Point Mutation at Single Tyrosine Residue of Novel Oncogene
NOK Abrogates Tumorigenesis in Nude Mice

Yue Chen, Ying-Hua Li, Xi-Ping Chen, Li-Min Gong, Shu-Ping Zhang, Zhi-Jie Chang, Xiu-Fang Zhang, Xin-Yuan Fu, and Li Liu

Abstract
Receptor protein-tyrosine kinases (RPTKs) are tightly regulated during normal cellular processes including cell growth, differentiation, and metabolism. Recently, a RPTK-like molecule named novel oncogene with kinase-domain (NOK) has been cloned and characterized. Overexpression of NOK caused severe cellular transformation as well as tumorigenesis and metastasis in nude mice. In the current study, we generated two tyrosine—phenylalanine (Y→F) point mutations (Y327F and Y356F) within the endodomain of NOK that are well conserved in many RPTK subfamilies and are the potential tyrosine phosphorylation sites important for major intracellular signaling. Using BaF3 cells stably expressing the ectodomain of mouse erythropoietin receptor, and the transmembrane and endodomain of NOK (BaF3-E/N), we were able to show that point mutations at either Y327 or Y356 dramatically blocked cellular transformation by NOK as examined by colony formation and cellular DNA synthesis. In addition, tumorigenesis induced by BaF3-E/N was completely abrogated upon the introduction of either single mutation. Importantly, signaling studies revealed that the activation of extracellular signal-regulated kinase was inhibited by Y356F and was significantly reduced by Y327F. Both mutations significantly impaired Akt phosphorylation. Interestingly, both mutations did not affect the kinase activity of NOK. Moreover, apoptotic analysis revealed that both mutations accelerated cell death by activating caspase-3-mediated pathways. Thus, our study shows that these potential tyrosine phosphorylation sites may play critical roles in NOK-mediated tumorigenesis both in vitro and in vivo. (Cancer Res 2005; 65(23): 10838-46)

Introduction
Receptor protein-tyrosine kinases (RPTKs) are composed of a large group of receptor subfamilies that mediate diverse cellular processes such as cell growth, cell differentiation, embryogenesis, angiogenesis, and metabolisms through specific ligand-receptor interactions (1–3). Mutagenesis studies indicate that tyrosine autophosphorylation sites of RPTK may play redundant roles in mediating downstream signaling cascades (2, 4–6). For example, a single mutation at the Tyr726 residue of fibroblast growth factor receptor-1 (FGFR1; corresponding to Tyr728 in FGFR3), although preventing the activation of phospholipase C-γ-mediated phosphatidylinositol hydrolysis and receptor internalization, still induces cellular mitogenesis and differentiation (7, 8). Single tyrosine residues (Tyr553, Tyr586, Tyr653, Tyr654, or Tyr730) of FGFR1 are dispensable not only for kinase activity, but for mitogenesis and cellular differentiation as well (4). In contrast, double mutations at the tyrosine residues (Tyr553 and Tyr554) of the activation loop completely abolish the FGFR1 kinase activity (4). However, only the combined mutations at multiple tyrosine autophosphorylation sites were able to eliminate the FGFR1-mediated mitogenesis and cellular differentiation (5). A similar situation has been observed for the autophosphorylation sites of other RPTKs, such as epidermal growth factor receptor (9). In contrast, an exceptional case has been found in the endodomain of FGFR3, in which a single Tyr724 residue (corresponding to Tyr730 in FGFR1) seemed to be responsible for the activation of multiple intracellular cascades (10).

The NOK is a newly identified RPTK-like molecule that possesses strong oncogenic potential and induces tumorigenesis and metastasis in nude mice (11). The NOK gene encodes a putative single transmembrane protein, but almost completely lacking ectodomain, is mainly distributed in the cytoplasmic compartment of the cell. NOK protein shares only 20% to 30% identity with FGFR/platelet-derived growth factor receptor (PDGFR) and belongs to a distinct subfamily of RPTK. In this study, we found that two tyrosine phosphorylation sites (Tyr327 and Tyr356) of NOK are well conserved in many RPTK molecules. In order to elucidate the potential functions of these tyrosine phosphorylation sites, we generated two tyrosine—phenylalanine (Y→F) mutations at the Tyr327 and Tyr356 sites of NOK. Interestingly, using BaF3 stable cells, we were able to show that single point mutations at either site were sufficient to abolish cellular transformation as well as tumorigenesis in nude mice. Mechanistic studies indicate that both mutations significantly impaired multi-intracellular mitogenic signals that are likely critical for NOK-induced tumor growth. However, both mutations did not prohibit the kinase activity of NOK. Therefore, Tyr327 and Tyr356 residues of NOK might serve as potential multisubstrate docking sites for the activation of downstream signal cascades.

Materials and Methods
Plasmid construction and site-directed mutagenesis. The construction of pcDNA3-NOK and pcDNA3-EPOR/NOK has been described
Cell culture, transfection, and generation of BaF3 stable cells.

Human embryonic kidney cells (293T) were grown in DMEM (HyClone Co., South Logan, UT) supplemented with 10% fetal bovine serum (FBS), 2 mmol/L glutamine, 50 units/mL penicillin, 100 µg/mL streptomycin, and 2 mmol/L glucose. Mouse pre-B cells (BaF3) were cultured in RPMI 1640 (HyClone) containing 10% fetal bovine serum (FBS), 2 mmol/L glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, and 10% WEHI-3B conditional medium. For transfection, ~1 × 10^6 cells/mL of 293T cells were plated onto each well of a six-well plate and were grown for an additional 2 days. Approximately 4 µg of plasmid DNA were delivered into 293T monolayer at ~80% confluency by using LipofectAMINE 2000 kit (Life Technologies Inc., Rockville, MD). For generation of BaF3 stable cells, ~20 µg of plasmid DNAs (pcDNA3, pcDNA3-EPOR/NOK (Y327F) or pcDNA3-EPOR/NOK (Y356F)) was electroporated into ~5 × 10^6 cells of BaF3 by using the electroporation system ECM399 (BTX, Inc., San Diego, CA) at 1,500 V and 235 to 240 V with a pulse time of 35 to 40 ms. Cells were first plated on 96-well dishes and selected in the presence of 1,000 µg/mL of G418 for 10 days. The resistant cells were then further expanded in 10-cm culture dishes and subsequently confirmed by Western blot analysis.

Cell proliferation assay, colony formation assay, and animal tumorigenesis.

Cellular DNA proliferation was monitored by [3H]thymidine incorporation assay. After overnight starvation (without serum and WEHI-3B conditional medium), ~1 × 10^5 BaF3 stable cells [BaF3-P3, BaF3-E/N, BaF3-E/N(Y327F) or BaF3-E/N(Y356F)] were added into each well of a 96-well culture plate. [3H]thymidine (1 µCi) was added into each well at 1, 2, and 3 days postinoculation just 6 hours before harvesting. Then, cells were washed with 1× PBS and resuspended into 150 µL of 5% trichloroacetic acid. Cell pellets were lysed in 150 µL of 0.5 N NaOH/0.5% SDS, collected into 96-well scintillation plate, and counted. The experiments were done in triplicate for each time point.

For colony formation assay, ~1 × 10^4 stable BaF3 cells [BaF3-P3, BaF3-E/N, BaF3-E/N(Y327F) or BaF3-E/N(Y356F)] were resuspended into 0.5% agar dissolved in RPMI 1640 without serum and WEHI-3B. Then, the suspension was layered over 5 mL of 0.4% top agar dissolved in RPMI 1640 plus 400 µg/mL of 293T cell culture medium. After incubation for 2 weeks, the anchorage-independent colonies were stained with RPMI 1640 containing 0.25 mg/mL of iodonitrotetrazonium for additional 2 days.

In order to test the oncogenic potentials of chimeric EPOR/NOK and its mutant derivatives in vivo, ~1 × 10^7 stable BaF3 cells [BaF3-P3, BaF3-E/N, BaF3-E/N(Y327F) or BaF3-E/N(Y356F)] were injected s.c. into six BALB/c nude mice aged 4 to 6 weeks. After 4 weeks of postinoculation, the animals were sacrificed, and their body weights were recorded. In addition, the weights of tumor, spleen, and liver from each animal were also determined.

Histologic examination and immunohistochemical analysis.

After sacrifice, the major organs of each animal such as liver, spleen, brain, lung, stomach, kidney, intestine, colon, and skeletal muscles were isolated and analyzed for the presence of tumor cell infiltration. The tissues were first fixed in 10% formalin, then dehydrated gradually in ethanol, followed by embedding in paraffin, and finally sectioned into 4 µm thickness. After overnight staining with hematoxylin followed by differentiation and destaining in acidic p-aminobenzoic acid, the sections were blued in bichromate, before being finally stained with eosin. Also, the paraffin-embedded specimens were sectioned and fixed on a glass slide for immunohistochemical analysis. The slides were first incubated with rabbit anti-NOK antibody at a dilution of 1:4,000 at room temperature for 1 hour. Subsequently, NOK expression was detected with an immunohistochemical polymer detection kit (Zymed, South San Francisco, CA).

Flow cytometry. Approximately 1 × 10^6 cells of BaF3-E/N or its mutant derivatives were harvested and fixed in cold 95% ethanol. Cell suspension was stained with two drops of 50 µg/mL propidium iodide and excited by a laser at 488 nm. A FACScan model flow cytometer was used to examine the collected cells. The WinMDI v2.9 program was used to analyze the processed data.

Western blot analysis. Cells were lysed in a reaction buffer containing 20 mmol/L Tris–Cl (pH 7.4), 150 mmol/L NaCl, 1 mmol/L EDTA, 1% Triton-100, 1 mmol/L Na3VO4, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerolphosphate, 1 mmol/L phenylmethylsulfonyl fluoride, 5 µg/mL aprotinin, and 5 µg/mL leupeptin (pH 7.5). Equal amounts of cell lysates were first separated onto 10% SDS-PAGE. The reaction products were then electrotransferred to a nitrocellulose membrane (Hybond enhanced chemiluminescence, Amersham Biosciences, Piscataway, NJ) at 100 V for 1.5 hours. After blocking with 10% nonfat milk, the transferred membrane was first probed with primary antibody, followed by horseradish peroxidase–conjugated secondary antibody. Finally, the reaction products were developed by enhanced chemiluminescence kit (Amersham Biosciences) and visualized with a Molecular Dynamic PhosphorImage (Molecular Dynamics, Sunnyvale, CA).

Results

Sequence alignment revealed two conserved tyrosine phosphorylation residues between NOK and the selected members of the receptor protein-tyrosine kinase subfamily.

A previous study indicated that 10 potential tyrosine phosphorylation sites are conserved in human versus mouse NOK (11). To further dissect which tyrosine residue of NOK is conserved among other RTK subfamilies, we did ClustalW alignment analysis using transmembrane plus endodomain isolated from FGFR1, FGFR2, FGFR3, FGFR4, PDGFRα, PDGFRβ, Met, Tiel1, Tek, and NOK with Genbank accession numbers NP_000595, CA964962, P22607, AA559389, P16234, P09619, AASA5991, P35290, NP_000450, and AAT01226, respectively, using the DAS program.7 Except for Met kinase (12), the listed RPTKs, including NOK, belong to the split tyrosine kinase family with two conserved kinase subdomains (subdomains 1 and 2) separated by an insert ranging in size from 12 amino acids in NOK to 100 amino acids in PDGFRβ (Fig. 1). Interestingly, two tyrosine residues (Tyr327 and Tyr356) from NOK located at subdomain 2 were found to be well conserved in all RPKTs examined (Fig. 1). These conserved sites have been shown to be important in FGFR1 and FGFR3 signaling (5, 10). For example, the Tyr724 residue of FGFR1 (corresponding to Tyr327 in NOK) is required for FRS2-mediated extracellular signal–regulated kinase (ERK) activation, whereas The Tyr724 residue of FGFR1 (corresponding to Tyr356 in NOK) is responsible for the activation of multiple signaling pathways (5, 10). Therefore, Tyr327 and Tyr356 residues may be important for the bioactivity of NOK.

Tyrosine—phenylalanine (Y→F) mutation at either Tyr327 or Tyr356 site was sufficient to block cell proliferation and transformation. To facilitate the study of NOK signaling, a chimeric receptor E/N was constructed by fusing the ectodomain of EPO receptor (E) and transmembrane and endodomain of NOK (N). Site-directed mutagenesis was done to convert Tyr327 or Tyr356 into phenylalanine to generate the chimeric mutant receptor E/N(Y327F) or E/N(Y356F) as shown in Fig. 2A. Stable BaF3 cells expressing E/N(Y327F), or E/N(Y356F) were obtained after G418 selection (Fig. 2B). The proliferation potentials of these stable cells were evaluated by [3H]thymidine incorporation assay at starvation conditions (without WEHI-3B and serum). Figure 2C shows that the replication of

7 http://www.sbc.su.se/~miklos/DAS/
BaF3-E/N cells doubled after 1 day of incubation and then remained at the platform level for at least 2 additional days. Single mutation at either Tyr327 or Tyr356 sites significantly reduced cellular proliferation as compared with BaF3-E/N, but maintained cellular proliferation at basal levels as compared with either wild-type BaF3 or BaF3-P3, indicating that mutation at either tyrosine site could severely impair NOK-induced mitogenesis. To further consolidate this result, a colony assay was employed. After overnight starvation, an equal number of wild-type chimeric and mutated chimeric BaF3 stable cells were plated onto soft agar supplemented with starved culture medium (minus serum and IL-3) for about 10 days. After staining with iodonitrotetrazonium for 2 additional days, colony diameters >0.1 mm were counted as positive. Figure 2 shows that a single mutation at either Tyr327 or Tyr356 dramatically inhibited anchorage-independent growth of mutated BaF3 stable cells. The number of positive colonies grown by BaF3-E/N(Y327F) and BaF3-E/N(Y327F) were reduced to 9.6 and 10, respectively, as compared with 450.5 for the BaF3-E/N cells (Fig. 2E). Overall, the data indicates that Tyr327 and Tyr356 sites likely play critical roles in NOK-mediated cellular transformation and mitogenesis in vivo.

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Tyrosine→phenylalanine (Y→F) mutation at either Tyr<sup>327</sup> or Tyr<sup>356</sup> site was sufficient to block NOK-induced tumorigenesis in nude mice. Previously, we have shown that the chimeric BaF3-E/N stable cells were able to induce tumorigenesis and metastasis in nude mice (11). In this study, we tried to address how potent these mutations are to influence tumorigenesis within a whole body environment. Stable cells (~1 × 10<sup>7</sup>) from BaF3-P3, BaF3-E/N, BaF3-E/N(Y327F), or BaF3-E/N(Y356F) were injected s.c. into the right flank of each nude mouse. Consistent with the previous report, BaF3-E/N-injected mice showed tumor growth at the injection sites after 4 weeks of inoculation. However, BaF3-E/N(Y327F) and BaF3-E/N(Y356F) mutant cells were completely unable to support tumor growth in nude mice (Table 1). The number of BaF3-E/N mice that survived were dramatically reduced from 10 to 3 by the 8th week of postinoculation (Fig. 3A). The majority of BaF3-E/N mice were deceased between 7 and 8 weeks of postinoculation. In contrast, all mice receiving either BaF3-E/N(Y327F) or BaF3-E/N(Y356F) mutant cells still had good survival rates 6 months after inoculation (data not shown), indicating that the Tyr<sup>327</sup> or Tyr<sup>356</sup> site may be crucial for NOK-induced tumorigenesis in nude mice. For the deceased animals, anatomic analyses revealed that the sizes of spleens and livers in BaF3-E/N mice were significantly enlarged (Fig. 3B). Some BaF3-E/N mice also presented prevalent penetration of metastatic foci in lung tissues (data not shown). Thus, BaF3-E/N mice deaths were mainly due to multiple organ failure induced by tumor invasion and metastasis.

In order to determine the mutation effect on E/N-induced pathogenesis, groups of mice injected with BaF3-P3, BaF3-E/N, BaF3-E/N(Y327F), and BaF3-E/N(Y356F) were sacrificed 7 to 8 weeks postinoculation. The animal weights as well as the weights of tumors, spleens, and livers were recorded. To make a better comparison, the ratio of the weight of tumor or isolated organ to the whole body weight was determined as shown in Table 1. The spleen and liver were significantly enlarged in animals receiving BaF3-E/N inoculation with a mean value of 2.02% versus 0.58% (BaF3-P3 control) and 12.1% versus 5.7% (BaF3-P3 control), respectively. However, no significant difference could be seen in the spleens and livers isolated from the animals injected with either BaF3-E/N(Y327F) or BaF3-E/N(Y356F) as compared with BaF3-P3 control (Table 1), indicating that either mutation was sufficient to block EPOR/NOK-induced tumorigenesis and subsequent pathogenesis. To further consolidate the results, various organs such as brain, lung, heart, liver, stomach, skeletal muscle, spleen, and colon from each injected mouse were prepared and stained with H&E. Similar to the effect of BaF3-NOK cells (11), histologic analysis confirmed that BaF3-E/N cells were also able to promote tumor metastasis at various distant organs such as liver, lung, spleen, kidney, skeletal muscle, and intestine that could be directly responsible for the fatal death of BaF3-E/N-injected animals (Fig. 3C). For the liver section of BaF3-E/N-injected mice, immunohistochemical analysis indicated that the invaded tumor cells were positive for the NOK expression (Fig. 4B). Examinations of the tissue sections of either BaF3-E/N(Y327F) (Fig. 4A) or BaF3-E/N(Y356F) mice (data not shown) did not reveal any apparent tissue abnormalities. Thus, the results indicate that Tyr<sup>327</sup> and Tyr<sup>356</sup> residues indeed play critical roles in NOK-mediated tumorigenesis and metastasis.

**Figure 2.** The effect of point mutation at the conserved tyrosine residue on NOK-mediated cellular proliferation and transformation. A, construction of a chimeric receptor by fusing the ectodomain of mouse EPOR with the transmembrane and endodomain of NOK. Solid boxes, ectodomain of mouse EPOR and the transmembrane (TM); open boxes, endodomain of NOK. Arrow, position that tyrosine (Y) has been mutated to phenylalanine (F). B, Western blot analysis on BaF3 stable cells. BaF3 cells were stably transfected with pcDNA3.0-E/N, pcDNA3.0-E/N(Y327F), or pcDNA3.0-E/N(Y356F) which were tagged with FLAG epitope under G418 selection. After separation, the reaction products were resolved onto 10% SDS-PAGE. The transferred membrane was probed with either anti-FLAG or anti-α-actin antibody. C, [<sup>3</sup>H]Thymidine-incorporation assay. After starvation, ~1 × 10<sup>5</sup> BaF3 stable cells [BaF3-E/N, BaF3-P3, BaF3-E/N(Y327F), or BaF3-E/N(Y356F)] were plated onto a 96-well plate and cultured for 1, 2, or 3 days under the stimulation of 1 Ci of [<sup>3</sup>H]thymidine. Points, mean from three independent experiments; bars, ± SD. D, colony formation assay. BaF3 stable cells [BaF3-P3 (control), BaF3-E/N, BaF3-E/N(Y327F), or BaF3-E/N(Y356F)] were grown in 0.4% soft agar in starvation conditions for about 10 days. After staining with 0.25 mg/mL iodonitrotetrazonium for 2 additional days, the colony formation was photographed under ×150 magnification. E, quantitation of colony numbers to BaF3 stable cells. Colony diameters >0.1 mm were counted as positive.
tumorigenesis in vivo. Abrogation at either residue was sufficient to inactivate the biological functions of NOK.

**Mutation at either Tyr327 or Tyr356 site inactivated NOK-mediated antiapoptosis and promotes cellular apoptosis through caspase-3 pathway.** Previous results indicate that at starvation conditions, BaF3-E/N cells reached a platform level of cellular proliferation after the first day of incubation (Fig. 2C), indicating that NOK may have an antiapoptotic effect. To directly test whether Y327F or Y356F mutation could sufficiently eliminate the antiapoptotic effect, ~ 1 × 10^6 of wild-type or mutant cells were collected, stained with propidium iodide, and analyzed by flow cytometry. Figure 4A shows that after 36 hours of starvation, only 10% of the BaF3-E/N cells were in the sub-G1 phase, whereas the rest of the cells remained at a normal cell cycle distribution with a high S phase population (~ 35%). In contrast, the apoptotic rates of BaF3-P3, BaF3-E/N(Y327F), and BaF3-E/N(Y356F) cells significantly increased to ≥43%, indicating that mutation at either Tyr327 or Tyr356 site sufficiently induced apoptosis.

However, the extent of apoptosis induced by these two mutant cell lines were different. The sub-G1 populations were significantly reduced in BaF3-E/N(Y327F) as compared with the BaF3-P3 control regardless of erythropoietin stimulation (42.7% versus 67.2-69.4%). However, in the absence of erythropoietin, both BaF3-P3 and BaF3-E/N(Y356F) underwent increased apoptotic processes with sub-G1 populations of 69.4% and 60%, respectively, whereas the addition of erythropoietin significantly reduced the sub-G1 population of BaF3-E/N(Y356F) to 43.6% (Fig. 4A).

A time course analysis shown in Fig. 4B revealed that, in the absence of erythropoietin, the apoptotic patterns of both BaF3-P3 and BaF3-E/N(Y356F) were parallel, whereas BaF3-E/N(Y327F) cells underwent a decreased apoptotic processes as compared with the other two. To further elucidate the possible apoptotic pathway involved, equal amounts of cell lysate prepared from each time point were loaded onto 10% SDS-PAGE (Fig. 4C). Western blot analysis was done to determine the total caspase-3 level in each cell line at starvation condition. Activation of apoptosis is represented by the decreased level of caspase-3. Figure 4C shows that the activation of caspase-3 in both BaF3-E/N(Y327F) and BaF3-E/N(Y356F) cells were accelerated after 3 hours of incubation, which was comparable to caspase-3 activation in BaF3-P3 cells (~2 hours). In contrast, BaF3-E/N remained at a constant level of caspase-3 for at least 3 hours. In addition, using the pan-caspase inhibitor Z-VAD-fmk to block intracellular caspase-mediated apoptosis, we observed a significant reduction (~ 3-fold) in the sub-G1 cell population of the each stable cell examined (Fig. 4D). Overall, the data indicates that, although the extent of apoptosis varied among different BaF3-stable cells, single mutations at either Tyr327 or Tyr356 sites were sufficient to eliminate E/N-induced antiapoptotic effects by activating caspase-3-mediated cell death.

**Mutation at either Tyr327 or Tyr356 site did not impair the intrinsic kinase activity of NOK.** Sequence alignment shown in Fig. 1 indicates that Tyr327 and Tyr356 sites may not fall into the activation loop region that has been characterized in other kinase receptors such as FGFR1 (13). To directly address the mutagenetic effect of these point mutations on NOK kinase activity, plasmid vectors carrying EPOR/NOK (E/N), or its mutant derivatives were individually transfected into 293T cells. Using an in vitro kinase assay system, we found that the kinase activity of the immunoprecipitated NOK was biologically active, indicating that this molecule has an intrinsic kinase activity (Fig. 5A, lane 2). Interestingly, point mutations at either Tyr327 or Tyr356 sites did not abolish their respective kinase activities, indicating that these two tyrosine sites are not in the activation loop region of NOK (Fig. 5A, lanes 3 and 4).

**Molecular mechanisms that Tyr327/Tyr356 sites may potentially control NOK-induced tumorigenesis.** The next question that we tried to address is how Tyr327 and Tyr356 residues control their downstream signals which are potentially important in NOK-induced tumorigenesis. Both RAS/mitogen-activated protein kinase and phosphoinositide-3-kinase (PI3K) pathways have been shown to be up-regulated by NOK (11). However, whether Tyr327/Tyr356 residues played stringent or redundant roles during these signaling activations are completely unknown. To address this question directly, cell lysates prepared from BaF3-P3, BaF3-E/N, BaF3-E/N(Y327F), and BaF3-E/N(Y356F) cells at starvation conditions were resolved onto 10% SDS-PAGE and probed with either anti-ERK or antiphosphorylated-ERK antibody (Fig. 5B). Like wild-type NOK, the chimeric E/N also significantly enhanced ERK phosphorylation. Mutation at Tyr327 (Y327F) severely reduced ERK activity, whereas Tyr356 mutation (Y356F) completely abolished ERK phosphorylation, indicating that the Tyr356 residue is critical for the full activation of the ERK pathway. In addition, to assay the mutation effect on the activation of PI3K pathway, the wild-type and mutant chimeric of the E/N expression cassette were individually transfected into 293T cells. Western blot analysis shows that, in the absence or presence of erythropoietin, wild-type E/N dramatically enhanced Akt phosphorylation, indicating that E/N might be constitutively active and might function in an erythropoietin-independent manner. However, both mutations significantly prevented Akt activation with a more severe inhibition being seen in E/N(Y356F) (Fig. 5C). Furthermore,

<table>
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<th>BaF3 stable cells</th>
<th>Deceased/ injected (no.)</th>
<th>Body weight (median), g</th>
<th>Tumor weight (median), %</th>
<th>Liver weight (median), %</th>
<th>Spleen weight (median), %</th>
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</thead>
<tbody>
<tr>
<td>P3</td>
<td>0/8</td>
<td>22.2-29.2 (24.9)</td>
<td>0 (0)</td>
<td>5.4-6.5 (5.7)</td>
<td>0.43-0.72 (0.58)</td>
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<td>E/N</td>
<td>8/8</td>
<td>17.0-29.2 (23.0)</td>
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<td>1.10-3.18 (2.02)</td>
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<tr>
<td>E/N(Y327F)</td>
<td>0/8</td>
<td>21.6-30.4 (25.6)</td>
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<td>5.5-6.9 (6.2)</td>
<td>0.34-0.83 (0.55)</td>
</tr>
<tr>
<td>E/N(Y356F)</td>
<td>0/8</td>
<td>21.5-31.1 (24.7)</td>
<td>0 (0)</td>
<td>5.4-7.1 (6.0)</td>
<td>0.31-1.00 (0.58)</td>
</tr>
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*aWeight of tumor divided by body weight for each animal.

bWeight of liver divided by body weight for each animal.

cWeight of spleen divided by body weight for each animal.
examination of the phosphorylated signal transducers and activators of transcription 5 (STAT5) revealed that STAT5 could be activated by E/N but not by E/N(Y327F) or E/N(Y356F) in the presence of 1% FBS, and this activation was independent of erythropoietin stimulation (Fig. 5D). Thus, a single mutation at either the Tyr327 or the Tyr356 site was sufficient to affect multiple downstream signaling pathways that might be critical for NOK-induced tumorigenesis.

To search for the possible mechanism controlling NOK-mediated tumor metastasis, we examined the expression level of endogenous E-cadherin. Studies have shown that E-cadherin plays an important role in controlling normal cell movement and proliferation (14).
Aberrant expression of E-cadherin causes tumor cell invasion and metastasis (15, 16). To explore the possible role of E-cadherin in NOK-induced metastasis, 293T cells were transfected with the wild-type or mutants of NOK. Western blot shows that overexpression of NOK reduced endogenous levels of E-cadherin as compared with the P3 control (Fig. 5E). However, single mutation at either Tyr327 or Tyr356 site did not significantly affect intracellular E-cadherin expression. Thus, the result indicates that the metastatic effect of NOK could be at least partially induced by the down-regulation of E-cadherin expression in tumor cells.

**Discussion**

Previously, it was shown that the novel RPTK-like molecule NOK could function as an oncogene to promote tumorigenesis and metastasis in nude mice (11). The NOK shares low homology with...
the members of FGFR/PDGFR superfamily, and probably stands out as a new subfamily. In this report, we further characterized the potential importance of NOK tyrosine phosphorylation sites using both biochemical and animal model approaches. We created two single mutations at Tyr327 (Y327F) and Tyr356 (Y356F) sites, respectively, which are proximal to the COOH terminus of NOK kinase domain. These two tyrosine sites are potentially phosphorylated and are well conserved in many RPTK subfamilies. BaF3 cells stably expressing a chimeric construct by fusing the ectodomain of EPOR with the endodomain of NOK (BaF3-E/N) had high levels of cellular DNA synthesis and transformation potential, whereas mutations at either site completely inhibited both effects. Similar to the wild-type NOK, the fusion gene EPOR/NOK also promoted tumorigenesis and metastasis in nude mice whereas either single mutation abrogated the malignancy. Interestingly, neither point mutations affected the intrinsic kinase activity of NOK. Mechanistic studies indicate that multiple signaling pathways can be influenced through both sites with a more potent effect exerted by Tyr356 residue.

The whole picture of signaling specificity by RPTK is complicated and remains largely elusive. Mutagenesis studies on numerous RPTKs has pointed out that redundancy of signaling pathways might be involved in RPTK-mediated cellular processes. Mutation at a single tyrosine phosphorylation site is inert to activate a specific cellular response, or the expression profile of immediate early gene expression upon RPTK activation (6, 17, 18). However, some RPTK molecules can signal multiple intracellular networks through a single tyrosine phosphorylation site that can serve as the multisubstrate docking site for the interaction of SH2-containing proteins. For example, The Tyr1100 residue of Tek receptor tyrosine kinase can be the common targeted site for the interaction by growth factor receptor binding proteins (Grb2, Grb7, Grb14), Shp2, and the p85 subunit of PI3K (19). More recently, the Tyr724 residue of FGFR3 has been identified as a potential multisubstrate docking site responsible for cellular transformation, PI3K, and mitogen-activated protein kinase activation, as well as the phosphorylation of Shp2, STAT1, and STAT3 (10).

Interestingly, sequence alignment in Fig. 1 uncovered two important NOK tyrosine residues (Tyr327 and Tyr356) that are well conserved in many subfamilies of RPTK such as FGFR, PDGFR, vascular endothelial growth factor receptor, MET, and Tie1. The kinase domain of RPTK is characterized by the dispersed network of two to three tyrosine residues along the receptor-intracellular domain and possesses an enzymatic activity that can hydrolyze ATP for autophosphorylation at these tyrosine sites (5, 20). Our study showed that a single mutation at either tyrosine site did not affect the kinase activity of NOK, indicating that the activation loop in NOK was not impaired. Because Tyr356 is directly.

![Image](https://example.com/image.png)

**Figure 5.** Single mutation at either Tyr327 or Tyr356 residue did not affect kinase activity, but significantly inhibited the activation of multiple mitogenic signals. A, kinase activities of E/N and its mutant derivatives. Approximately 15 μg of FLAG-tagged pcDNA3.0, pcDNA3.0-E/N, pcDNA3.0-E/N(Y327F), or pcDNA3.0-E/N(Y356F) were individually transfected into 293T cells. Cell lysates were immunoprecipitated with anti-FLAG antibody. Approximately 15 μg of cell lysate prepared from BaF3-P3, BaF3-E/N, BaF3-E/N(Y327F), or BaF3-E/N(Y356F) stable cells were resolved onto 10% SDS-PAGE. The transferred membrane was probed with anti-ERK or anti-phosphorylated ERK antibodies. B, effect of point mutations on the ERK pathway. After overnight starvation, ~ 20 μg of cell lysate prepared from BaF3-P3, BaF3-E/N, BaF3-E/N(Y327F), or BaF3-E/N(Y356F) stable cells were resolved onto 10% SDS-PAGE. The transferred membrane was probed with anti-ERK or anti-phosphorylated ERK antibodies. C, effect of point mutation on the Akt pathway. After overnight starvation, BaF3-P3, BaF3-E/N, BaF3-E/N(Y327F), or BaF3-E/N(Y356F) stable cells were stimulated with or without 20 units/mL erythropoietin for 30 minutes before harvesting. Cell lysates were then resolved and subjected to immunoblot analysis by using anti-Akt or anti-phosphorylated Akt antibody. D, effect of point mutations on the activation of STAT5. After overnight starvation, BaF3-P3, BaF3-E/N, BaF3-E/N(Y327F), or BaF3-E/N(Y356F) stable cells were cultured in RPMI 1640 containing 1% FBS and stimulated with or without 20 units/mL erythropoietin for 30 minutes before harvesting. Cell lysates were then resolved and subjected to immunoblot analysis by using anti-STAT5 antibody. E, NOK reduced endogenous E-cadherin expression. The monolayer of 293T cells was transfected with an equal amount of pcDNA3.0, pcDNA3-NOK, pcDNA3-NOK(Y327F), or pcDNA3-NOK(Y356F). Afterwards, cell lysates were subjected to immunoblot analysis by using anti-E-cadherin, anti-β-actin, or anti-α-actin.
corresponding to the Tyr724 phosphorylation site in FGFR3, it might also have a similar potential to serve as a multisubstrate docking site for NOK-mediated cellular processes. Indeed, our experimental results highlight the critical importance of Tyr356 phosphorylation site for the induction of multiple mitogenic signals that mediate cellular transformation, tumorigenesis, and metastasis. A similar effect has also been found for the Tyr327 site of NOK that corresponds to Tyr701 of FGFR1 (or Tyr95 of FGFR3). Although the effect of single mutation at Tyr701 to FGFR1 has not been directly addressed, systematical mutagenesis showed that both Tyr724 and Tyr701 are required for FGFR1-induced neurite formation (5). Crystallographic analysis of FGFR1 provides some valuable hints for our understanding on the biological roles of Tyr327 and Tyr356 in NOK (13). Tyr701 (corresponding to Tyr327 in NOK and Tyr695 in FGFR3) is partially buried in the subdomain 2 of FGFR1 kinase, therefore, might not be directly phosphorylated. However, this tyrosine residue likely functions structurally through the hydrogen bond formation with the side chain of nearby residues such as His377. On the other hand, Tyr720 (corresponding to Tyr724 in NOK and Tyr701 in FGFR3) closely parallels the Tyr701 residue and lies in an opened α-helix chain that is readily accessible to various cellular proteins. Based on this structural model, we propose that the aromatic ring of Tyr724 might be in a good spatial position to form a stable scaffold with its surrounding residues through H-bond formation. This scaffold will, in turn, serve as a supporting core to push the Tyr724-containing α-helix chain open for signaling substrate access. Thus, mutation at Tyr327 could cause a structural collapse that leads to the burial of the Tyr356 site, whereas mutations at the Tyr356 site may retain the correct structural conformation but prevent the docking signals for multisubstrate access.

Our study showed that both the Tyr327 and Tyr356 sites are critical in mediating NOK-induced cellular and tumoral effects. Similar to the effect of wild-type NOK, a chimeric construct expressing the ectodomain of EPOR and the endodomain of NOK is also constitutively active, as shown in the colony assay at starvation conditions in Fig. 2D. Both Tyr327 and Tyr356 residues seem to be required for the activation of multiple signal cascades such as ERK, Akt, and STAT5. Although the mutant BaF3 cells expressing E/N (Y327F) or E/N(Y356F) were unable to enhance the examined mitogenic signals upon erythropoietin stimulation, we could not completely exclude the possibilities of alternative mitogenic pathways involved that may account for the reduced apoptosis in BaF3-E/N(Y356F) as shown in Fig. 4A. However, these mitogenic signals may function in a minor and transient way and might not support cellular proliferation for a long period of time. In addition, the animal experiments further highlight the functional importance of both Tyr327 and Tyr356 residues of NOK in a whole body environment. Overall, in this study, we identified and in vivo characterized two conserved tyrosine residues located at the NOK kinase subdomain 2 (Fig. 1) which is shared by many RTK family subfamilies. These conserved tyrosine sites may not only play a central role in NOK functions, but may also be a common target site for the activation of other RTKs.

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