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NOK Activates STAT3 Signaling by a JAK2-Dependent Mechanism*

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Abstract Novel oncogene with kinase-domain (NOK) can activate multiple mitogenic signaling pathways including the janus kinases (JAK) and signal transducer and activator of transcription proteins (STAT). It was showed that NOK specifically and physically interacted with STAT3 in human embryo kidney 293T (HEK293T) cells. In addition, NOK could directly interact with most of the STAT3 subdomains except coiled-coil and C-terminal domains. Removing ectodomain and transmembrane domain of NOK markedly enhanced its intermolecular interaction with STAT3. Also, NOK could co-immunoprecipitate with JAK2 *in vivo*. Importantly, co-expression of NOK and JAK2 produced a synergistic effect on NOK-mediated STAT3 activation, while inactivating the kinase domain of JAK2 completely prevented this synergistic effect. Overall, the results indicated that NOK might complex with both STAT3 and JAK2 and activate STAT3 signaling by a JAK2-dependent mechanism.

Key words NOK, JAK2, STAT3, synergistic effect, co-immunoprecipitation, signaling activation

STAT signaling pathway can be activated by diverse receptors including cytokine receptors and receptor protein tyrosine kinase (RPTK) molecules[1~3]. In mammals, JAK-STAT signaling network is mainly consisted of four non-receptor tyrosine kinases (JAK1, JAK2, JAK3 and tyrosine kinase 2 (Tyk2)) and seven downstream STAT (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6) proteins[4,5]. Commonly, cytokine mediated receptor dimerization induces the juxtaposition of JAK associated with the receptors and trans- or auto-phosphorylation of JAK. The activated JAK then phosphorylates the receptor at particular tyrosine residues that in turn serve as the docking sites for the binding of SH2 containing proteins such as STAT. Subsequently, the latent STAT is switched to an active form upon phosphorylation by JAK. Finally, the activated STATs dimerize and translocate to cell nucleus to turn on downstream target gene expressions.

Different from the JAK-STAT activation by type Ⅰ and type Ⅱ cytokine receptors, RPTK mediated STAT activation is apparently more complicated. By now, at least three mechanisms could account for this process: the JAK-dependent [6~8], the JAK-independent [9~12] and the involvement of both [7, 13, 14]. Studies indicate that at least a portion of RPTKs activates STAT in a JAK-dependent manner as demonstrated by the facts that dominant negative or chemical inhibitor of JAK could block STAT signaling [6,8]. Two different pathways may account for JAK-independent STAT activation by RPTK. First, the kinase domain of RPTK is able to directly phosphorylate STAT[9]. Alternatively, RPTK-mediated STAT activation can be induced by recruiting a non-JAK tyrosine kinase such as Src [15]. However, genetic alterations such as gene amplification or mutations often lead to RPTK constitutively active in certain cancer tissues in which the endogenous STAT also expresses constitutively [13,14]. In this circumstance, both Src and JAK but not RPTK kinase domains are required for constitutive STAT activation [13, 14].

NOK is a unique RPTK that expresses intracellularly and is able to constitutively activate multiple mitogenic signals [16~18]. Using a chimeric

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EPOR/NOK in which the ectodomain of mouse erythropoietin receptor was fused to the transmembrane and endodomain of NOK, we demonstrated that STAT5 was constitutively active in BaF3-EPOR/NOK stable cells [17]. Point mutation at either tyrosine 327 or 356 residue of NOK inhibited or significantly reduced STAT5 activation [17]. In addition, over-expressing NOK in HEK293T cells could activate both STAT1 and STAT3 [18]. Mutagenic study further pointed out that the tyrosine 417 residue of NOK possessed an autoinhibitory effect on both STAT1 and STAT3 activations [18].

Although NOK could induce several STAT activations, the detailed mechanisms underlying these activations were still not clear. In this study, we intended to focus our study on the elucidation of the molecular mechanism(s) responsible for NOK-mediated STAT3 activation. We used co-immunoprecipitation, deletion mapping and luciferase approaches to dissect the potential intermolecular interactions among NOK, JAK2 and STAT3. Overall, we found that NOK and JAK2 could produce a synergistic effect on NOK-mediated STAT3 activation in a JAK2 indispensable manner.

1 Materials and methods

1.1 Plasmid constructs

NOK-Myc, NOK-HA or NOK-ICD-Myc constructs were described previously [18].

1.2 Chemicals and reagents

Mouse anti-Myc (9E10), mouse anti-HA (F-7), mouse anti-β-actin (C-2) and rabbit anti-STAT3 (C-20) were purchased from Santa Cruz Biotechnology. Mouse anti-Flag (M2) antibody was purchased from Sigma. 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, MDCK and KO2F4 cells were grown in DMEM containing 10% calf serum, 100 U/ml penicillin and 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 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 grown at 80% confluency were transfected with VigoFect. Cells were co-transfected with plasmid vectors containing NOK-Myc and Flag-STAT3. 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 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.

1.6 Luciferase assay

HEK293T cells grown in 24 well plates were co-transfected with the STAT3 luciferase reporter plasmid pGL3- (APRE)4-luc or STAT5 luciferase reporter plasmid pUC18-LHRE/TK 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).

2 Results

2.1 NOK specifically interacts with STAT3 *in vivo*

It was showed previously that NOK could activate multiple downstream cascades including STAT3 signaling pathway. To detect if NOK was able to directly interact with STAT3, co-immunoprecipitation assay was employed. NOK-HA was co-transfected with Flag-STAT3 or Flag-STAT5a or empty vector into HEK293T cells (Figure 1a). The reaction product was first immunoprecipitated with anti-Flag antibody and then blotted with anti-HA antibody. Figure 1a

demonstrates that NOK was detected in the STAT3 but not STAT5a immunoprecipitated products, indicating that NOK could directly and specifically interact with STAT3 in HEK293T cells.

To further confirm this result, we tried to detect the possible interaction between NOK and endogenous STAT3. To that end, a tet-off inducible MDCK cell line stably expressing NOK was constructed. The cells were treated with or without doxycycline for 24 h. The co-immunoprecipitation assay demonstrated that in the absence of doxycycline NOK could physically interact with endogenous STAT3, while addition of doxycycline could efficiently block intracellular NOK expression, indicating that the interaction between NOK and endogenous STAT was specific (Figure 1b).

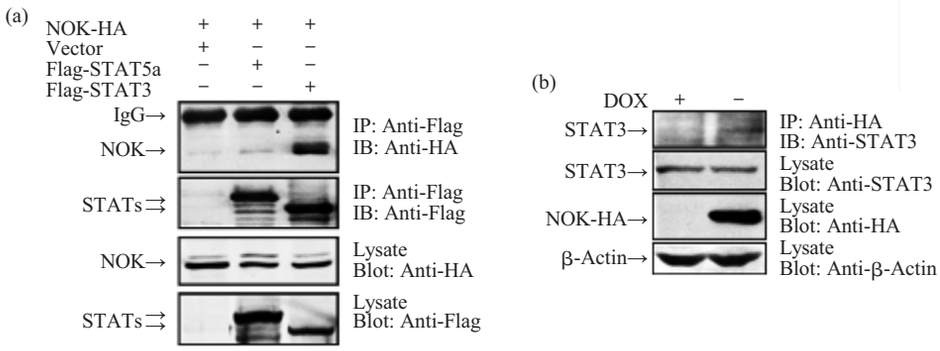

Fig. 1 NOK specifically interacts with STAT3 *in vivo*

(a) The interaction of NOK and STAT3 in transfected HEK293T cells. NOK-HA was co-transfected with Flag-STAT3 or Flag-STAT5a or empty vector into HEK293T cells. Cell lysates were first immunoprecipitated with anti-Flag antibody followed by Western blot analysis with anti-HA antibody. The expression levels of NOK-HA, Flag-STAT3 and Flag-STAT5a were also detected. (b) NOK interacted with endogenous STAT3. A Tet-off MDCK cell line stably transfected with NOK-HA was constructed. The cells were maintained in the presence or absence of doxycycline for 24 h. The cell lysates were first immunoprecipitated with anti-HA antibody followed by Western blot analysis with antibody to STAT3. The expression levels of NOK-HA and STAT3 were also detected. β-Actin was served as an internal control.

2.2 NOK has directly physical contacts with the multiple domains of STAT3

The next question we tried to answer was which domain (s) of NOK and STAT3 was responsible for this intermolecular interaction. STAT3 can be subdivided into six domains: N-domain (ND), coiled-coil domain (CC or 4H), DNA binding domain (DB), linker domain (LD), Src homology 2 domain (SH2) and C-terminal domain (CT) as shown in Figure 2a [19]. The individual domains of Flag-tagged STAT3 were co-transfected with NOK-Myc into HEK293T cells. The results demonstrated that NOK could be detected in the reaction products immunoprecipitated with Flag-ND, Flag-DB, Flag-LD and Flag-SH2 but not Flag-4H and Flag-CT (Figure 2b, the top panel). The result was further confirmed by reciprocal co-immunoprecipitation with anti-Flag antibody and subsequent Western blot analysis with anti-Myc antibody (Figure 2b, the second panel).

To further elucidate this intermolecular interaction between NOK and STAT3, we used a previously defined NOK mutant (NOK-ICD) that only contained NOK endodomain to assay if the N-terminus of NOK had a role in NOK-STAT3 interaction. Figure 3b shows that, in a co-immunoprecipitation assay, the amount of STAT3 proteins immunoprecipitated by antibody to Myc-tagged NOK-ICD was more than that by antibody to Myc-tagged NOK, indicating that STAT3 had higher *in vivo* affinity with NOK-ICD than with NOK.

2.3 NOK specifically activates STAT3 through JAK2 pathway

Studies showed that RPTKs could activate STAT3 through JAK2-dependent and / or - independent manner [7, 12, 14]. To test the effect of JAK2 on NOK-mediated STAT3 activation, NOK-Myc was co-transfected with JAK2-HA or empty vector into HEK293T cells. Cell lysates were first immunoprecipitated with anti-HA and then blotted with anti-Myc antibody. NOK was only detected in the reaction product co-transfected with JAK2 (Figure 4a), indicating that a direct interaction between JAK2 and NOK occurred *in vivo*.

To assay if NOK could activate STAT3 through

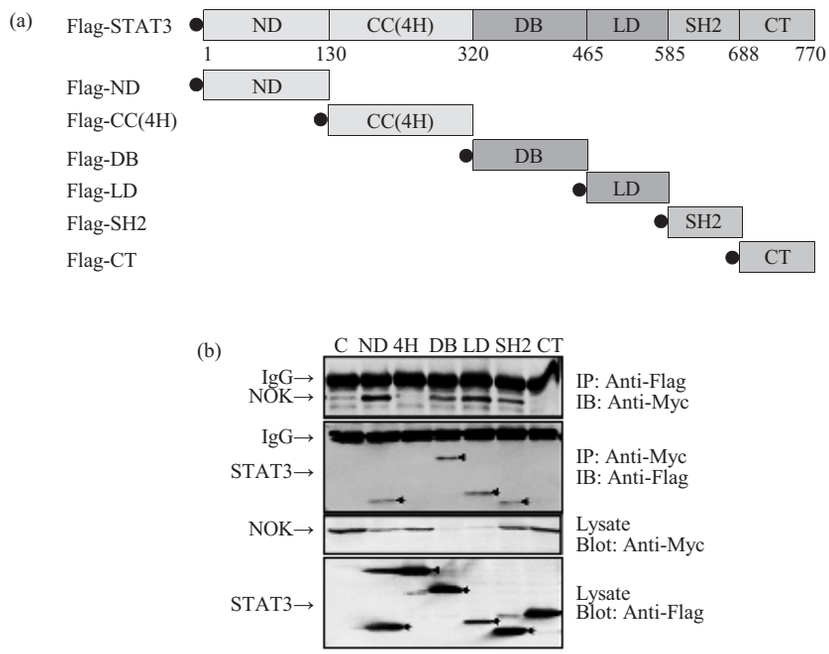

Fig. 2 Mapping the interaction domains of STAT3 with NOK

(a) Schematic diagram showing the domain structure of STAT3. Individual domains such as N-terminal domain (ND), the Coil-coiled domain (4H), the DNA-binding domain (DB), the linker domain (LD), the SH2 domain (SH2) and the C-terminal domain (CT) are shown. A Flag tag was fused with the N-terminuses of the full length STAT3 and its individual domains. (b) Identification of the STAT3 domains interacting with NOK. The domain truncation mutants of Flag-STAT3 were transfected with NOK-Myc into HEK293T cells. The cell lysates were first immunoprecipitated with anti-Flag or anti-Myc followed by Western blot analysis with antibody to Myc or Flag. The expression levels of NOK and individual STAT3 domains were also detected.

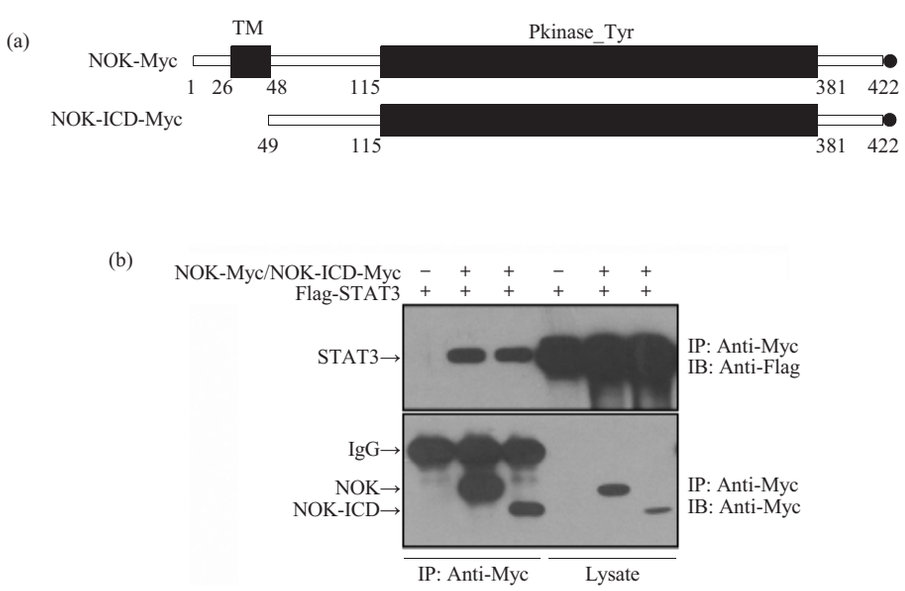

Fig. 3 Mapping the interaction domains of NOK with STAT3

(a) Schematic diagram showing the protein structures of human NOK and the endodomain mutant of NOK (NOK-ICD). A Myc tag was fused with the C-terminuses of these proteins. (b) Identification of the NOK domains interacting with STAT3. NOK-Myc and NOK-ICD-Myc were co-transfected with Flag-STAT3 into HEK293T cells. The cell lysates were first immunoprecipitated with anti-Myc followed by Western blot analysis with antibody to Flag. The expression levels of NOK-Myc, NOK-ICD-Myc and Flag-STAT3 were also evaluated.

JAK2 pathway, NOK and/or JAK2 were co-transfected with STAT3-responsive reporter (pGL3-(APRE)4-luc) into HEK293T cells. After 24 h, the cells were grown in the absence or presence of serum for additional 20 h. JAK2 or NOK alone could induce more than 4 fold increases in STAT3 activation (Figure 4b). More importantly, co-transfection of JAK2 and NOK produced a synergistic effect on STAT3 activation (Figure 4b). To further confirm this result, a STAT3 deficient cell line (KO2F4) was employed. Similarly, NOK and/or JAK2 were co-transfected with STAT3 and 4× APRE-luciferase into KO2F4 cells. In agreement with Figure 4b, co-transfection of JAK2 and NOK also produced a synergistic effect on STAT3 activation (Figure 4c) in KO2F4 cells. Thus, the results indicated that JAK2 should be a critical element in NOK-mediated STAT3 activation.

To detect the specificity of JAK2 in NOK-mediated STAT3 activation, NOK and/or JAK2 were co-transfected with STAT5 plus STAT5-responsive reporter (pUC18-LHRE/TK) into HEK293T cells. Different from Figure 4b and Figure 4c, only JAK2 but not NOK induced STAT5 activation (Figure 4d). Addition of NOK into JAK2 did not enhance JAK2-mediated STAT5 activation (Figure 4d), indicating that NOK specifically induced STAT3 but not STAT5 activation in HEK293T cells.

To further define the specificity of JAK2 in NOK-mediated STAT3 activation, the kinase dead mutant of JAK2 (JAK2KD) was included in the assay system with a similar transfection array as shown in Figure 4c. Either NOK or JAK2 but not JAK2KD significantly enhanced STAT3 activity in HEK293T cells, while co-tansfecting NOK and JAK2 induced a synergistic effect on NOK-mediated STAT3 activation (Figure 4e). Differently, co-tansfection of NOK and JAK2KD abolished NOK-mediated STAT3 activation (Figure 4e). Thus, our results strongly indicated that NOK-mediated STAT3 activation was indeed induced through a JAK2-dependent mechanism.

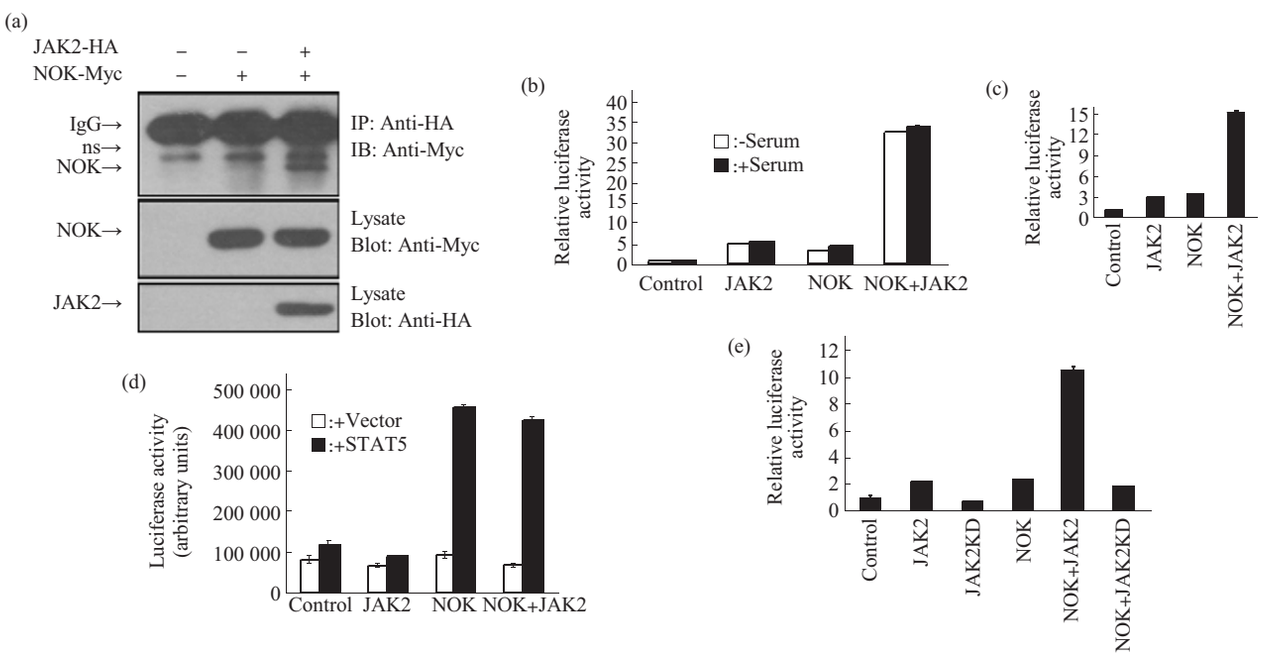

Fig. 4 NOK specifically activates STAT3 through JAK2 pathway

(a) The intermolecular interaction of NOK and JAK2. NOK-Myc was transfected alone or co-transfected with JAK2-HA into HEK293T cells. 24 h later, the cell lysates were immunoprecipitated with anti-HA antibody. The reaction products were resolved onto 10% SDS-PAGE. The transferred membrane was then probed with antibodies against HA or Myc. (b) HEK293T cells were transfected with indicated constructs plus the STAT3-responsive reporter construct (pGL3-(APRE)4-luc). After 24 h posttransfection, the cells were either starved or maintained in DMEM contained 10% FBS for additional 20 h. The lysates were analyzed by using dual luciferase reporter system. The results represented as one test assayed in triplicate. Similar results were obtained in three independent experiments. (c) The KO2F4 (STAT3 knocked out) cells were transiently transfected with those plasmids as shown in (b) plus STAT3 expression construct. Cell lysates were analyzed by using the dual luciferase reporter system. The results represented as one test assayed in triplicate. Similar results were obtained in two independent experiments. (d) HEK293T cells were co-transfected with the indicated plasmids plus the STAT5-responsive reporter construct (pUC18-LHRE/TK). 24 h later, the cell lysates were analyzed by using the dual luciferase reporter system. The results represented as one test assayed in triplicate. (e) HEK293T cells were co-transfected with the indicated plasmids plus the STAT3-responsive reporter construct (pGL3-(APRE)4-luc). After 24 h posttransfection, the cell lysates were analyzed by using dual luciferase reporter system. The results represented as one test assayed in triplicate. Similar results were obtained in three independent experiments.

3 Discussion

Previou sly we showed that NOK could activate multiple STATs such as STAT1, STAT3 and STAT5 [17, 18]. However, the mechanism (s) controlling these STAT activations have not been explored. It is known that RPTK could activate STATs by either JAK-dependent or JAK-independent pathway or both. In this study, we provided convincing evidences to show that NOK could physically interact with both STAT3 and JAK2. Our data further demonstrated that the cooperation between NOK and JAK2 could produce a synergistic effect on STAT3 activation. Moreover, NOK-mediated STAT3 activation was induced in a JAK2-dependent manner.

JAK2 can be a common upstream effector for the activation of both STAT3 and STAT5. Our current data demonstrates that NOK only activated STAT3 but not STAT5 by a JAK2-dependent mechanism in transfected HEK293T cells (Figure 4). This result is different from our previous observation that NOK could constitutively activate STAT5 in BaF3-EPOR/NOK stable cells[17]. The contradiction may be mainly due to the different assay systems established in these two studies. It is likely that NOK induces a particular STAT activation in a given tissue. For example, in mouse pre-B cell line BaF3 cells, over-expression of NOK could induce STAT5 activation[17]. Study on Flt3, a receptor protein tyrosine kinase, might shield some lights on this issue[10]. Stable expression of Flt3 in BaF3 cells only induced the activation of STAT5a but not other STATs, indicating the tissue specificity of STAT5a activation by Flt3. Thus, we propose that the specificity of NOK-mediated STAT activation may also differ from one tissue to another.

Similar to Src, NOK is also an intracellular tyrosine kinase associated with JAK-STAT pathway. However， different from Src, NOK has a transmembrane helix but does not contain Src homology 2 (SH2) domain. Studies indicate that Src can directly activate STAT in a JAK-independent fashion. Olayioye, *et al* [11] showed that Src but not JAK was required for ErbB-mediated STAT activation. Alternatively, RPTK-mediated STAT activation can be cooperatively induced by both Src and JAK [7, 20]. This type of cooperation can eventually lead to the constitutive STAT activation and abnormal cell growth in certain tumor cells [13]. Based on the available data, we propose that the functional properties of NOK may be similar to that of Src. It is known that NOK is constitutively active inside the cells and can activate multiple downstream signals independent of ligand stimulation [17, 18]. Thus, NOK is likely deregulated *in vivo*.

This deregulation may be partially due to the fact that intracellular NOK and JAK2 can function cooperatively to produce a synergistic effect on STAT3 activation. The ability that NOK physically interacts with both STAT3 and JAK2 might provide NOK molecule a priority to make a synergistic effect on STAT3 signaling. We found that NOK could physically associate with all the subdomains of STAT3 except the CC (4H) and CT domains. Interestingly, study indicates that CC and CT domains can physically associate with each other, and this type of intramolecular interaction is important for receptor binding, tyrosine phosphorylation and subsequent activation of STAT3[19]. Hence, the physical interaction of NOK and STAT3 may directly induce and account for the enhanced levels of STAT3 activation (Figure 4). Moreover, current data implies that NOK may function upstream of JAK2 in STAT3 activation pathway since the kinase dead mutation of JAK2 could effectively block this synergistic effect. Thus, NOK may serve as a bridge to connect both JAK2 and STAT3 to form a multi-interactive and cooperative signaling complex. Overall, our study demonstrates for the first time that NOK is an important effector on the activation of STAT3 signaling pathway, and this activation may contribute greatly to NOK-mediated cellular transformation *in vivo*.

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NOK 通过 JAK2 依赖性方式激活 STAT3 信号通路 *

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摘要 NOK 能激活包含 JAK-STAT 信号通路在内的多种促细胞有丝分裂信号通路．研究发现，在人胚肾细胞(HEK293T)中，NOK 与 STAT3 具有直接的相互作用．进一步的实验表明，NOK 能同 STAT3 蛋白除螺旋结构域及 C 端结构域外的其他 4 个结构域发生相互作用，而 NOK 的胞内区则介导了 NOK 同 STAT3 的相互作用．同时，免疫共沉淀实验显示，NOK 能与 JAK2 发生相互作用．重要的是，共同表达 NOK 与 JAK2 蛋白对 STAT3 信号通路能产生一种非常显著的协同激活作用，但当共同表达 NOK 和 JAK2 的激酶活性缺失突变体时，并不产生这种协同激活效应．综上，实验结果显示，NOK 可能同 STAT3 和 JAK2 形成一个复合物，通过 JAK2 依赖性方式激活 STAT3 信号通路．

关键词 NOK, JAK2, STAT3, 协同效应, 免疫共沉淀, 信号激活
学科分类号 R73

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