PIBB 生物化学与生物物理进展
Progress in Biochemistry and Biophysics
2008, 35(1): 29~34
www.pibb.ac.cn

NOK Interacts With Akt and Enhances Its Activation*

LI Ying-Hua [1)], CHANG Zhi Jie [1)], LIU Li [1, 2)**]

([1)] *School of Medicine, Department of Biological Sciences and Biotechnology, State Key Laboratory of Biomembrane and Membrane Biotechnology, Tsinghua University, Beijing* 100084, China;
[2)] *Institute of Basic Medical Sciences Chinese Academy of Medical Sciences, School of Basic Medicine Peking Union Medical College, Beijing* 100005, China)

Abstract NOK is a newly identified receptor protein-tyrosine kinase (PRTK) molecule that can promote tumorigenesis and metastasis. Previous data showed that NOK could activate the phosphatidylinositol 3'-kinase (PI3K) pathway in stable BaF3 cells. But how does NOK activate PI3K in cells remains unknown. It was showed that NOK physically interacted with the PI3K downstream effector Akt and enhanced its activation in human embryo kidney 293T (HEK293T) cells. Deletion mapping indicated that protein kinase B (Akt) was able to directly contact with the kinase domain of NOK. Inactivating the Akt kinase domain significantly reduced the intermolecular interaction between NOK and Akt, while constitutively active mutant of Akt apparently had a stronger interaction with NOK. NOK did not have an additive effect on insulin-mediated Akt activation. Overall, the results indicate that NOK might complex with Akt and directly activate PI3K/Akt signaling pathway.

Key words RPTK, NOK, PI3K, protein kinase B (Akt)

Receptor protein-tyrosine kinases (RPTKs) are able to activate multiple cellular processes such as cell growth, differentiation, migration, and anti-apoptosis that may subsequently result in tumor development and metastasis [1~5]. One of the key signal-transduction molecules activated by RPTK is the PI3K. Emerging evidences have suggested that PI3K is one of the key mediators to control cellular transformation and cancer development [6~8]. The protein kinase Akt (also known as protein kinase B (PKB)) is a major downstream effector of PI3K that is most often associated with the malignant development of human cancers. The activation of Akt can phosphorylate a multitude of diverse signaling substrates that have great impacts on cell survival, cell-cycle progression, cell growth and cell metabolism [9, 10].

The novel oncogene with kinase-domain (NOK) belongs to a distinct member of the RPTK superfamily that can promote tumorigenesis and metastasis in animal model [11, 12]. NOK could activate the phosphatidylinositol 3'-kinase (PI3K) pathway in stable BaF3 cells, and LY294002, a specific inhibitor of PI3K could dramatically inhibited colony formation of BaF3-NOK in soft agar, indicating that blocking the PI3K pathway was sufficient to prevent NOK induced cellular transformation [12]. Furthermore, Chen, *et al* [11] found that a single tyrosine mutant of NOK (NOK-Y356F) could prevent the activation of Akt in stable BaF3 cells.

In the present report, we explore the potential role of NOK in mediating PI3K/Akt activation in human embryo kidney 293T (HEK293T) cells. Our results indicate that NOK might promote the activation of PI3K/Akt signaling pathway by directly targeting Akt, the downstream signaling molecule of PI3K.

1 Materials and methods

1.1 Plasmid constructs

pcDNA3.1/NOK-Myc-His and pcDNA3 .1/NOK-HA-His constructs were described previously [13]. The various NOK deletion mutants were constructed by

*This work was supported by grants from National Basic Research Program of China (2001CB510006, 2002CB513007), Beijing Scientific and Technological Program (H020220020420A-02), The National Natural Science Foundation of China (30671944) and Tsinghua-Yue-Yuen Fund.
**Corresponding author.
Tel: 86-10-62773625, E-mail: liu_li@mail.tsinghua.edu.cn
Received: November 21, 2007 Accepted: December 12, 2007

using PCR directed approach with Pyrobest DNA polymerase (Takara Biotechnology, Dalian, China) by using specific primers and subsequently verified by DNA sequencing analysis. All constructs were subcloned into pcDNA3.1/Flag-His (Invitrogen, Carlsbad, CA, USA).

1.2 Chemicals and reagents

Mouse anti-Myc (9E10), mouse anti-HA (F-7) and mouse anti-β-actin (C-2) were purchased from Santa Cruz Biotechnology. Mouse anti-Flag (M2) antibody was purchased from Sigma. Rabbit anti-phospho-Akt (Thr308) (#9275) was purchased from Cell Signaling. Protein G PLUS-Agarose (sc-2002) was from Santa Cruz Biotechnology. Secondary antibodies such as goat anti-mouse and goat anti-rabbit Ig labeled with horseradish peroxidase were from Vigorous, Inc.

1.3 Cell culture and transfection

HEK293T cells were grown in DMEM containing 10% calf serum, 100 U/ml penicillin, 100 mg/L streptomycin at 37℃ supplemented with 5% CO_2. Transfection was performed by using VigoFect (Vigorous, Inc.).

1.4 Western blot analysis

Transfected HEK293T cells were lysed with gentle rotation in a lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA pH 8.0, 0.5% NP40) in the presence of protease inhibitors. After centrifugation at 4℃ for 20 min at 12 000 r/min, the cell lysates were resolved on 10% SDS-PAGE followed by blotting onto nitrocellulose membranes. The transferred membranes were then probed with primary antibodies followed by relevant secondary antibodies conjugated to horseradish peroxidase. Detection was enhanced by chemiluminescence (PIERCE).

1.5 Co-immunoprecipitation assay

HEK293T cells were co-transfected with plasmid vectors containing NOK-Myc and HA-AKT. Single transfection with one of the plasmids alone was served as control. 24 h later, cells were lysed on ice by using 500 μl lysis buffer mentioned above. The detergent soluble fraction was recovered by centrifugation at 4℃ for 20 min at 12 000 r/min and the supernatants were subjected to immunoprecipitation with mouse anti-Myc antibody. Immune complexes were isolated with protein G PLUS-Agarose beads. The immunoprecipitated products were washed four times with lysis buffer, eluted with 2×SDS-PAGE loading buffer and analyzed by Western blotting.

2 Results

2.1 NOK directly interacted with Akt and enhanced its activation *in vivo*

It was showed previously that NOK could activate PI3K pathway in stable BaF3 cells. However, the mechanism underlying this activation is unknown. To detect whether NOK was able to directly interact with Akt, co-immunoprecipitation assay was employed. HA-Akt was co-transfected with NOK-Myc or empty vector into HEK293T cells (Figure 1a). The reaction product was first immunoprecipitated with anti-Myc antibody and then blotted with anti-HA antibody. Figure 1a demonstrates that Akt was detected in the NOK immunoprecipitated products, indicating that NOK could directly interact with Akt in HEK293T cells. This interaction was further confirmed by the result of the reciprocal co-immunoprecipitation as demonstrated in Figure 1b.

The next question we tried to ask was whether the intermolecular interaction between NOK and Akt could activate PI3K/Akt signaling pathway in HEK293T cells. Figure 1c demonstrates that over-expressing NOK did not significantly enhance endogenous Akt phosphorylation in HEK293T cells. In contrast, delivering exogenous Akt construct into HEK293T cells could activate intracellular Akt phosphorylation. Furthermore, co-transfecting NOK and Akt into HEK293T cells markedly increase the level of Akt phosphorylation by about 2～3 folds as compared with the single Akt transfection (Figure 1c, compare lane *4* with lane *3*), indicating that NOK could activate Akt signaling in different types of cells.

2.2 Akt had a directly physical contact with the kinase domain of NOK

It is still unclear that which domain(s) of NOK was responsible for interacting with Akt. To solve this issue directly, a series of NOK deletion mutants was used to define the domain(s) responsible for NOK-Akt intermolecular interaction. HEK293T cells were single transfected with HA-Akt or co-transfected with HA-Akt and NOK-Flag, NOKΔECD-Flag, NOK-ICD-Flag or NOK-KD-Flag expression constructs. The cell lysates were first immunoprecipitated with anti-Flag antibody and then subjected to Western blot analysis with antibody to HA. Figure 2 demonstrates that the Akt proteins could be effectively co-immunoprecipitated by all three NOK deletion mutants, indicating that the

kinase domain but not the N-terminus (1～115 aa) and C-terminus (381～422 aa) of NOK was required for this intermolecular interaction.

2.3 NOK-mediated Akt activation has less effect on insulin-mediated Akt activation

As NOK could direct interact with Akt *in vivo*,

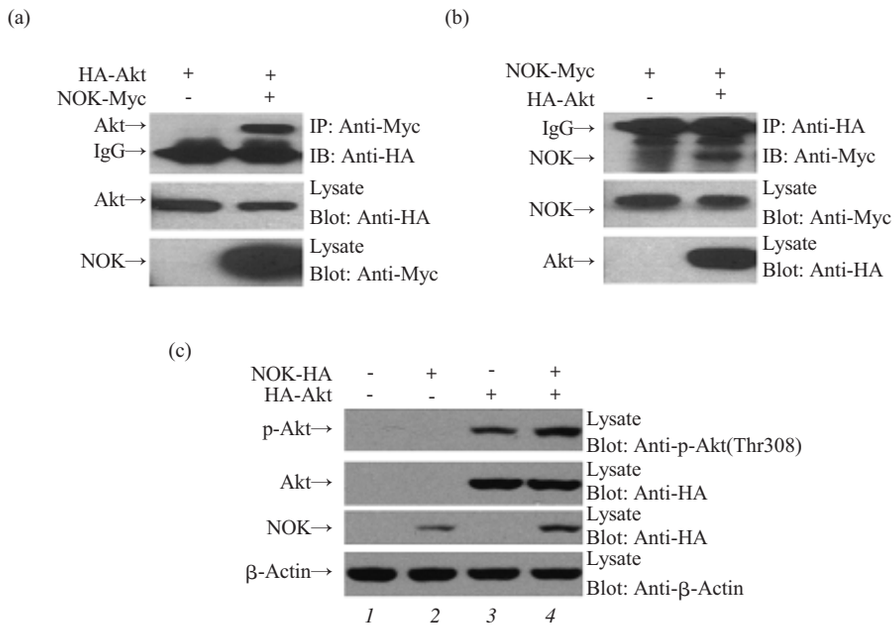

Fig. 1　NOK directly interacted with Akt and enhanced its activation *in vivo*

(a) Co-immunoprecipitation assay. HEK293T cells were transfected with plasmids encoding NOK-Myc or co-transfected with HA-Akt and NOK-Myc. Cell lysates were immunoprecipitated with anti-Myc antibody. The transferred membrane was then probed with antibodies against HA. (b) Reciprocal co-immunoprecipitation assay. HEK293T cells were transfected with plasmids encoding HA-Akt or co-transfected with HA-Akt and NOK-Myc. Cell lysates were first immunoprecipitated with anti-HA antibody. The transferred membrane was then probed with antibodies Myc. (c) NOK activated exogenous Akt activity. HEK293T cells were transfected with the empty construct (lane *1*), NOK-HA (lane *2*), and HA-Akt (lane *3*) or co-transfected with NOK-HA plus HA-Akt (lane *4*). The reaction products were then probed with anti-p-Akt antibody. The expression levels of NOK and Akt were also evaluated with anti-HA antibody. β-Actin protein was used as internal control.

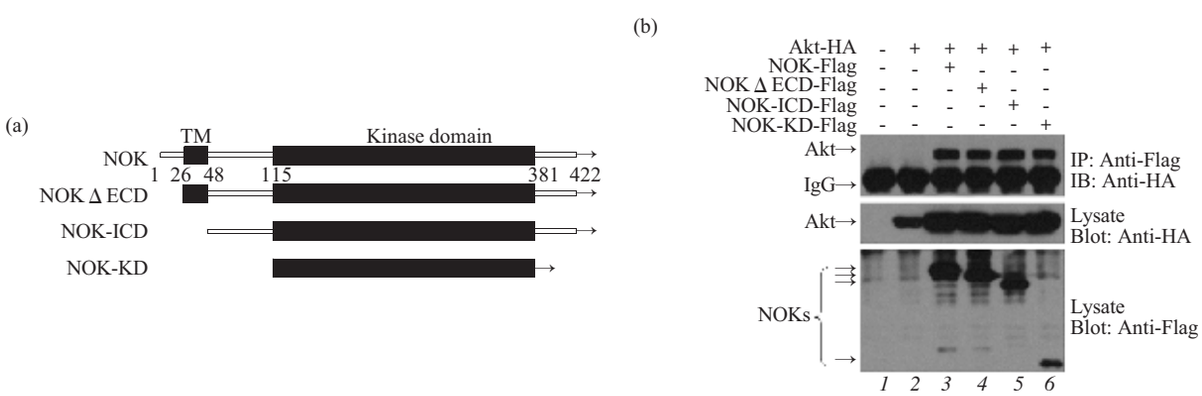

Fig. 2　Akt had directly physical contacts with the kinase domain of NOK

(a) Schematic diagram showing the protein structures of human NOK and its deletion mutants: the ectodomain deletion mutant (NOK ECD), the endodomain mutant (NOK-ICD) and the kinase domain mutant (NOK-KD). A Flag tag was fused with the C terminuses of these proteins. (b) Identification of the NOK domain interacting with Akt. NOK-Flag, NOKΔECD-Flag, NOK-ICD-Flag and NOK-KD were co-transfected with HA-Akt into HEK293T cells. The cell lysates were first immunoprecipitated with anti-Flag antibody followed by Western blot analysis with antibody to HA. The expression levels of NOK-Flag, NOK ECD-Flag, NOK-ICD-Flag, NOK-KD and HA-Akt were also evaluated.

we want to know whether the kinase activity of Akt is necessary for this intermolecular interaction between Akt and NOK. A kinase dead Akt (HA-Akt-DN) carrying a lysine (K) → methionine (M) mutation at 179 aa and a constitutively active Akt mutant (HA-Akt-CA) which only expresses the myristoylated catalytic domain of Akt (deleted pleckstrin homology (PH) domain) were used. Figure 3b shows that the kinase dead Akt bound to NOK as effective as the wild-type Akt did. In contrast, the constitutively active form of Akt exhibited an enhanced binding to NOK (Figure 3b, compare lane *3* with lanes *1* & *2*).

Since Akt can be activated by insulin stimulation, we tried to ask whether the phosphorylation level of insulin activated Akt could be enhanced by NOK *in vivo*. Figure 3c shows that insulin could markedly induce the activation of Akt. In the absence of insulin, additionally co-transfecting NOK could further promote the Akt phosphorylation (Figure 3c, compare lanes *3* and *5*). However, in the presence of insulin stimulation, co-transfecting NOK could not greatly promote the Akt phosphorylation (Figure 3c, compare lanes *4* and *6*), indicating that NOK-mediated Akt activation might have less effect on insulin mediated signaling cascade.

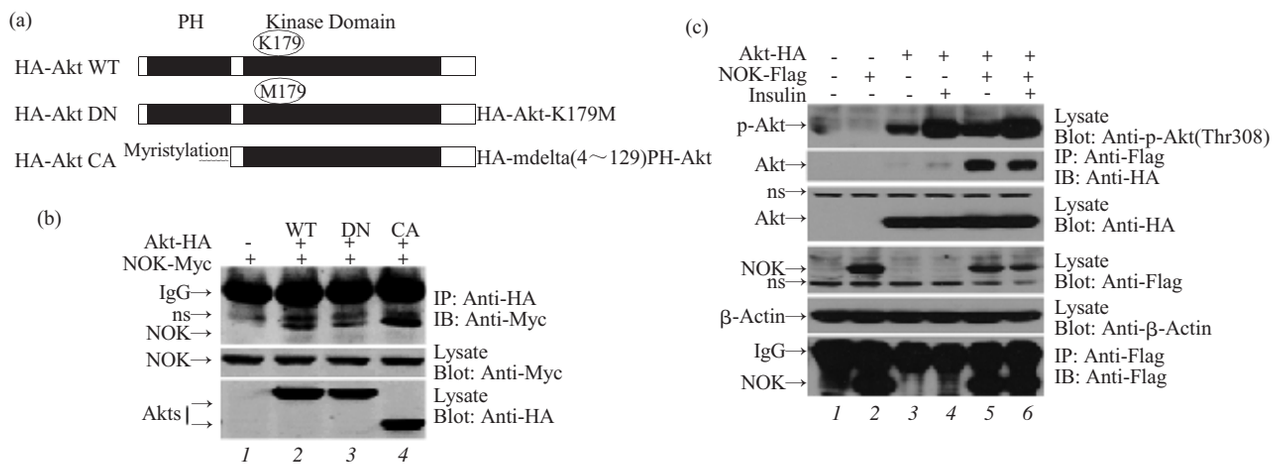

Fig. 3　Interaction of NOK and Akt is not regulated by insulin stimulation

(a) Schematic diagram showing the protein structures of mouse Akt and its mutant derivatives: the wild type Akt, the kinase dead mutant (Akt-K179M) and the constitutive active mutant (mdelta(4～129)PH-Akt). (b) HA-Akt, HA-Akt-K179M and HA-mdelta(4～129)PH-Akt were co-transfected with NOK-Myc into HEK293T cells. The cell lysates were first immunoprecipitated with anti-Flag antibody followed by Western blot analysis with antibody to HA. The expression levels of HA-Akt WT, HA-Akt-K179M, HA-mdelta(4～129)PH-Akt and NOK-Myc were also evaluated. (c) HEK293T cells were co-transfected with the indicated plasmids. Twenty-four hours later, the transfected cells were starved for additional 24 h, and then treated with or without insulin (100 nmol/L) for 15 min. Equal amounts of cell lysates were co-immunoprecipited by using anti-Flag antibody followed by Western blot analysis with antibody to HA. Cell lysates were also probed with anti-p-Akt antibody. The protein expression of NOK and Akt was also evaluated with anti-Flag or anti-HA antibody. β-Actin was served as internal control.

3　Discussion

Previously we showed that NOK could activate PI3K/Akt pathway [11, 12]. However, the mechanism controlling the Akt activation has not been explored. In this study, we provided important evidence to show that NOK could physically interact with Akt and enhanced Akt activation. NOK did not give an additive effect on Akt activation in responding to insulin stimulation.

Our study demonstrates for the first time that the pathway associated with NOK-mediated Akt activation may be different from that used by most of PTKs. Commonly, the ligand mediated RPTK dimerition activates its own kinase activity and subsequently phosphorylates its C-terminal tail which can serve as docking site to directly or indirectly interact with the p85 regulatory subunit of PI3K [10, 14]. The indirect activation on p85 can be achieved by adoptor proteins such as members of insulin receptor substrate (IRS)

family. For intracellular non RPTK molecule like the oncogene BCR/ABL, the activation of PI3K can also be mediated by a variaty of adaptor proteins such as Grb2-Gab2, Crkl-c-Cbl, IRS-1 and Src [15]. The activated PI3K then leads to the production and accumulation of the membrane associated second messenger phosphatidylinositol-3,4,5-trisphosphate (PIP3) that is able to interact with the PH domain of Akt to initiate downstream signaling cascades. Interestingly, our results indicates that, unlike most of PTKs, NOK might function at or immediate upstream of Akt to turn on Akt signaling. This is due to the fact that NOK could physically interact with Akt and promote Akt phosphorylation. In the inactive form, it is believed that the PH domain usually held constrains to prevent Akt activation [16]. In consistent with this concept, we demonstrated that removing the PH domain of Akt significantly enhanced the intermolecular interaction between NOK and Akt. Moreover, the kinase activity of NOK but not Akt might be required for this intermolecular interaction. These results provide additional evidences to support that NOK could likely be an immediate upstream modulator to control Akt activity. However, these results do not completely exclude the possibility that NOK may have alternative targeting sites such as the p85 subunit of PI3K since in our previous report the addition of PI3K specific inhibitor LY290042 could effectively block colony formation in NOK transformed BaF3 cells [12]. Whether this discrepancy is due to the tissue specific activation or multi-targeting by NOK is still needed to be elucidated.

The activation of PI3K/Akt signaling pathway is mainly responsible for insulin-mediated glucose metabolism [17]. Our data demonstrated that NOK apparently did not have major impact on insulin-mediated Akt activation. We propose that NOK might be a weak kinase that was barely able to compete out the strong signal generated by insulin receptor upon insulin stimulation. Thus, insulin-mediated Akt activation might override the NOK-mediated Akt activaiton in transfected HEK293T cells.

Acknowledgements We thank Dr. Michael Greenberg (Division of Neuroscience, Children's Hospital, Boston) for the mammalian expression vectors HA-AKT WT, HA-AKT DN and HA-AKT CA. We also would like to thank Professor ZHAO Nan- Ming, Professor ZHANG Xiu-Fang and Professor PEI Zhao-Hong for their encouragements and supports during the course of the study.

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NOK 与 Akt 相互作用并增强 Akt 的活化 *

李颖华[1)]　常智杰[1)]　刘　力[1, 2)**]

([1)]清华大学医学院，清华大学生物科学与技术系，生物膜与膜生物技术国家重点实验室，北京 100084；
[2)] 中国医学科学院基础医学研究所 北京协和医学院基础学院 微生物系，北京 100005)

摘要　NOK 是一个新近鉴定的受体型蛋白酪氨酸激酶分子，它能够促进肿瘤的形成和转移．前期的研究表明，NOK 在小鼠前 B 细胞(BaF3)中能够激活磷脂酰肌醇 3- 激酶(PI3K)信号通路．但是，人们并不清楚 NOK 在细胞内是如何激活 PI3K 信号通路的．研究发现，NOK 与 PI3K 下游的效应分子蛋白激酶 B(Akt)具有直接的相互作用．并且，在人胚肾细胞(HEK293T)中，NOK 能明显增强 Akt 的活性．通过 NOK 缺失突变体的免疫共沉淀实验，确定了 Akt 能直接结合 NOK 的激酶结构域．同时，Akt 的激酶活性缺失体并不影响其与 NOK 的结合，但也观察到，持续活化的 Akt 跟 NOK 具有更强的相互作用．最后，发现 NOK 对 胰岛素介导的 Akt 激活并没有产生叠加效应．实验结果显示，NOK 可以与 Akt 直接相互作用并增强 PI3K/Akt 信号通路的活化．

关键词　受体型蛋白酪氨酸激酶，NOK，磷脂酰肌醇 3- 激酶，蛋白激酶 B (Akt)
学科分类号　R73

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* 国家重点基础研究发展规划项目(2001CB510006，2002CB513007)，北京市科委重大项目(H020220020420A-02)，国家自然基金项目(30671944)和清华大学 - 裕元医学基金资助．

** 通讯联系人．
Tel:010-62773625, Fax:010-62773624, E-mail: liu_li@mail.tsinghua.edu.cn

收稿日期：2007-11-21，接受日期：2007-12-12