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The carboxyl terminal tyrosine 417 residue of NOK has an autoinhibitory effect on NOK-mediated signaling transductions

Ying-Hua Li [a], Shan Zhong [a], Zhi-Li Rong [a], Yong-Ming Ren [a], Zhi-Yong Li [a], Shu-Ping Zhang [a], Zhijie Chang [a], Li Liu [a,b,*]

[a] *Tsinghua Institute of Genome Research, Institute of Biomedicine & School of Medicine, Tsinghua University, Beijing 100084, China*
[b] *Department of Microbiology and Etiology, Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing 100005, China*

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Abstract

Receptor protein tyrosine kinases (RPTKs) are essential mediators of cell growth, differentiation, migration, and metabolism. Recently, a novel RPTK named NOK has been cloned and characterized. In current study, we investigated the role of the carboxyl terminal tyrosine 417 residue of NOK in the activations of different signaling pathways. A single tyrosine to phenylalanine point mutation at Y417 site (Y417 F) not only dramatically enhanced the NOK-induced activation of extracellular signal-regulated kinase (ERK), but also markedly promoted the NOK-mediated activation of both signal transducer and activator of transcription 1 and 3 (STAT1 and 3). Moreover, the proliferation potential of NIH3T3-NOK (Y417F) stable cells were significantly elevated as compared with that of NIH3T3-NOK. Overall, our results demonstrate that the tyrosine Y417 residue at the carboxyl tail of NOK exhibits an autoinhibitory role in NOK-mediated signaling transductions.
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The protein kinase is involved in a broad range of fundamental cellular processes such as metabolism, splicing, migration, protein modification, tumorigenesis, and metastasis etc. [1–4]. Studies on kinome reveal that the available number of protein kinase molecules is limited in human genome [5], and the expressions of these molecules are often tightly regulated [4]. By now, about 518 protein kinases have been documented [5]. Among them, only 90 molecules belong to protein tyrosine kinases in which 58 molecules are receptor protein tyrosine kinases (RPTKs) and 32 are non-RPTKs [5]. The novel oncogene with kinase-domain (NOK) is a newly identified receptor like tyrosine kinase that promoted tumorigenesis and metastasis and may stand out as a distinct member within RPTK superfamily [6,7].

Many protein kinases subtly regulate their intracellular kinase activities by an autoinhibitory/activation mechanism [8–10]. For RPTK molecule, it has been shown that the juxtamembrane region (JM) and the carboxy-terminal tail (CT) are critical segments in regulating the autoinhibitory process [11–16]. In the absence of extracellular ligand stimulation, the JM and CT domains are usually folded over the kinase domain to prevent RPTK activation, whereas ligand-mediated receptor dimerization stimulates the conformational change on protein structure upon *trans* phosphorylations at some critical tyrosine residues and results in opening up the otherwise 'buried' kinase domain to turn on downstream signaling cascades [10,17]. However, the extent of the autoinhibition may vary from one molecule to another. For example, Chiara et al. showed that removing the last 46 amino acids of platelet-derived growth factor receptor β (PDGFRβ) could activate its kinase activity but not transformation ability [12]. In

* Corresponding author.
E-mail address: Liu_Li@mail.tsinghua.edu.cn (L. Liu).

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contrast, deleting the last 15 residues of the endothelial receptor tyrosine kinase Tie2 not only enhanced the kinase activity and receptor autophosphorylation but also markedly induced Akt and ERK activations and anti-apoptotic effect [14]. For a subset of non-RPTKs such as Brk (breast tumor kinase) that contains Src homology 2 (SH2) and Src homology 3 (SH3) domains, the autoinhibition of Brk is probably due to the intramolecule interactions between the C-terminal tyrosine 447 (Y447) residue and the SH2 domain as well as between the proline-rich sequence within the SH2-kinase linker region and the SH3 domain [18]. Although the terminal Y447 residue of Brk may not be phosphorylated, a single tyrosine → phenylalanine point mutation at Y447 (Y447F) dramatically increased the Brk kinase and autophosphorylation properties [18], indicating that phosphorylation at Y447 may not be prerequisite for its autoinhibitory function.

Structurally, NOK protein has many unique features. NOK is a receptor like protein kinase with a single transmembrane helix that is located at a region proximal to its N-terminus, does not contain a signal peptide and lacks nearly a complete receptor ectodomain [7]. Previously, we found that point mutation at either Y327 or Y356 site was sufficient to abrogate tumorigenesis in nude mice by impairing multiple downstream mitogenic signaling pathways [6]. The activity of RPTK inside the cells can sometimes be balanced by an autoinhibitory/activation mechanism. However, how NOK gene is auto-regulated is currently unknown. It has been demonstrated that the NOK protein is distributed in cell cytoplasm rather than on cell surface [7]. From a structural point of view, the regulation of NOK may be similar to that of Brk. In the present study, we provide important evidences to show that the terminal tyrosine 417 residue of NOK has an autoinhibitory effect on the activations of NOK-mediated downstream signaling pathways, and point mutation at the Y417 residue could rescue this inhibition and dramatically promoted cellular signaling and proliferation.

Materials and methods

Antibodies. Anti-HA (F-7), anti-p-ERK (E-4), anti-ERK1 (K-23), anti-p-STAT1 (A-2), anti-STAT1 (E-23), anti-β-Actin, and goat anti-α-Tubulin (E-19) antibodies were purchased from Santa Cruz Biotechnology.

Plasmid construction and site-directed mutagenesis. To generate the mutant NOK construct, the NOK cDNA was first released from pcDNA3-NOK [7] by *Hin*dIII/*Xba*I double digestion and subcloned into pUC18 vector to form pUC18-NOK. Then, pUC18-NOK-Y417F was generated by using MutantBEST Kit (Takara Biotechnology). The polymerase chain reaction (PCR) was carried for 30 cycles at 94° C for 45 s, 55 °C for 45 s, and 72 °C for 4 min with the primers 5′-gagcctcttcttcaa ctatagcatgc-3′ and 5′-tccactctgatgccggccacag-3′. After confirmed by sequencing analysis, the mutated cDNA was subcloned into pcDNA3.0 to form pcDNA3.0-NOK(Y417F). Elk1-dependent luciferase reporter system including pFA-Elk1 and pFR-Luc was kindly provided by Dr. Akihiko Yoshimura (Kyushu University, Fukuoka, Japan).

Cell culture, transient transfection, and the generation of NIH3T3 stable cells. Murine embryo fibroblast cells NIH3T3 and modified human embryonic kidney cells 293T (HEK293T) were grown in DMEM containing 10% calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin at 37 °C in 5% CO_2-containing atmosphere. Transfection was performed by using VigoFect (Vigorous, Inc.). For stable cell selection, the transfected NIH3T3 cells were first plated onto 96-well dishes and selected in the presence of 1 mg/ml G418 for 10 days. Then, selected resistant clones were expanded in 10 cm culture dishes for additional analysis.

Luciferase assay. Luciferase assays were carried out in HEK293T cells grown in 12 well plates by co-transfecting the Elk1 luciferase reporter system (1.0 μg pFA-Elk1 and 1.0 μg pFR-Luc for each well) or STAT3 luciferase reporter system plus the indicated constructs. After 24-h transfection, the cells were cultured with or without serum for another 24 h, and then the cell lysates were assayed by Dual Luciferase Assay system (Promega) and detected by Top Count (Packard).

[^{3}H]Thymidine incorporation assay. Stable NIH3T3 cell lines were plated onto 24-well plates at 3.0×10^4 cells/well. After 24 h, the cells were starved overnight. [^{3}H]Thymidine (1 μCi/ml) was added into each well 6 h before harvesting. The reaction products were fixed with 5% trichloroacetic acid and solubilizing in 0.5 M NaOH/0.5% SDS prior to scintillation counting.

Western blot analysis. Transfected cells were lysed with gentle rotation in a lysis buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, pH 8.0, and 0.5% NP40) in the presence of protease inhibitors. After centrifugation at 4 °C for 20 min at 12,000 rpm, the cell lysates were resolved by SDS–PAGE and the reaction products were subjected to immunoblotting analysis.

Results

The tyrosine 417 residue of NOK had an autoinhibitory role in NOK-mediated MAPK signaling pathway

The carboxyl terminuses of protein tyrosine kinases (PTKs) often present autoinhibitory role [10]. It has been shown that the terminal tyrosine residue of PTK could contribute to the autoinhibitory effect of PTK [18,19]. The terminal tyrosine 417 (Y417) residue of human NOK is among the aligned ten tyrosine residues conserved between human and mouse NOKs [7], and has a very similar structural location as with the tyrosine 447 of Brk. Thus, we assumed that the Y417 of NOK might also have an autoinhibory effect. To this end, we generated a tyrosine → phenylalanine mutation at this point to construct NOK-Y417F mutant (Fig. 1A). In order to test whether the Y417F mutant had an autoinhibitory effect, we first used the transient transfection approach to evaluate the activation of NOK-mediated RAS/MAPK signaling pathway by using Elk1 luciferase assay system. Previous study indicated that the point mutation at either Y327 or Y356 site significantly impaired NOK-mediated ERK activation [6]. In order to make a better comparison, the wild-type NOK and its mutant derivatives (Y327F, Y356F, and Y417F) were all individually co-transfected with the Elk1 luciferase reporter system into HEK293T cells. In consistent with our previous observation, the over-expression of wild-type NOK dramatically enhanced Elk1-mediated luciferase activity by 3- to 4-folds as compared with that of the empty vector alone (P3), whereas point mutation at either Y327 or Y356 site markedly reduced the activation of RAS/MAPK signaling (Fig. 1B). Intriguingly, point mutation at Y417 residue did not reduce but further promoted NOK-mediated luciferase activation (Fig. 1B),

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