 negatively regulates NF-κB signaling pathway activated by TNFα dependent on its kinase activity and NF-κB signaling pathway activated by caspase-10 independent of its kinase activity.

Keywords RIOK3 · NF-κB activation · TNFα · Caspase-10

Introduction

Atypical protein kinases, which are a set of enzymes that have kinase properties but lack sequence similarity with typical protein kinases, include proteins known to be involved in a wide variety of cellular processes and proteins functionally unclear. Among these atypical protein kinases, the family of RIO kinases, which was first identified in yeast and named based on the founding member, yeast RIO1 (right open reading frame 1) [1], conservatively exists from archaea to human. All the organisms contain at least two RIO proteins, which were homologous to yeast RIO1 and RIO2, respectively [2]. RIO3, a third group of RIO proteins, was initially identified as a homolog of *Aspergillus nidulans* sudD [3] and was discovered only in multicellular eukaryotes. In yeast, RIO1 is required for proper cell cycle progression and chromosome maintenance [4]. Furthermore, RIO1 and RIO2 were identified as non-ribosomal factors in late 18S rRNA processing [2, 5]. These previous studies of the RIO family were restricted to yeast and the function of human RIOK3 gene remains largely elusive, although it was cloned 10 years ago [3].

It has been reported that the expression of RIOK3 was significantly lower at the invasive front than in the tumor center in malignant melanomas, which suggests that RIOK3 may be involved in tumor metastasis [6]. The transcription factor NF-κB is proposed to be at the center of multiple pathways that promote an invasive phenotype [7–10]. Suppression of metastasis upon blockage of NF-κB activity has been reported in human prostate cancer cells

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[7], melanoma cells [8], and breast cancer cells [9]. Moreover, NF-κB is a major regulator of inflammation and carcinogenesis by promoting the expression of genes related to apoptosis, cell cycle, cell proliferation, and differentiation [11, 12]. In resting cells, NF-κB is predominantly sequestered in the cytoplasm and associated with members of the inhibitory IκB family. In response to various signals, the IKK complex phosphorylates IκB proteins and targets them for proteasome-mediated degradation [13]. Free NF-κB then translocates to the nucleus and activates transcription.

In this report, we describe the role of RIOK3 in the NF-κB signaling pathway and demonstrate the physical interaction and the functional interrelation between RIOK3 and caspase-10, which may provide clues to the involvement of RIOK3 in pathogenesis.

Materials and methods

Reagents and antibodies

Recombinant human TNFα was a product of Genentech. The mouse monoclonal antibody against RIOK3 was purchased from Abnova. The mouse monoclonal antibody against caspase-10 was purchased from Medical & Biological Laboratories. The mouse monoclonal antibody against phospho-IκBα and GAPDH, rabbit anti-mouse secondary antibodies conjugated with HRP, mouse IgG and protein G-Agarose were purchased from Santa Cruz Biotechnology. The rabbit polyclonal antibody against IκBα was purchased from Kangchen Bio-tech. The mouse monoclonal antibodies against GST, 6×His, Myc, and Flag epitopes were products of Sigma-Aldrich. siRNA duplexes were chemically synthesized by Shanghai GenePharma Co., Ltd. The RIOK3 siRNA sequences were as follows: 746, UGUUGGAGA CAAUCACUGGdTdT; 1203, GCUUGUCCAUGCUGAC CUCdTdT. The control siRNA sequence was UUCUCCGA ACGUGUCACGUdTdT.

Plasmids

The full length cDNA of RIOK3 and caspase-10g were amplified, respectively, from the human fetal brain cDNA library (Clontech) and the human Jurkat cDNA library (Shanghai Genomics Inc.). The full length cDNAs of RIP1 and NIK were purchased from Proteintech Group, Inc. For the yeast two-hybrid screen, RIOK3 was cloned into pGBKT7 plasmid and fused to the Gal4 DNA binding domain. For in vitro binding assays, RIOK3 was cloned into pGEX-6P-1 vector with an N-terminal GST tag and caspase-10g was cloned into pET32a vector with an N-terminal 6×His tag. For transfection, RIOK3 and its truncation cDNAs, RIP1, and NIK were cloned into a mammalian expression vector pEF with an N-terminal Flag tag. Caspase-10g and its truncation cDNAs were cloned into a pCDEF3 vector with an N-terminal Myc tag. RIOK3 (K290A) was generated by site-directed mutagenesis using the Quickchange^{TM} kit (Stratagene).

Cell culture and transfection

HEK293T cells were maintained in Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% Fetal Bovine Serum (Hyclone). All transfections were performed using LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions.

Luciferase reporter assay

HEK293T cells were seeded in 24-well plates and were transfected in the following day with NF-κB luciferase reporter plasmid (pNF-κB-Luc, 100 ng) and other constructs (300 ng each) or 60 pmol siRNA as indicated. When necessary, vector plasmid was supplemented to ensure that the total amount of DNA was kept constant. pRL-TK *Rellina* plasmid (10 ng) was cotransfected to normalize transfection efficiency. Twenty-four hours or 48 h after transfection, cells were treated with TNFα (10 ng/ml) or left untreated. Luciferase activity was analyzed using the Dual Luciferase Reporter Assay System (Promega). Data in results represent means ± SD (error bars) of at least three independent experiments.

Yeast two-hybrid analysis

pGBKT7-RIOK3 was used as the bait to screen a human fetal brain cDNA library in Matchmaker II yeast two-hybrid system (Clontech). $Leu^+/Trp^+/His^+$ clones were verified by β-galactosidase activity. Prey plasmids from $Leu^+/Trp^+/His^+/LacZ^+$ clones were isolated and then sequenced and analyzed. Positive clones were tested for interaction along with either pGBKT7-RIOK3 or pGBKT7 by cotransformation in yeast Y190.

In vitro binding assay

The fusion protein GST-RIOK3, expressed in *E. coli* BL21, was induced by 0.1-mM IPTG at 28°C for 1 h and then purified with glutathione-Sepharose4B beads. His-caspase-10g, expressed in *E. coli* BL21 (DE3), was induced by 0.1-mM IPTG at 22°C for 6 h and then purified by Ni-NTA Agarose (QIAGEN). The following GST pull-down assay was performed as previously described [14].

Immunoprecipitation and immunoblotting

Immunoprecipitation and Western blot were carried out as described [15]. For the detection of endogenous interaction between RIOK3 and caspase-10, 1×10^8 HEK293T cells were lysed in 1.5-ml lysis buffer. Lysates were precleared with Protein G-Agarose beads and 2 μg mIgG for 2 h at 4°C. The precleared lysates were immunoprecipitated with anti-RIOK3 or mIgG as the control. The immunoprecipitated samples were washed three times with lysis buffer, resolved by SDS–PAGE, and then subjected to immunoblotting analysis with the indicated antibodies.

In vitro kinase assay

Immunoprecipitated RIOK3 and RIOK3 (K290A) were obtained from transfected HEK293T cells and subjected to an auto-kinase assay performed as previously described [16].

Results

RIOK3 inhibits TNFα-induced NF-κB activation

The expression pattern of RIOK3 in malignant melanoma suggests that RIOK3 possibly inhibits NF-κB activation. It is well known that TNFα triggers the activation of NF-κB signaling pathway. Here, we investigated whether RIOK3 is a putative repressor of NF-κB activation induced by TNFα. Interestingly, luciferase reporter assays in HEK293T cells revealed that overexpression of RIOK3, not RIOK1, substantially inhibited TNFα-induced NF-κB transcriptional activity (Fig. 1a) in a dose-dependent manner (Fig. 1b). We next examined the phosphorylation of IκBα which is quickly phosphorylated upon NF-κB activation [17]. TNFα rapidly induced the phosphorylation of IκBα after a 5-min or 10-min treatment and overexpession of RIOK3 obviously attenuated the phosphorylation (Fig. 1c), which further confirmed the effect of RIOK3 on NF-κB activation.

Knockdown of RIOK3 potentiates TNFα-induced NF-κB activation

In order to make it more physiologically relevant, we examined whether RIOK3 regulates TNFα-induced NF-κB activation when endogenous RIOK3 expression was reduced via RNAi. Two RIOK3 siRNAs were fished out, which are designated as 746 and 1203. In addition, a non-specific siRNA was used as the negative control. The effectiveness of them against RIOK3 was confirmed by monitoring the protein level of RIOK3 in HEK293T cells (Fig. 2a). In reporter assays, knockdown of endogenous

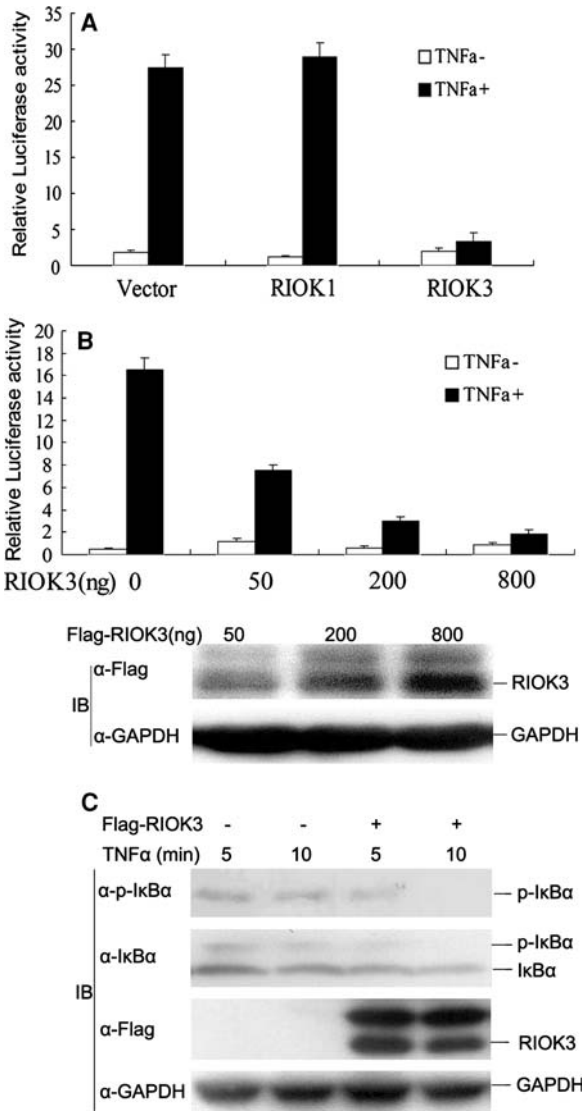

Fig. 1 RIOK3 inhibits TNFα-induced NF-κB activation. **a** RIOK3, not RIOK1, inhibits TNFα-induced NF-κB activation. HEK293T cells were transfected with pNF-κB-Luc and pRL-TK, together with empty, Flag-RIOK1 or Flag-RIOK3 plasmid. Twenty-four hours after transfection, cells were treated with TNFα (10 ng/ml) or left untreated for 6 h and then processed for luciferase reporter assays. **b** Dose–response effects of RIOK3 on TNFα-induced NF-κB activation. HEK293T cells were transfected with indicated amounts of Flag-RIOK3. Twenty-four hours after transfection, cells were treated with TNFα (10 ng/ml) or left untreated for 6 h and then processed for reporter assays. Western blotting with anti-Flag antibody demonstrates the expression level of RIOK3 and GAPDH serves as a control. **c** Effects of RIOK3 on the phosphorylation of IκBα. HEK293T cells were transfected with empty vector or Flag-RIOK3 empty vector. Twenty-four hours after transfection, cells were treated with TNFα (10 ng/ml) for indicated time. Western blotting was performed on the cell extracts to check the protein level of IκBα and phosphorylation of IκBα

RIOK3 by siRNA considerably potentiated TNFα-induced NF-κB activation (Fig. 2b) and phosphorylation of IκBα (Fig. 2c and Supplementary Table 1), suggesting that

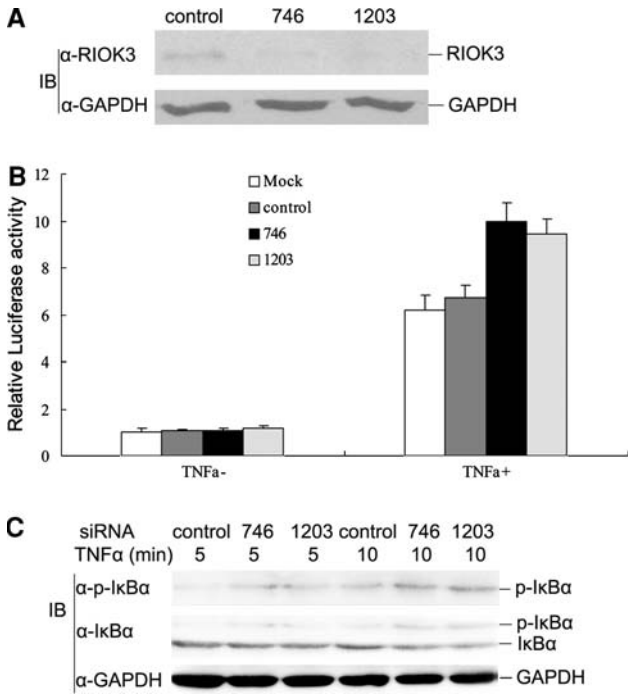

Fig. 2 Knockdown of RIOK3 potentiates TNFα-induced NF-κB activation. **a** RNA interference of RIOK3. HEK293T cells were transfected with the indicated siRNAs. Forty-eight hours after transfection, cell lysates were subjected to immunoblotting for determining endogenous RIOK3 protein levels. **b** Reduced RIOK3 expression potentiates TNFα-induced NF-κB activation. HEK293T cells were transfected with the indicated siRNAs. Forty-eight hours after transfection, cells were treated and assayed as in Fig. 1a. **c** Effects of RIOK3 knockdown on the phosphorylation of IκBα. HEK293T cells were transfected with the indicated siRNAs. After 48 h, cells were treated and assayed as in Fig. 1c

RIOK3 is a physiological inhibitor of the TNFα-induced NF-κB activation pathway.

Identification of caspase-10 as a RIOK3-interacting protein

The regulation of NF-κB activation is often based upon specific protein–protein interactions. In order to investigate the molecular mechanism underlying the effect of RIOK3 on NF-κB activation, we performed a yeast two-hybrid screen to identify RIOK3-associated proteins. We obtained four positive clones from 1×10^7 transformants. Sequence analysis showed that one of these clones encoded the N-terminal prodomain of caspase-10.

The prodomain of caspases-10, composed of two tandem death effector domains (DEDs), was shown to be capable of recruiting the NF-κB signaling molecules such as receptor-interacting protein 1 (RIP1) and NF-κB inducing kinase (NIK), eventually leading to NF-κB activation [18]. Recently, a new prodomain-only isoform of caspase-10, caspase-10g, was found to enhance NF-κB activity and only to promote slight apoptosis [19]. The caspase-10g isoform was therefore employed in the following experiments for convenience. The interaction between RIOK3 and caspase-10 was validated by a pull-down assay and coimmunoprecipitation. We first purified GST-RIOK3 and His-caspase-10g, and performed a GST pull-down assay. This showed His-caspase-10g was copurified with GST-RIOK3 (Fig. 3a). In order to examine their physiological association, we performed an endogenous coimmunoprecipitation experiment, which indicated that RIOK3 was associated with caspase-10 in untransfected HEK293T cells (Fig. 3b).

Domains involved in the association of RIOK3 and caspase-10g

RIOK3 is composed mostly of an RIO domain and caspase-10g is composed almost exclusively of N-DED and C-DED. In order to further map the interacting region of RIOK3 and caspase-10g, we constructed Flag-RIOK3 derivatives (Fig. 3c, right), including RIOK3N1 (1–222a.a.), RIOK3N2 (1–470a.a.), and RIOK3C1 (222–519a.a.), and caspase-10g derivatives (Fig. 3d, right), including caspase-10gN1 (1–113a.a.) and caspase-10gC1 (114–247a.a.). Coimmunoprecipitation assays showed that the RIO domain was crucial for the interaction, the flanked protein sequence most likely helped the binding (Fig. 3c, left), and RIOK3 interacted with each DED of caspase-10g (Fig. 3d, left).

RIOK3 suppresses caspase-10g-mediated NF-κB activation by preventing the association of caspase-10 with other proteins

Interaction analysis demonstrated the association of RIOK3 with caspase-10. Therefore, we decided to examine the effect of RIOK3 on caspase-10-mediated NF-κB activation. Reporter assays indicated that RIOK3 suppressed the caspase-10g-mediated NF-κB activation in HEK293T cells (Fig. 4a). RIOK3N2 had a similar effect, but RIOK3N1, which could not bind to caspase-10g, had no such effect (Fig. 4a). It has been demonstrated that RIP1 and NIK directly bind to the prodomain of caspase-10 and they are essential for caspase-10-mediated NF-κB activation. Both modules of DED are required for these interactions as well as NF-κB activation [18]. Truncation derivative analysis showed that RIOK3 simultaneously bound to each single DED of caspase-10. We speculated that RIOK3 is likely to inhibit caspase-10-mediated NF-κB activation due to its blockade of caspase-10 binding to RIP1 and NIK. Competitive binding assays validated our hypothesis: when the concentration of RIOK3 increased, the RIP1 and NIK signals were reduced (Fig. 4b, c).

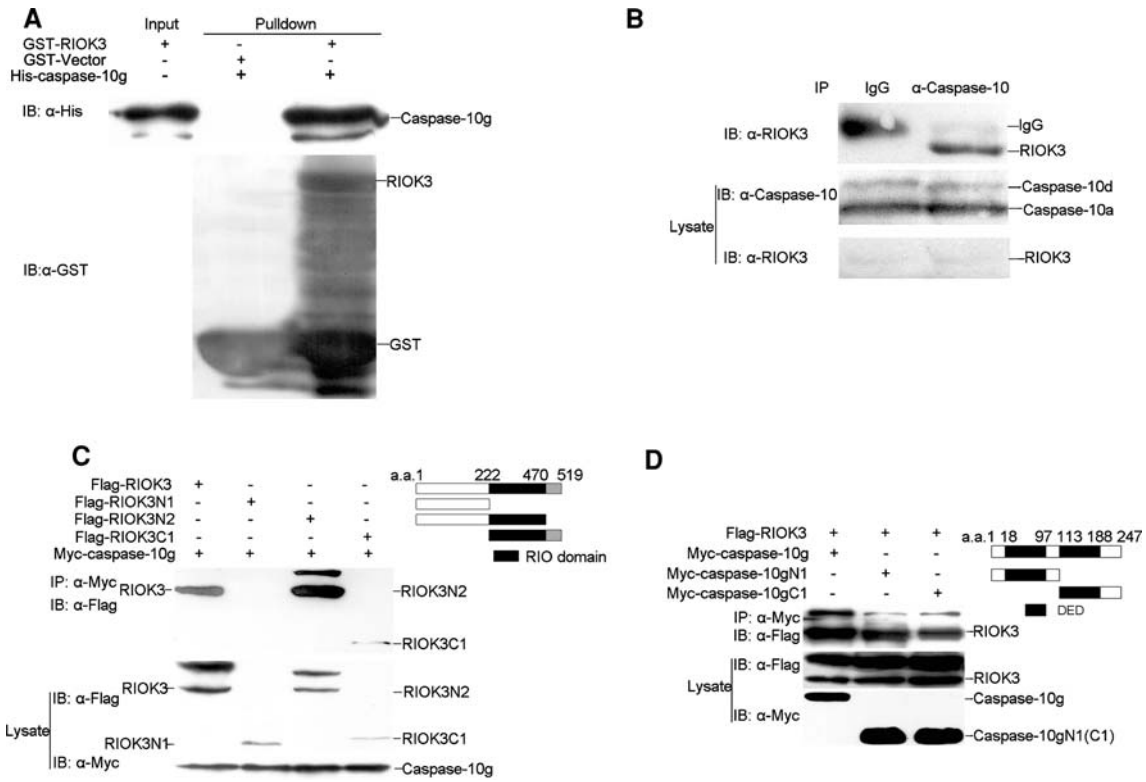

Fig. 3 RIOK3 interacts with caspase-10. **a** GST pull-down assay. GST and GST-RIOK3 proteins expressed in *E. coli* BL21 were immunoblotted with anti-GST antibody (*bottom panel*). His-caspase-10g expressed in *E. coli* BL21 (DE3) was purified and then incubated with the purified GST or GST-RIOK3 in the presence of glutathione beads. The bound proteins were immunoblotted with anti-His antibody (*upper panel*). **b** In vivo association of endogenous RIOK3 and caspase-10. Equal amount of HEK293T cell lysates was immunoprecipitated with anti-caspase-10 antibody or mouse IgG and analyzed by anti-RIOK3 antibody. **c** Left, caspase-10g was coimmunoprecipitated with full length, N1, N2 and C1 of RIOK3. HEK293T cells were co-transfected with the indicated constructs. Right, schematic representation of Flag-RIOK3 truncations designed to identify the interaction region with Myc-caspase-10g. **d** Left, RIOK3 was coimmunoprecipitated with full length, N1 and C1 of caspase-10g. Right, schematic representation of Myc-caspase-10g truncations designed to identify the interaction region with Flag-RIOK3

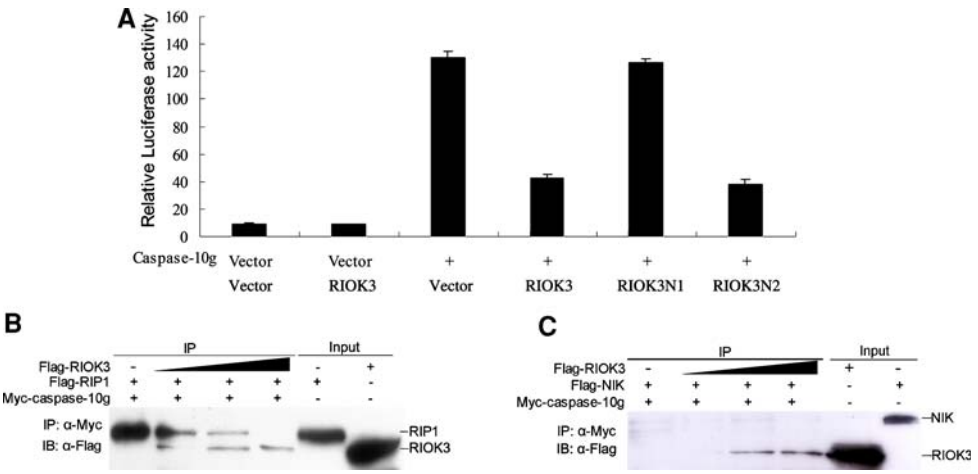

Fig. 4 RIOK3 inhibits caspase-10g-mediated NF-κB activation. **a** Effects of RIOK3 and its derivatives on caspase-10g-mediated NF-κB activation. HEK293T cells were transfected with indicated plasmids. Twenty-four hours after transfection, reporter assays were performed. **b** RIOK3 and RIP1 bind competitively to caspase-10g. HEK293T cells were transfected with Flag-tagged RIOK3, RIP1 and Myc-tagged caspase-10g, respectively. We mixed each quarter of the cell lysates of RIP1 and caspase-10g, and then added the cell lysates of RIOK3 to each mixture by the ratio of 1:2:4 or none. The final cell lysate mixtures were immunoprecipitated with anti-Myc and immunoblotted with anti-Flag. **c** RIOK3 and NIK bind competitively to caspase-10g. The experiment was performed similarly as described in **b**

A RIOK3 kinase inactive mutant does not markedly affect TNFα-induced NF-κB activation

Since RIOK3 possesses a kinase signature, it is indispensable to address the relevance between the kinase activity of RIOK3 and NF-κB activity. We first examined the kinase activity of RIOK3. HEK293T cells were transfected with Flag-RIOK3 or RIOK3 (K290A), in which the invariant lysine in subdomain II that is critical for ATP binding was mutated [20]. Immunoprecipitated RIOK3 or RIOK3 (K290A) was subjected to an in vitro kinase assay, followed by SDS–PAGE and visualized by autoradiography. A ^{32}P-labeled band corresponding to RIOK3 was observed only when the wild-type protein was immunoprecipitated (Fig. 5a). The observation showed RIOK3 (K290A) could act as a kinase dead mutant, hereafter be shortened as RIOK3KD. Reporter assays showed that the kinase inactive version of RIOK3, RIOK3KD, did not inhibit TNFα-induced NF-κB activation (Fig. 5b), but the enhancing effect of caspase-10g was repressed by RIOK3KD (Fig. 5c). These data suggest that RIOK3 inhibits TNFα-induced NF-κB activation relying on its kinase activity, although this activity seems not to be dealt with caspase-10-mediated NF-κB activation.

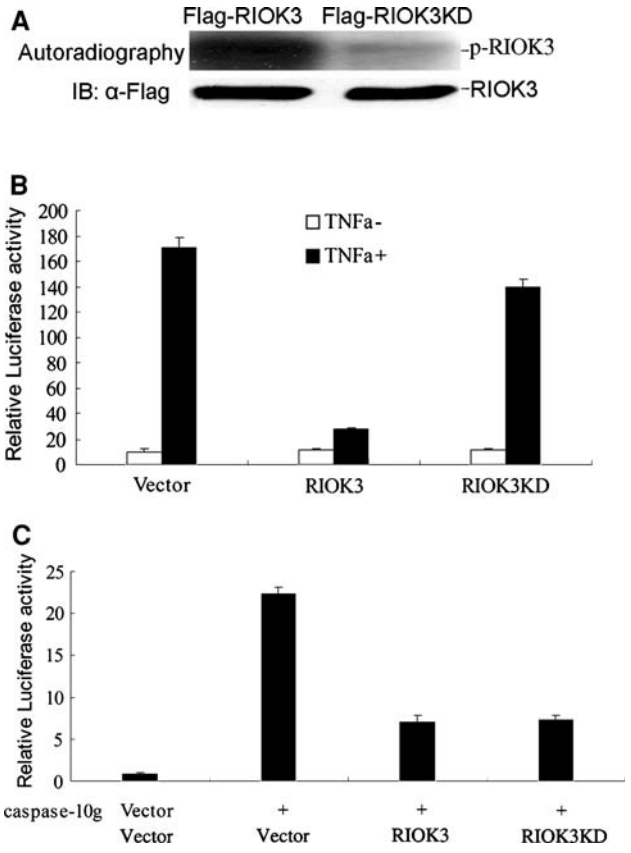

Fig. 5 Effects of RIOK3KD on NF-κB activation. **a** RIOK3 in vitro kinase assay. HEK293T cells (~1 × 10^7) transfected with Flag-RIOK3 or Flag-RIOK3KD were lysed, and lysates were immunoprecipitated with anti-Flag. Half of the precipitates were blotted with anti-Flag, and the other half were used for kinase assay. **b** RIOK3KD did not inhibit TNFα-induced NF-κB activation. HEK293T cells were transfected with pNF-κB-Luc and pRL-TK, together with empty, Flag-RIOK3 or Flag-RIOK3KD plasmid. Cells were treated and assayed as in Fig. 1a. **c** RIOK3KD inhibited caspase-10-mediated NF-κB activation. Transfection and reporter assays were performed similarly as described in Fig. 4a

Discussion

In a screen of the complete protein kinase for identifying somatic mutations in human breast cancer, no mutations of RIOK3 protein were found [21], which suggests that functional RIOK3 protein is necessary for cell survival. This result also suggests a possible explanation why RIOK3-involved diseases are rarely found.

Our findings support a correlation between the expression pattern of RIOK3 in malignant melanoma and the effect of RIOK3 on NF-κB activation. NF-κB activation has been proven as an event that promotes melanoma tumor progression [8, 22]. A number of NF-κB-regulated chemokines are highly expressed in melanoma. For example, overexpression of IL8 causes metastatic tumor growth in normal primary melanoma cells [23] and is associated with the transition from radial growth phase to vertical growth phase in melanoma [24]. Inhibition of NF-κB in highly metastatic melanoma xenografts in nude mice resulted in a decrease in angiogenesis which was correlated with down-regulation of the expression level of IL8 [8]. Some gene mutations promote the IKK complex-mediated degradation of IκB family members, and then NF-κB becomes activated in malignant melanoma [25]. We suppose overexpression of RIOK3 impairs the phosphorylation of IκBα and then suppresses its degradation, which leads to reduced NF-κB activation; conversely, down-regulation of the expression of RIOK3 results in NF-κB activation and makes melanoma cells invasive.

Dependent on its prodomain, caspase-10 activates NF-κB through RIP1 and NIK. Our results demonstrate that by binding to both DEDs of the prodomain of caspase-10, RIOK3 prevents the interaction of caspase-10 with RIP1 and NIK, and then blocks NF-κB signal transduction mediated by caspase-10. Caspase-8 is structurally and functionally homologous to caspase-10 and can also activate NF-κB by a similar mechanism [18, 26], which suggests that caspase-8 may substitute for caspase-10 in the NF-κB signaling pathway. But intriguingly, RIOK3 did not interact with caspase-8 (data not shown). It is reported that caspase-10 cannot replace caspase-8 in TRAIL (TNF-related apoptosis inducing ligand)-induced apoptosis [27] and TNFα-induced NF-κB activation [28]. The specific

interaction between caspase-10 and RIOK3 further suggests that caspase-10 and caspase-8 have distinct roles in NF-κB signaling.

Our results suggest that RIOK3, which may be an emerging paradigm of protein kinases that also function as adapters, inhibits caspase-10-mediated NF-κB activation independent of its kinase activity but functions as a kinase in TNFα-induced NF-κB activation pathway. Thereby, it is possible that RIOK3 affects NF-κB signaling pathway by a mechanism independent of caspase-10. It is well known that regulation of NF-κB signaling by phosphorylation is highly versatile [11, 29]. Identification of the substrate of RIOK3 kinase will more clearly illuminate the mechanism that leads to repression of NF-κB activation by RIOK3. In previous studies, seemingly contradictory results had been reported that caspase-10 was involved in TNFα-induced apoptosis in intestinal epithelial cells [30] but not in polymorphonuclear neutrophils [31]. The physiological role of caspase-10 in TNFα-induced NF-κB activation remains to be proven. Also, the establishment of the in vivo relevance of NF-κB activation by caspase-10 will help us to understand the mechanism underlying the negative effects of RIOK3 on NF-κB activation.

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