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Molecules and Cells

Transmembrane Helix of Novel Oncogene with Kinase-Domain (NOK) Influences Its Oligomerization and Limits the Activation of RAS/MAPK Signaling

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Ligand-dependent or independent oligomerization of receptor protein tyrosine kinase (RPTK) is often an essential step for receptor activation and intracellular signaling. The novel oncogene with kinase-domain (NOK) is a unique RPTK that almost completely lacks an ectodomain, expresses intracellularly and activates constitutively. However, it is unknown whether NOK can form oligomer or what function oligomerization would have. In this study, two NOK deletion mutants were generated by either removing the ectodomain (NOKΔECD) or including the endodomain (NOK-ICD). Co-immunoprecipitation demonstrated that the transmembrane (TM) domain of NOK was essential for its intermolecular interaction. The results further showed that NOK aggregated more closely as lower order oligomers (the dimer- and trimer-sized) than either deletion mutant did since NOK could be cross-linked by both Sulfo-EGS and formaldehyde, whereas either deletion mutant was only sensitive to Sulfo-EGS. Removing the NOK TM domain (NOK-ICD) not only markedly promoted higher order oligomerization, but also altered the subcellular localization of NOK and dramatically elevated the NOK-mediated constitutive activation of extracellular signal-regulated kinase (ERK). Moreover, NOK-ICD but not NOK or NOKΔECD was co-localized with the upstream signaling molecule RAS on cell membrane. Thus, TM-mediated intermolecular contacting may be mainly responsible for the constitutive activation of NOK and contribute to the autoinhibitory effect on RAS/MAPK signaling.

INTRODUCTION

The oligomerization of protein tyrosine kinase (PTK) is complicatedly modulated by diverse elements or modules (Hubbard, 2002; Li and Hristova, 2006; Schlessinger, 2002). The ectodomain, transmembrane domain and endodomain of a receptor PTK (RPTK) are all capable of mediating receptor oligomerization, while other structural components such as coiled-coil (McWhirter et al., 1993; Smith et al., 2003), leucine zipper (Bartkiewicz et al., 1999; Tong et al., 1997), and cysteine-rich motif (Ozaki et al., 2005) are also key mediators to PTK oligomerization. Although the oligomerization was thought to be the prerequisite step for PTK activation and subsequently cellular transformation, recent studies indicate that it does not always hold true (Jiang and Hunter, 1999; Kani et al., 2005; Yu et al., 2002).

Numerous studies have pointed out that a monomer-oligomer model is one of the central mechanisms to regulate PTK kinase activity and intracellular signaling. It is believed that the signaling strength of PTK is tightly regulated at multiple levels. Genetic alterations such as mutation, deletion or gene amplification could result in unregulated activation of PTK which usually causes inappropriate cell growth (Brennan et al., 2000). The specific ligand-receptor interaction provides the RPTK molecule a key means to regulate its own functions. The equilibrium of the monomer-dimer or the dimer-higher oligomer transitions can be dynamically regulated in the presence of specific ligands to ensure that a particular steric constraint will be adjusted for the generation of appropriate signaling strength. Ligand-mediated higher order oligomerization (e.g. tetramerization) of RPTK is most often in its highest activated state (Brennan et al., 2000; Clayton et al., 2005). Using multidimensional microscopy techniques, Clayton et al. (2005) clearly demonstrated that ligand-mediated higher order oligomerization of EGFR might be an important mechanism for receptor transactivation (Clayton et al., 2005). In contrast, ligand-in-dependent oligomerization may sometimes confer to signaling inhibition. Studies revealed a fine regulation between ErbB2 (HER2) and ErbB3 (EGFR3) during receptor activation versus inactivation. In the absence of ligand, ErbB3 had the propensity to aggregate as homo-oligomer on cell surface to prevent heterodimerization and signaling. Addition of ligand destabilized homo-oligomerization but not heterodimerization with ErbB2,

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therefore, promoted mitogenic activation (Guy et al., 1994; Kani et al., 2005).

NOK was initially identified as a novel RPTK molecule (Blume-Jensen and Hunter, 2001; Manning et al., 2002; Robinson et al., 2000; Simpson et al., 2000; Ye et al., 2003), and later found to be a potent oncogene to induce tumorigenesis and metastasis in nude mice (Chen et al., 2005; Liu et al., 2004). Like other tyrosine kinases, NOK is also strictly regulated at multiple levels. For instance, the Y417 residue located at the carboxyl terminal of NOK had an autoinhibitory role in NOK-mediated signaling transductions (Li et al., 2007), whereas Y327 and Y356 could potentially serve as multi-substrate docking sites for downstream signaling (Chen et al., 2005). Intracellularly, NOK may form multi-protein complex with diverse molecules such as STAT3, JAK2 and Akt to constitutively activate multiple signaling pathways (Li et al., 2008a; 2008b). Recent studies also showed that NOK gene was over-expressed in certain lung and breast cancer tissues, indicating the potential value of NOK in disease diagnosis (Amachika et al., 2007; Kimbro et al., 2008; Moriai et al., 2006).

Most PTKs can oligomerize during their activation in the presence or absence of specific ligands. However, it is unknown whether NOK can form an oligomer or how NOK oligomerization is regulated intracellularly. In this study, we demonstrate for the first time that the propensity of NOK aggregation might make it constitutively active inside cells. The TM domain of NOK not only induced lower order oligomerization, but also autoinhibited NOK-mediated activation of RAS/MAPK signaling pathway.

MATERIALS AND METHODS

Chemicals

Mouse anti-Myc, anti-HA, and anti-p-ERK as well as rabbit anti-GFP and Protein G PLUS-Agarose were purchased from Santa Cruz Biotechnology (USA). Mouse anti-Flag was purchased from Sigma (USA). Mouse anti-STYK1 was purchased from Abnova (Taiwan). Rabbit anti-NOK antibody was raised by immunizing rabbits with purified GST-NOK (49AA-159AA) and subsequently affinity-purified. Goat anti-mouse and goat anti-rabbit Ig labeled with horseradish peroxidase were from Vigorous, Inc. (China).

Plasmid constructs

All PCR amplification reactions were performed with Pyrobest DNA polymerase (Takara Biotechnology, China) and verified by DNA sequencing. All constructs for expression in HEK293T cells were subcloned into pcDNA3.1/Myc-His, pcDNA3.1/HA-His and pcDNA3.1/Flag-His (Invitrogen, USA). pFA-Elk1 and pFR-Luc was kindly provided by Dr. Akihiko Yoshimura (Kyushu University, Japan). FGFR1 was kindly provided by Dr. D. Ornitz.

Cell culture, transient transfection, and luciferase assay

HEK293T or COS7 cells were grown in DMEM containing 10% calf serum. Transfection was performed by using VigoFect (Vigorous, Inc., China). Luciferase assays were carried out in HEK293T cells grown in 24 well plates by co-transfecting the Elk-1 luciferase reporter system (1.0 μg pFA-Elk1 and 1.0 μg pFR-Luc plasmid for each plate) plus the indicated constructs. After 24-h transfection, the cell lysates were assayed by Dual Luciferase Assay system (Promega Corp., USA) and detected by Packard Top Count (GMI, Inc., USA).

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, 0.5% NP40) in the presence of protease inhibitors. The cell lysates were resolved onto 10% SDS-PAGE. The transferred membrane was probed with a primary antibody followed by a secondary antibody conjugated to horseradish peroxidase. Reaction was enhanced by using chemiluminescence (Pierce, USA).

Co-immunoprecipitation

Cells were co-transfected with plasmid vectors containing NOK-Myc and NOK-HA. Single transfection with one of the plasmids alone was served as control. The 24 h later, the cells were lysed 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 and analyzed by Western blotting.

Chemical cross linking

HEK293T cells were transfected with NOK-Flag construct. 36 h later, the cells were lysed with TGH buffer (0.1% Triton X-100, 10% glycerol, 20 mM Hepes, pH 7.5). The reaction products were incubated with or without 10mM Sulfo-EGS (Pierce) or 25 mM formaldehyde (Sigma, USA). At each interval (5′, 15′, 30′ and 60′), an aliquot was taken from each reaction. The reaction products were then analyzed by Western blotting.

Immunostaining and confocal microscopy

Cos7 cells growing on glass coverslips were fixed with 4% paraformaldehyde, and then permeabilized with 0.3% Triton X-100. After blocking, the reaction products were incubated with primary antibodies and then detected with FITC- or TRITC-conjugated goat anti-mouse IgG. The coverslips were mounted in glycerol and analyzed using a laser scanning confocal microscope.

RESULTS

The intermolecular interaction of NOK *in vivo*

Since NOK is constitutively active inside the cells, we reasoned that an intermolecular interaction of NOK might exist. To address this issue directly, we performed a co-immunoprecipita-tion analysis by using two different tagged NOK constructs: NOK-HA and NOK-Myc. If NOK molecules could interact intermolecularly, we should be able to detect the presence of one tagged NOK protein in the immunoprecipitated product with another tagged antibody. Indeed, NOK-HA could be detected in the reaction product immunoprecipitated with anti-Myc antibody against NOK-Myc only under the co-transfection condition (Fig. 1A). In contrast, mixing the reaction products derived from separated transfections *in vitro* did not promote the intermolecular interaction of NOK (Fig. 1A). The intermolecular interaction of NOK was further confirmed by the result of reciprocal co-immunoprecipita-tion as demonstrated in Fig. 1B.

The TM domain is essential for the intermolecular interaction of NOK

The next question we tried to ask was which structural domain of NOK was responsible for this intermolecular interaction. Two deletion mutants, NOKΔECD and NOK-ICD, were generated by removing either the ectodomain or both the ectodomain and TM domain of NOK, respectively (Fig. 2A). After co-transfection, cell lysates were co-immunoprecipitated with anti-Flag antibody and then blotted with anti-HA antibody. The result presented in Fig. 2B demonstrated that NOKΔECD could efficiently interact

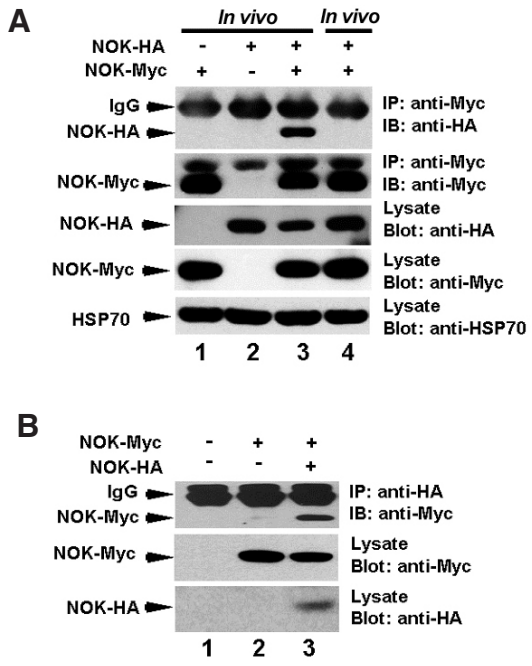

Fig. 1. The detection of the intermolecular interaction of NOK. (A) Co-immunoprecipitation assay. HEK293T cells were co-transfected with both NOK-HA and NOK-Myc. Single transfection with either NOK-HA or NOK-Myc was served as control. The reaction products were immunoprecipitated with anti-Myc antibody and then probed with anti-HA antibody. '*In vitro*' denotes the mixing control from singly transfected cells before immunoprecipitation. (B) The reciprocal co-immunoprecipitation. HEK293T cells were transfected with indicated plasmids. The reaction products were immunoprecipitated with anti-HA antibody and then probed with anti-Myc antibody.

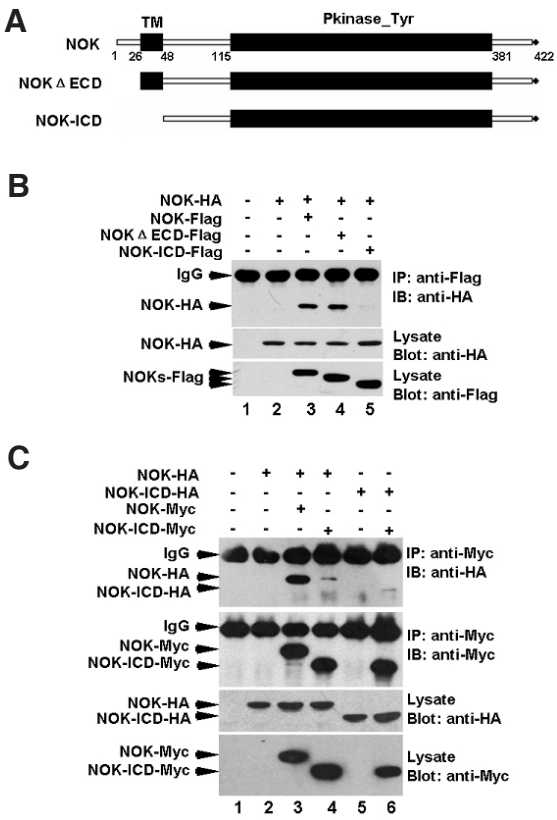

Fig. 2. The TM domain was critical for the intermolecular interaction of NOK. (A) The structures of NOK and its mutant derivatives (NOKΔECD and NOK-ICD). (B) NOK-HA was co-transfected with NOK-Flag, NOKΔECD-Flag or NOK-ICD-Flag into HEK293T cells. Cell lysates were first immunoprecipitated with anti-Flag and then probed with anti-HA or anti-Flag. (C) HEK293T cells were co-transfected with NOK-Myc and NOK-HA, NOK-ICD-Myc and NOK-HA, or NOK-ICD-Myc and NOK-ICD-HA. The cell lysates were first immunoprecipitated with anti-Myc and then probed with anti-HA or anti-Myc antibody.

with NOK when both proteins were co-expressed. In contrast, NOK-ICD failed to interact with NOK protein in the same assay condition, indicating that the TM domain of NOK might be critical in mediating the intermolecular interaction of NOK.

To further confirm this result, NOK-HA was transfected alone or co-transfected with either NOK-Myc or NOK-ICD-Myc into HEK293T cells (Fig. 2C). Similarly, NOK-ICD-HA was transfected alone or co-transfected with NOK-ICD-Myc into the same type of cells (Fig. 2C). After co-immunoprecipitation with anti-Myc antibody, the reaction products were probed with anti-HA antibody. In consistent with the result of Fig. 2B, removing the ectodomain and transmembrane domain of NOK sharply reduced the intermolecular interaction of NOK (Fig. 2C). Thus, the TM domain is indeed an important element in mediating the intermolecular interaction of NOK.

The TM domain is critical for NOK induced oligomerization

Since intermolecular interaction is an important step for the oligomerization of RPTK, it is possible that NOK can form oligomer inside the cells. To test this hypothesis, we employed chemical cross linking technique to capture the oligomer complex from transfected HEK293T cells. Two different cross linking reagents, Sulfo-EGS and formaldehyde**,** were utilized and confirmed to work effectively in detecting FGFR1 dimerization (Fig. 3A). The distance between the reactive groups for the cross-linking reagents is 16.1 Å for Sulfo-EGS and 2-3 Å for formaldehyde. These cross-linking chemicals could distinguish how compact the NOK induced oligomer was. Our result demonstrated that NOK could form lower order oligomers (dimer- and trimer-sized, Fig. 3B). A time dependent alteration in the amount of oligomerization was not observed in the reaction products by using both cross-linking reagents, with a relative weak reaction found in formalinized samples (Fig. 3B). In addition, removing the ectodomain of NOK did not affect the dimer-sized but likely distabilized the trimer-sized formation in some instances (Fig. 3C). The trimer-sized was completely absent in formalinized samples, indicating that a more relaxed protein complex could be formed (Fig. 3C). Intriguingly, NOK-ICD mutant was only sensitive to Sulfo-EGS in forming a higher order oligomer (tetramer-sized), indicating that NOK-ICD induced higher order oligomer was also preferentially folded into a relaxed configuration (Fig. 3D).

To further confirm the results, the transfected HEK293T cells were first immunoprecipitated with anti-Flag antibody after chemical cross-linking with Sulfo-EGS, and subsequently blotted with anti-NOK antibody. Figure 4 demonstrated that both NOK and NOKΔECD could form strong dimer-sized and trimer-sized products in the presence of Sulfo-EGS. Interestingly, in the absence of Sulfo-EGS, NOK and NOKΔECD could induce weak oligomerizations, indicating that the forced aggregation

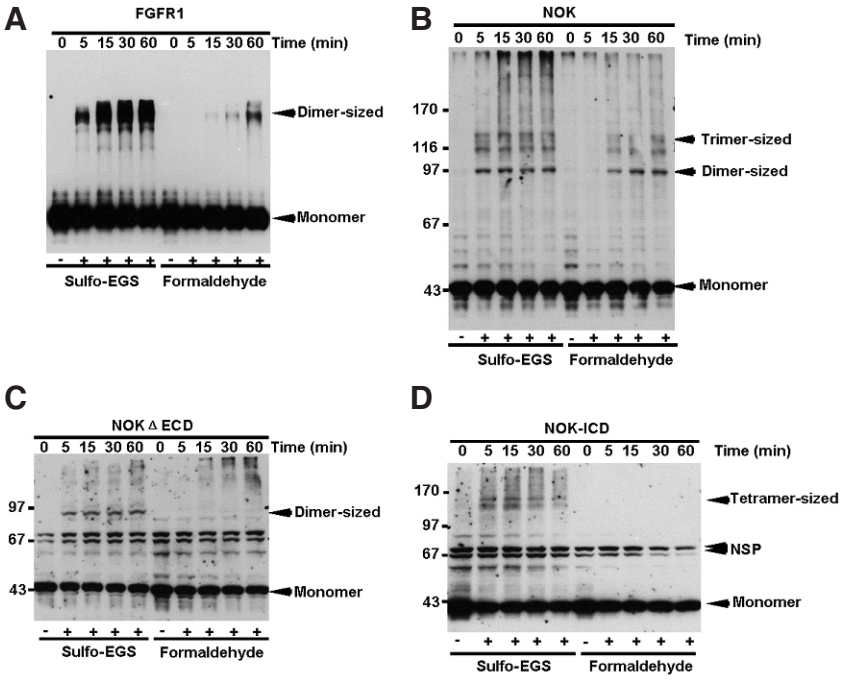

Fig. 3. The TM domain of NOK mediated oligomerization. HEK293T cells were transiently transfected with FGFR1-Myc (A), NOK-Flag (B), NOKΔECD-Flag (C), or NOK-ICD-Flag (D). After 36 h transfection, cell lysates were treated with or without Sulfo-EGS or formaldehyde for 0′, 5′, 15′, 30′ and 60′. The reaction products were probed with anti-Myc (A), anti-STYK1 (B), or anti-NOK (C and D) antibody and analyzed by Western blot. NSP indicated the non-specific band.

was able to induce some degrees of covalent interaction. In contrast, no oligomer was found in the immunoprecipitated products of NOK-ICD at either before or after Sulfo-EGS treatment (Fig. 4), indicating that the tetramer-sized product detected by Western blotting in Fig. 3D might not be stable enough under this assay condition. Thus, the intermolecular interaction of NOK preferentially produced the dimer-sized and trimer-sized products, and removing the TM domain of NOK could only induce higher order oligomerization (tetramer-sized) but not lower order oligomerization (dimer-sized or trimer-sized).

The subcellular localization of NOK and its mutant derivatives

Previous data showed that the NOK-EGFP fusion protein was predominantly expressed in the cytoplasm of NIH3T3 cells (Liu et al., 2004). In order to define the subcellular localizations of NOK mutant derivatives, immunostaining analysis was performed. The result demonstrated that both NOK and NOKΔECD proteins had a similar staining pattern with a punctate cytoplasmic distribution, indicating that removing the N terminus of NOK did not alter the pattern of subcellular distribution (Fig. 5A). Intriguingly, when deleting both the ecotodomain and TM domain, a significant portion of NOK proteins was detected on the plasma membrane of transfected cells (Fig. 5A). Consistently with the result of NOK transfection, the endogenous NOK also had a punctuated cytoplasmic distribution in COS-1 cells (Fig. 5B). Thus, the TM domain of NOK may not only directly mediate NOK oligomerization but also be responsible for its subcellular localization.

The TM domain of NOK exhibits an autoinhibitory effect on NOK-mediated activation of RAS/MAPK signaling pathway

It has been shown that the TM domain of NOK could mediate both lower order oligomerization and subcellular localization. However, how NOK functioned constitutively is remained unsolved. To this end, we used Elk-1 luciferase assay system to evaluate the NOK-mediated RAS/MAPK signaling pathway. In consistent with our previous observation, over-expressing wild-

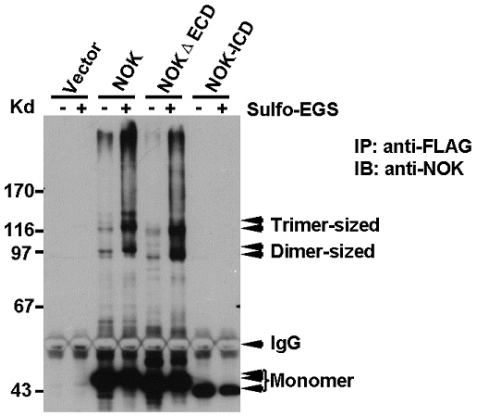

Fig. 4. Immunoprecipitation analysis on oligomerization of NOK and its mutant derivatives after chemical cross-linking in HEK293T cells. The cell lysates were first cross-linked with Sulfo-EGS for 20 min, immunoprecipitated and subsequently analyzed by Western blotting.

type NOK dramatically enhanced Elk-1-mediated luciferase activity by 3- to 4-folds as compared with that of the vector alone (Fig. 6A). To our surprise, NOK-ICD mutant did not reduce but rather promoted NOK-mediated Elk1 activation (Fig. 6A), indicating that the TM domain of NOK possessed an autoinhibitory effect on the NOK-induced RAS/MAPK activation. To further confirm this observation, we co-transfected either NOK or its mutant derivative (NOKΔECD or NOK-ICD) together with GFP-ERK2 into HEK293T cells, and assayed both the exogenous GFP-ERK and endogenous ERK phosphorylation levels. Figure 6B showed that deleting the ectodomain of NOK barely increased both GFP-ERK2 and endogenous ERK phosphorylations. However, additionally removing the TM domain of NOK dramatically enhanced both types of ERK activity. Moreover, the subcellular localizations of RAS and NOK & its mutant derivatives were analyzed by co-transfecting their plasmid

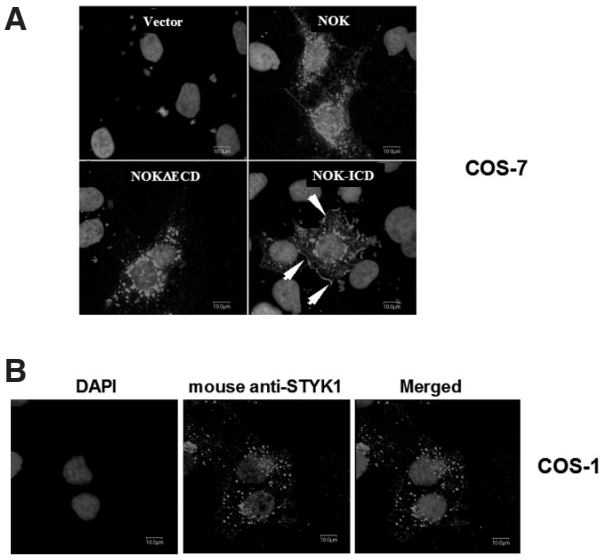

Fig. 5. Subcellular localizations of NOK and its mutant derivatives. (A) COS-7 cells were transiently transfected with empty vector, NOK-Flag, NOKΔECD-Flag, or NOK-ICD-Flag. The transfected cells were immunostained with anti-Flag antibody followed by TRITC-conjugated secondary antibodies (red). The nuclear were counterstained with Hoechst (blue). The solid arrow indicates the cell membrane distribution of NOK-ICD. (B) Immunostaining analysis on endogenous NOK expression in COS-1 cells by using anti-STYK1 antibody.

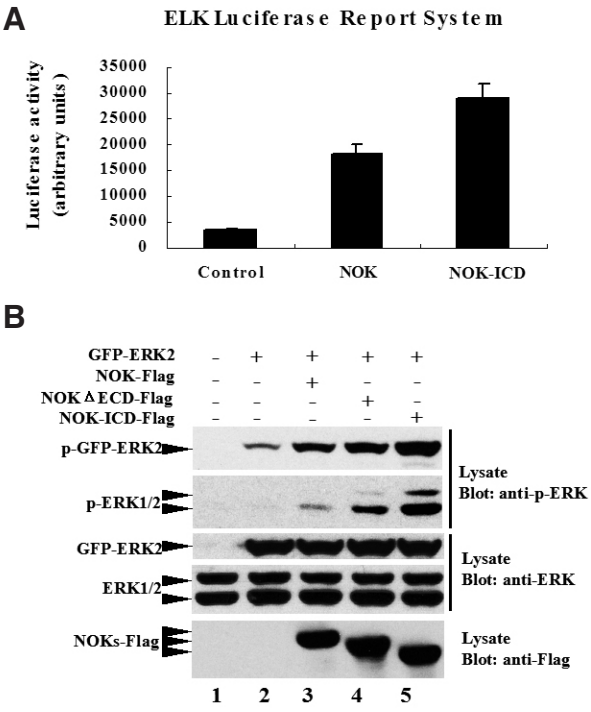

Fig. 6. The TM domain of NOK autoinhibited NOK-mediated activation of RAS/MAPK signaling pathway. (A) The activation of Elk-1 luciferase activity by NOK and its mutant derivatives. Values are represented as means ± SEM of triplicated tests from one experiment. Similar results were obtained in three independent experiments. (B) HEK293T cells were co-transfected with the indicated plasmids. The 24 h later, the transfected cells were harvest. The reaction products were probed with anti-p-ERK, anti-ERK, anti-GFP or anti-Flag antibody and analyzed by Western blot.

DNAs into 293T cells (Fig. 7). Figure 7 clearly shows that NOK-ICD but not NOK or NOKΔECD was co-localized well with the upstream signaling molecule RAS on cell membrane. Overall, our results strongly indicate that the N terminal portion, mainly the TM domain, of NOK plays an autoinhibitory role in NOK-mediated constitutive activation of RAS/MAPK signaling.

DISCUSSION

NOK is a unique RPTK like molecule that lacks almost complete receptor ectodomain, therefore, can not be regulated through ligand-receptor interaction. Previous studies indicated that NOK protein was constitutively active inside the cells (Chen et al., 2005; Liu et al., 2004). However, how NOK functioned constitutively is unknown. In this study, we provided the first evidence to show that NOK could form oligomer *in vivo*, and the N-terminal portion of NOK was critical in mediating NOK oligomerization and exhibited an autoinhibitory role in NOK-mediated constitutive activation of RAS/MAPK signaling pathway.

Our current results indicate that the monomer-oligomer model may at least partially account for NOK-mediated cellular events. The data shows that a steric constraint is held at the N-terminal portion (especially the TM domain) of NOK to prevent the formation of high order oligomer (tetramer-sized). Simply removing the ectodomain did not change the pattern of NOK aggregation (Fig. 4) but might reduce the trimer stability (Fig. 3C). The TM domain of NOK seemed to be essential to mediate lower but not higher order oligomerization since additionally deleting the TM domain eliminated the aggregation of both dimer- and trimer-sized products but increased the tetramer-sized products (Fig. 3D). Thus, the TM domain of NOK likely provides a level of control by preventing high order oligomerization.

Cross-linking experiment demonstrated that NOK could simultaneously exist as monmer, dimer- and trimer-sized forms which might be critical to make it constitutively active inside the cells (Fig. 3B). Unlike typical RPTK, the activation of NOK does not rely on specific ligand recognition. This type of feature might provide NOK an unregulated character. Thus, what makes NOK constitutively active should be a fascinating subject to be pursued. The great transforming potency induced by NOK might be largely due to its propensity to aggregate *in vivo*. Mohammadi et al. (1993) demonstrated that the aggregation of the intracellular domain of EGFR might be an important step for receptor activation and auto-phosphorylation. However, different from the forced aggregation of EGFR that may represent a regulated event, NOK could naturally aggregate into monomer, dimer- and trimer-sized products upon over-expression, indicating that NOK might be unregulated and therefore function constitutively active inside the cells. The unregulated aggregation of NOK may contribute greatly to the NOK-induced malignancy and inappropriate cell growth. The oligomerization of NOK may structurally facilitate the *trans* phosphorylation and eventually intracellular activation. Many studies indicate that the higher order oligomerization, especially the tetramer form, of PTK often represents as an energetically favorable state which is capable to generate the maximal activity (McWhirter et al., 1993; Mohammadi et al., 1993). However, a constitutive active PTK molecule like NOK may not be neces sary always in its highest active state.

Unlike BCR-ABL oncogenic protein in which the BCR domain

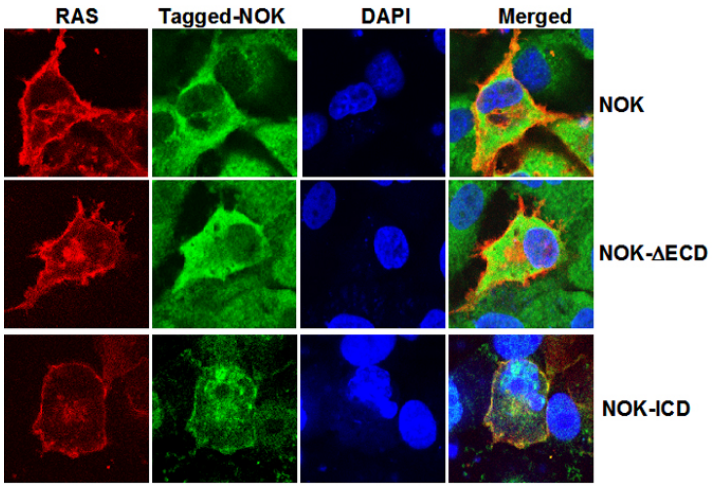

Fig. 7. NOK-ICD co-localized with RAS on cell membrane. COS-7 cells were transiently co-transfected with RAS-HA plus NOK-Flag, NOKΔECD-Flag, or NOK-ICD-Flag. The transfected cells were immunostained with anti-HA and anti-Flag antibodies followed by TRITC (red) and FITC (green) conjugated secondary antibodies, respectively. The nuclear were counterstained with DAPI (blue).

1 itself could mediate tetramerization (McWhirter et al., 1993), NOK aggregated as a tetramer-sized product upon removing its N-terminus (mainly the TM domain) which resulted in the further activation of the intracellular RAS/MAPK signaling pathway, indicating that the TM domain of NOK played an autoinhibitory role in the activation of NOK-mediated signaling transduction. Another example of N-terminus mediated autoinhibition is the pro-oncogene c-Abl. Removing the N-terminal residues of c-Abl promoted its homo and hetero oligomerization and in turn resulted in the conformational changes that might be critical for *trans* phosphorylation and subsequently released the autoinhibition (Fan et al., 2003; Pluk et al., 2002). Similarly, the TM domain of NOK was capable of limiting the enhanced activation of NOK-mediated intracellular signaling by restricting the formation of higher order oligomerization.

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