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NOK/STYK1 has a strong tendency towards forming aggregates and colocalises with epidermal growth factor receptor in endosomes

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ABSTRACT

Our previous studies showed that the overexpression of *N*ovel *O*ncogene with *K*inase-domain (NOK)/STYK1 led to cellular transformation, tumorigenesis and metastasis. This report characterises the subcellular distribution of NOK in HeLa cells and its localisation in early endosomes. Confocal immunolocalisation studies indicated that NOK had structural subtypes and was distributed into two distinct expression patterns: a dot pattern (DP) and an aggregation pattern (AP). The results of an immunohistochemistry (IHC) analysis of pathological tissues also showed that high expression level of endogenous NOK was expressed in an aggregate-like structure *in vivo*. Importantly, we found that NOK was localised in endosomes and colocalised with epidermal growth factor receptor (EGFR) in activated endosomal vesicles. However, as the stimulation time increased, NOK and EGFR began to progress through different pathways. EGFR was gradually degraded after treatment with EGF for approximately 20 min, whereas NOK levels were not reduced. This result suggests that NOK mainly plays a role in facilitating the trafficking of EGFR from early endosomes to later endosomes/lysosomes. Taken together, NOK has a strong tendency towards forming aggregates, which may have physiological implications and provide the first evidence that this novel receptor kinase is colocalised with EGFR in endosomes to participate in a post-internalisation step of EGFR.

1. Introduction

Receptor protein tyrosine kinases (RTKs) play critical roles in the regulation of cell growth, differentiation, proliferation, metabolism and apoptosis. RTKs form a large group of receptor subfamilies, and their typical structure is composed of three domains: a diverse ectodomain specific for ligand binding, a single transmembrane domain and an intracellular tyrosine kinase domain for activating downstream signalling cascades [1]. Most RTKs are located in the cytoplasmic membrane and require ligand-induced receptor oligomerisation, leading to the tyrosine autophosphorylation of receptor subunits [2,3]. The activities of RTKs are normally tightly regulated, and the dysfunction of their signalling through mutations or other genetic alterations results in abnormal kinase activity and malignant transformation [1,4–7]. RTKs are involved in multiple steps during tumorigenesis and metastasis, including the detachment of metastatic cells from neighbouring cells, cell motility and invasion to other tissues [1]. Therapeutics targeting the RTK family has demonstrated clinical success in the treatment of diverse epithelial cancers [8,9].

NOK (also known as a putative serine/threonine and tyrosine receptor protein kinase, STYK1) was identified as a member of the RTK family and has a short ectodomain, a putative single transmembrane domain and a conserved intracellular tyrosine kinase domain [10]. The overexpression of NOK in BaF3 cells induced tumorigenesis and metastasis in nude mice, suggesting that the NOK gene acts as an oncogene to promote tumour formation [10]. NOK mRNA has also been found to be upregulated in breast, ovarian, prostate and lung cancers [11–13]. A single tyrosine mutation in NOK (Y327F or Y356F) is sufficient to abolish its activities in transformation and tumorigenesis, indicating that its cellular functions are mediated by tyrosine-dependent signalling [14]. However, as a newly discovered gene, the subcellular localisation of the NOK protein is largely unknown.

Cellular distribution studies generally serve as an initial step toward a deeper understanding and a more detailed characterisation of the functions of a novel protein. Because the NOK protein has one putative transmembrane domain, we initially hypothesised that NOK would most likely localise to the plasma membrane. However, previous studies demonstrated that NOK was predominantly expressed in the cytoplasm and mainly detected in the microsomal fraction [10,15]. Therefore, further analysis of the cellular distribution of NOK is essential. In this report, we determined that NOK was expressed in particular structural isoforms

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and localised to the early endosomes. NOK was colocalised with EGFR and involved in the post-internalisation step of EGFR. These findings provide a deeper understanding of NOK functional activities and a direction for future NOK studies.

2. Materials and methods

2.1. Antibodies and reagents

Restriction and modification enzymes were obtained from TaKaRa Biotechnology (Dalian, China). The mouse monoclonal HA probe (F-7) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, USA). The rabbit monoclonal anti-EEA1 and anti-EGFR antibodies were purchased from Cell Signalling Technology (Boston, USA). Recombinant human EGF was purchased from R&D Systems (Minneapolis, USA). The fluorescent secondary antibodies TRITC goat anti-rabbit or anti-mouse IgG and FITC goat anti-mouse IgG were purchased from ZSGB-BIO (Beijing, China). Anti-GAPDH and AMCA goat anti-rabbit IgG were purchased from Proteintech Group, Inc. (Chicago, USA). Hoechst 33342 was purchased from Sigma (St. Louis, USA).

2.2. Cell culture

Human cervical carcinoma HeLa cells were grown in DMEM (Hyclone, Thermo Scientific, Beijing, China) supplemented with 10% foetal bovine serum (ExCell Bio, Shanghai, China), 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% glutamine. For indirect immunofluorescence detection, the cells were plated onto glass coverslips. A total of 2.5 μg of plasmid was used for transfection using the Vigofect Transfection Reagent (Vigorous Biotechnology, Beijing, China). Immunofluorescent staining was performed 36 h after transfection.

2.3. Immunofluorescent staining

Cells grown on glass coverslips in 6-well plates were washed twice with ice-cold PBS, fixed with 4% paraformaldehyde in PBS for 20 min at room temperature, and permeabilised with 0.2% Triton X-100 in PBS for 10 min at room temperature. The cells were blocked with 1% BSA in PBS for 1 h at 37 °C. Primary antibodies diluted in 0.2% BSA in PBS were incubated for 1 h at 37 °C and followed by incubation with FITC-, TRITC-, or AMCA-conjugated goat anti-rabbit or anti-mouse IgG. The nuclei were or were not counterstained with Hoechst 33342. The coverslips were mounted in a glycerol-based anti-fade mounting medium and analysed with a Zeiss LSM 710 confocal microscopy.

2.4. EGF stimulation

HeLa cells were plated on glass coverslips in 6-well plates and cultured for 24 h. Then, 2.5 μg of the NOK-HA plasmids was transfected into HeLa cells. After 12 h, the cells were washed twice with PBS and starved for 24 h without serum. The starved cells were incubated with 100 ng/ml EGF at 4 °C for 60 min in binding buffer (20 mM Hepes-NaOH, pH 7.5, 150 mM NaCl, and 0.1% bovine serum albumin). The cells were then either fixed with 4% paraformaldehyde or allowed to internalise EGF at 37 °C for 5 min, 20 min, 30 min and 60 min. Then, the cells were fixed and stained with anti-HA and anti-EGFR primary antibodies followed by TRITC- and FITC-conjugated secondary antibodies. The results were analysed with a Zeiss LSM 710 confocal microscopy.

2.5. Statistical analysis

All of the data were analysed with the Excel programme and are expressed as the mean ± standard error of at least three independent experiments. Significant differences were determined with Student's t test. Statistical significance is represented as $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

3. Results

3.1. Expression patterns of NOK in HeLa cells and cancer tissues

To study NOK cellular distribution, a NOK-HA expression plasmid was transfected into HeLa cells followed by detection with an anti-HA antibody. As indicated in Fig. 1A, there were two types of structural patterns of NOK distribution in the cells: one was evenly distributed in the cytoplasm with a typical dot pattern (DP), and the other formed an aggregation pattern (AP) (Fig. 1A), but no distinct localisation of NOK to the cell membrane was observed. The percentage of DP-containing and AP-containing cells was 50.46% and 49.54%, respectively (Fig. 1D). To further define the subcellular distribution and expression patterns of NOK, two deletion derivatives of NOK were generated: NOKΔECD, with the N-terminal ectodomain deleted, and NOK-ICD, with both the N-terminal ectodomain and transmembrane domain deleted (Fig. 1E). The immunostaining analysis indicated that NOKΔECD proteins had a similar staining pattern as full-length NOK. The percentage of AP-containing cells was 47.64% (Fig. 1B, D). However, for the NOK-ICD proteins, the percentage of AP-containing cells was dramatically reduced to 10.51% (Fig. 1C, D). Overall, our results strongly indicate that the transmembrane domain of NOK plays a critical role in forming aggregates.

Because the above findings were based on transiently transfected cells, we also analysed the distribution pattern of endogenous NOK in cancer tissues. A parallel immunohistochemical (IHC) analysis of cervical and breast adjacent and cancerous samples was performed with the anti-NOK antibody. The results indicated that NOK expression was much higher in the cancerous tissues of cervical and breast cancers than in the adjacent normal tissues. The highly expressed NOK isoform in the tumours tended to be the AP form (Supplementary Fig. 1). Taken together, these findings suggest that an AP isoform of NOK exists both *in vitro* and *in vivo*.

3.2. NOK localises to early endosomes, and its transmembrane domain is essential for its localisation

Because most RTKs undergo endocytosis to direct their functions [16] and NOK was demonstrated to mainly be distributed in the cytoplasm, we next investigated whether NOK was localised to the endosome. Ectopically expressed NOK-HA and endogenous EEA1, which is an early endosome marker protein [17], were co-stained. The results demonstrated that NOK was colocalised with endogenous EEA1 (Fig. 2A). The total percentage of cells in which NOK and EEA1 were colocalised was 61.73% (Fig. 2D). However, the percentage of cells in which NOK and EEA1 were colocalised was different between the two structural subtypes of NOK: the AP form (84.11%) was twofold more likely to colocalise with EEA1 than the DP form (39.75%) (Fig. 2A). These results indicated that a population of NOK proteins localised to the early endosomes and that the AP isoforms were favoured in this distribution.

We next examined which domain (s) of NOK was essential for its localisation to the early endosome. Two deletion derivatives of NOK, NOKΔECD and NOK-ICD, were transfected into HeLa cells. As shown in Fig. 2B, NOKΔECD proteins had a similar colocalisation pattern as full-length NOK. In total, 54.91% of NOKΔECD-

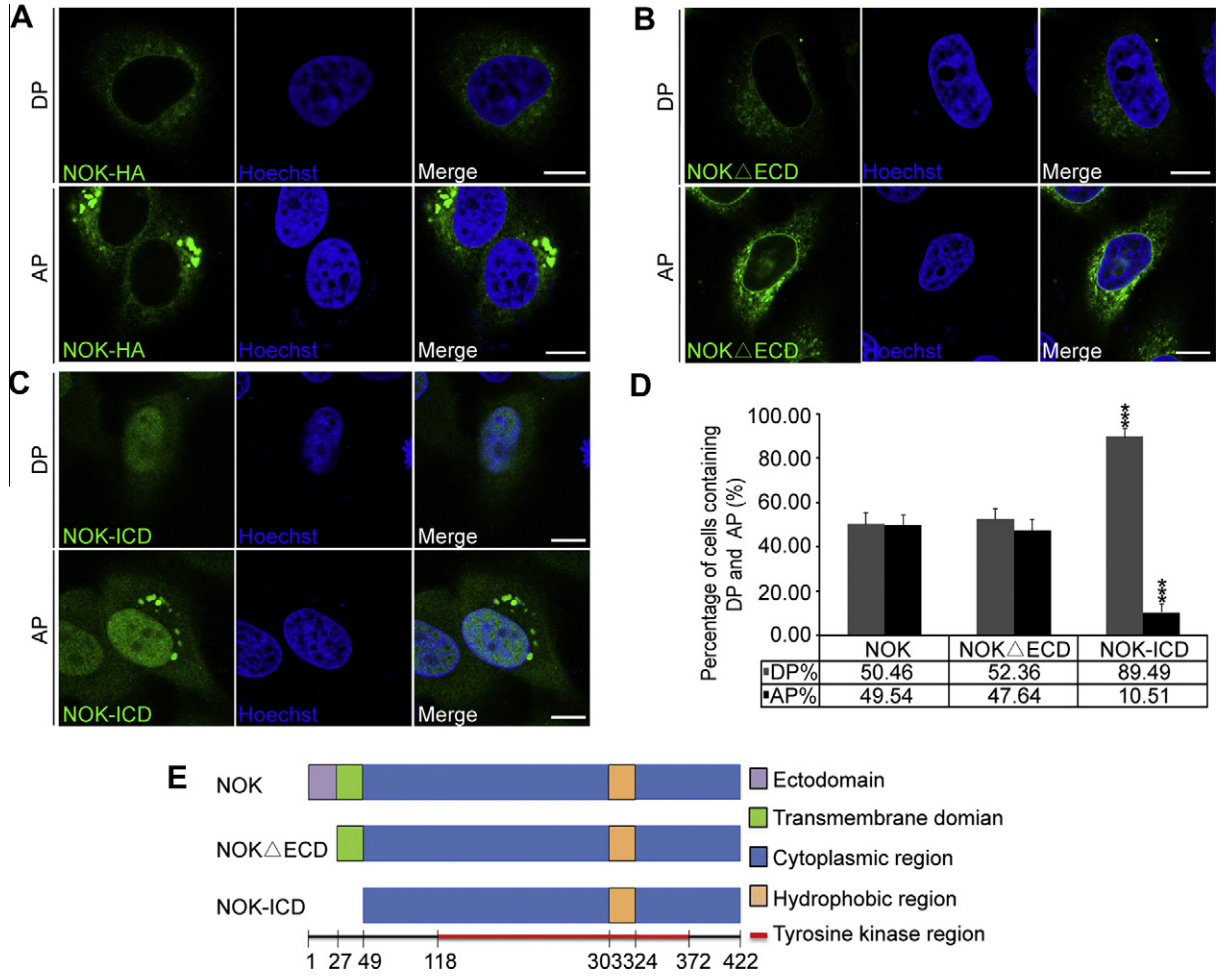


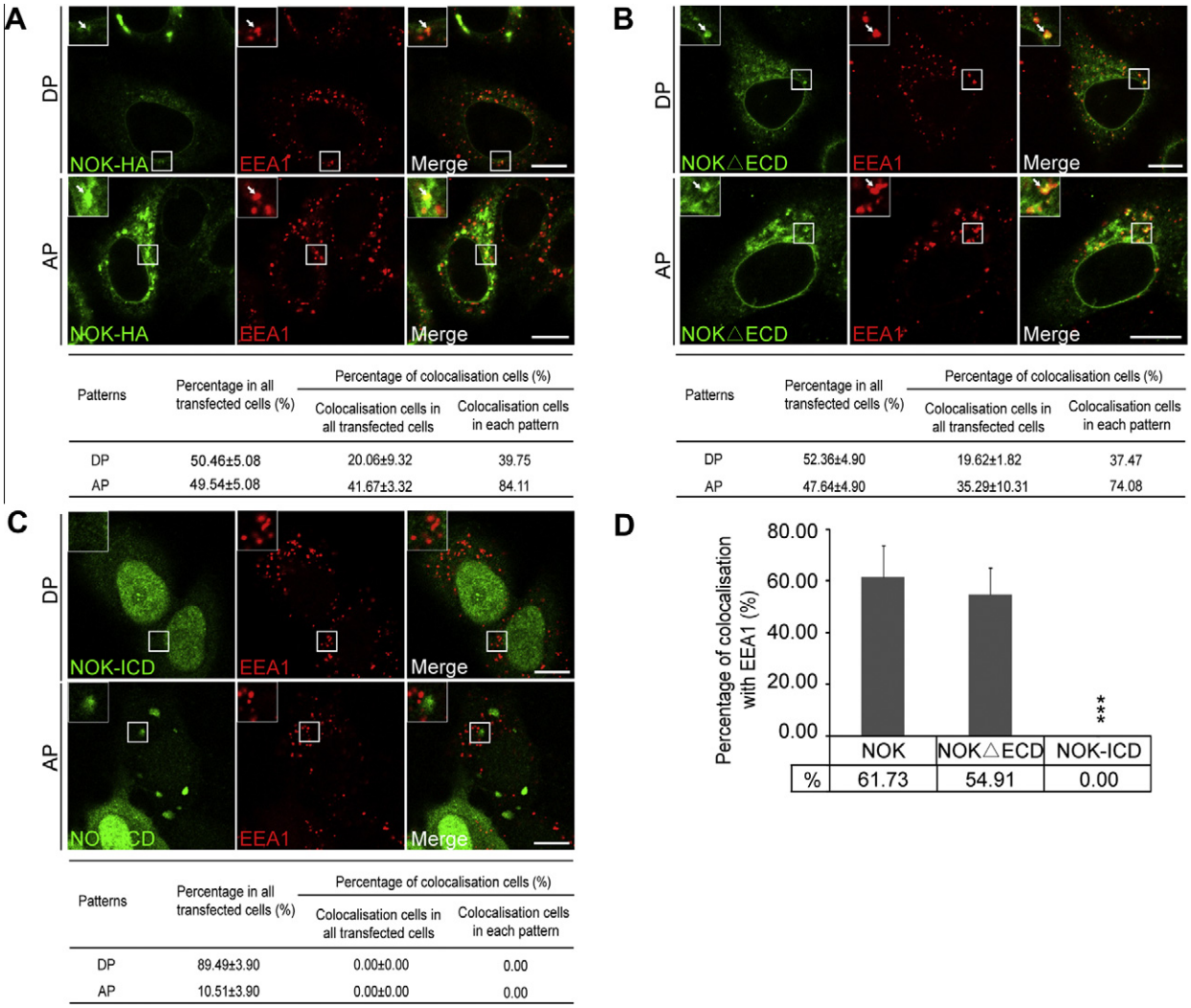
Fig. 1. The expression patterns of exogenous NOK and its deletion variants in HeLa cells. HeLa cells transfected with NOK-HA (A), NOKΔECD-HA (B) and NOK-ICD-HA (C) plasmids were immunostained with the anti-HA antibody followed by staining with a FITC-conjugated secondary antibody (green). The nuclei were counterstained with Hoechst 33342 (blue). Scale bars, 10 μm. There are two expression patterns in each group: the dot pattern (DP) and the aggregation pattern (AP). (D) The bar graph is based on cell counting and calculation and indicates the percentage of DP and AP of NOK and its deletions in transfected cells. Each value represents the average of three independent experiments, and the error bar is the standard deviation. $^{***}P < 0.001$ vs NOK. (E) A schematic representation of the NOK protein structure and the deletion variants tested. NOKΔECD: a N-terminal ectodomain deletion variant; NOK-ICD: an ectodomain and transmembrane domain deletion variant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

transfected cells showed colocalisation of NOK with EEA1, and 37.47% of DP isoform- and 74.08% of AP isoform-containing cells showed co-localisation of NOK with EEA1. Notably, the deletion of both the N-terminal ectodomain and the transmembrane domain (NOK-ICD) totally eliminated the colocalisation of NOK with EEA1 (Fig. 2C, D). The ratio of the NOK-ICD variant was sharply decreased to 0.00% in both DP- and AP-containing cells (Fig. 2D). These findings suggest an essential function of the NOK transmembrane domain in regulating the distribution of NOK to the early endosome.

3.3. NOK is colocalised with EGFR and participates in the post-internalisation step of EGFR after stimulation with EGF

EGFR is a typical member of the RTK family that enacts its function through the endocytic process. The above data showed that NOK was localised in early endosomes. Moreover, NOK was predicted to interact with EGFR (data not shown). Therefore, we hypothesised that NOK might participate in the endocytic process of EGFR. To test this hypothesis, immunofluorescence assays were performed in NOK-transfected HeLa cells that were stimulated with EGF. After treatment at 4 °C for 60 min (no treatment at 37 °C), we observed faint colocalisation of NOK with EGFR in some small dots, and there were few typically activated endosome-like vesicles (Fig. 3A). When the cells were stimulated with EGF at 37 °C for 5 min after treatment at 4 °C, some EGFR-positive endosomal vesicles were activated (Fig. 3B), and NOK and EGFR were clearly colocalised in all of these activated vesicles (Fig. 3B). In other words, all of the EGFR-positive vesicles were also NOK positive, suggesting that NOK proteins were recruited to these activated EGFR-positive vesicles. As the stimulation time increased, the colocalisation of NOK with EGFR was progressively changed. At 20 min, more and larger activated endosomal vesicles were formed, and NOK was more distinctly colocalised with EGFR in these vesicles (Fig. 3C), but the localisation of NOK began to deviate from EGFR in some cells at 20 min, and the EGFR-positive vesicles were no longer all NOK positive. From 20 to 60 min after treatment at 4 °C, the percentage of the cells showing the complete colocalisation of NOK in all of the EGFR-positive vesicles was gradually decreased; in contrast, the percentage of the cells showing the partial colocalisation of NOK in some of the EGFR-positive vesicles was significantly increased (Fig. 3C–F).

To determine whether NOK traffics through the degradation pathway similar to EGFR, western blotting was performed. The results indicated that the level of EGFR was gradually decreased after stimulation with EGF at 37 °C from 20 to 180 min, whereas the



Patterns	Percentage in all transfected cells (%)	Percentage of colocalisation cells (%)	
		Colocalisation cells in all transfected cells	Colocalisation cells in each pattern
DP	50.46±5.08	20.06±9.32	39.75
AP	49.54±5.08	41.67±3.32	84.11

Patterns	Percentage in all transfected cells (%)	Percentage of colocalisation cells (%)	
		Colocalisation cells in all transfected cells	Colocalisation cells in each pattern
DP	52.36±4.90	19.62±1.82	37.47
AP	47.64±4.90	35.29±10.31	74.08

Patterns	Percentage in all transfected cells (%)	Percentage of colocalisation cells (%)	
		Colocalisation cells in all transfected cells	Colocalisation cells in each pattern
DP	89.49±3.90	0.00±0.00	0.00
AP	10.51±3.90	0.00±0.00	0.00

Fig. 2. NOK localises to the early endosome, and its transmembrane domain is essential for its localisation. (A) Co-staining of NOK-HA with EEA1 (an early endosome marker) in NOK-HA-transfected HeLa cells. (B) Co-staining of NOKΔECD-HA with EEA1 in NOKΔECD-HA-transfected HeLa cells. (C) Co-staining of NOK-ICD-HA with EEA1 in NOK-ICD-HA-transfected HeLa cells. (D) The percentage of the total colocalisation of EEA1 with NOK, NOKΔECD or NOK-ICD. The cells were stained with anti-HA and anti-EEA1 antibodies followed by staining with FITC (green)- and TRITC (red)-conjugated secondary antibodies, respectively. The results were obtained with confocal microscopy, cell counting, and calculation. The top panels show the colocalisation of EEA1 with NOK-HA or its derivatives in DP-containing cells, and the bottom panels show their colocalisation in the AP-containing cells. The insets show the areas with higher magnifications, and the areas of colocalisation are indicated with arrowheads. Scale bars: 10 μm. The tables below each group of figures indicate the calculated percentage of the colocalisation of NOK in each pattern, and each value is the average of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

level of NOK showed no reduction (Fig. 3G), suggesting that NOK did not progress through the same degradation pathway as EGFR. To confirm whether these EGFR-positive vesicles were both NOK and EEA1 positive, we chose one time point to perform triple co-staining with antibodies against anti-HA, EEA1 and EGFR–GFP. The results indicated that NOK, EEA1 and EGFR were colocalised in these activated vesicles (Fig. 3H), suggesting that the vesicles with NOK and EGFR colocalisation were endosomes. Together, these data indicated that NOK is actively involved in the progression of EGFR after internalisation and that NOK deviates from the pathway through which EGFR is further trafficked.

4. Discussion

Previous studies have demonstrated that NOK, a new member of the RTK superfamily, has potential to function in the regulation of cell proliferation, malignancy and cancer development [10]. However, the localisation of NOK *in vivo* is largely unknown. In this report, we used HA-tagged NOK to characterise its subcellular characteristics with confocal immunofluorescence. We performed the majority of the tests in transient expression systems because the presently available anti-NOK antibodies did not provide adequately sensitive detection of the endogenous protein through immunofluorescent staining. Using this approach, we determined that NOK exists subcellularly in two distinct isoforms: DP and AP isoforms. Multiple lines of evidence indicate that these particular structural organisations are neither a randomised phenomenon nor an artificial observation that simply resulted from overexpression: (1) these findings were persistent and reproducible across various tests and statistically consistent; (2) a selectivity of these two isoforms existed for distribution to specific organelles; (3) the deletion of the NOK N-terminal domain(s) perturbed the subcellular distribution of these two patterns; and (4) these patterns were identified *in vivo* with endogenous NOK in clinical samples. Additionally, NOK lacking the transmembrane domain almost completely lost the AP isoform, indicating that this structural subtype represents clustered NOK-containing complexes that require

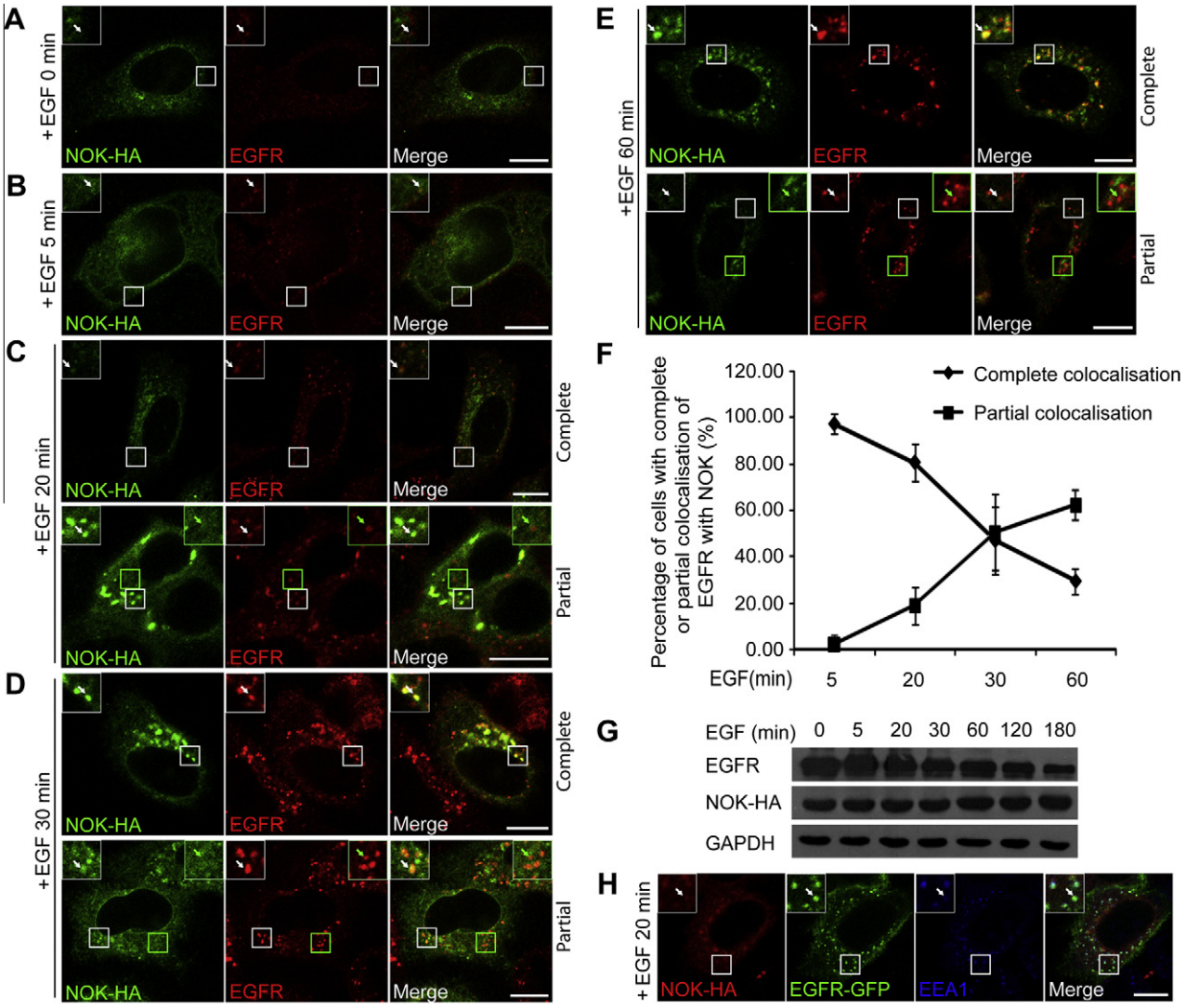


Fig. 3. NOK is colocalised with EGFR and participates in the endocytic process of EGFR after stimulation with EGF. HeLa cells transfected with the NOK-HA plasmid were stimulated with EGF for the indicated times (A for 0 min, B for 5 min, C for 20 min, D for 30 min and E for 60 min). The stimulation method is described in Section 2. The cells were stained with anti-HA and anti-EGFR antibodies followed by staining with FITC (green)- and TRITC (red)-conjugated secondary antibodies, respectively. The results were obtained with confocal microscopy. The insets show the areas with higher magnifications. For C, D and E, the top panels show the total colocalisation of internalised EGFR with NOK, and the bottom panels show the partial colocalisation of internalised EGFR with NOK-HA. The EGFR- and NOK-positive colocalisation dots are indicated with white arrowheads, and the EGFR only-positive (not NOK-positive) localisation dots are indicated with green arrowheads. Scale bars: 10 μm. (F) Statistical analysis of the percentage of cells with the total or partial colocalisation of EGFR with NOK-HA after EGF stimulation. Each value represents the average of three independent experiments, and the error bar is the standard deviation. (G) Western blotting result showing the difference in NOK-HA and EGFR protein levels after EGF stimulation. The cell lysates of HeLa cells transfected with NOK-HA treated with or without EGF were immunoblotted with anti-EGFR and anti-HA antibodies. Endogenous GAPDH is used as loading control. (H) Colocalisation of NOK, EEA1 and EGFR in endosomes. HeLa cells transfected with NOK-HA and EGFR–GFP plasmids were incubated with 100 ng/ml EGF at 4 °C for 60 min followed by warming to 37 °C for 20 min. The transfected cells were co-stained with antibodies against HA and EEA1 followed by staining with TRITC (red)- and AMCA (blue)-conjugated secondary antibodies, respectively. The green fluorescence indicates the expression of EGFR–GFP. The experiment was performed with confocal microscopy. The insets show the areas with higher magnifications. Areas of colocalisation are indicated with white arrowheads. Scale bar: 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

the transmembrane domain for engagement and assembly. Additional explanations can be concluded from our previous co-immunoprecipitation results. We previously showed that the transmembrane domain of NOK influences its oligomerisation [18]. A cross-linking experiment demonstrated that NOK could simultaneously exist as a monomer, dimer and trimer. The high transforming potency induced by NOK might largely be due to its propensity to aggregate *in vivo* [18]. Regardless, the abundant expression of AP isoforms in tumours and their preference for distribution in endosomes indicate that this structural subtype may have profound biological and physiological implications.

Endosomes are considered to be a legitimate platform of the signalling pathway [3]. Receptor-mediated endocytosis is a mechanism used by eukaryotic cells to internalise ligands, and the internalised ligand–receptor complexes further activate downstream pathways [19]. Emerging evidence shows that RTKs play their roles through endocytic membrane trafficking within mammalian cells, and aberrant signal processing and feedback regulation can lead to defects associated with pathologies such as cancer [20–22]. In the present study, we demonstrated that NOK was localised to early endosomes. The transmembrane domain is required for effective NOK distribution to early endosomes, indicating that the hydrophobic transmembrane domain is actively involved in endocytosis. AP isoforms showed a higher preference for distribution in endosomes than the DP isoforms, further suggesting the biological relevance of AP isoforms. In fact, aggregation inducing the

activation of the EGFR receptor protein tyrosine kinase *in vitro* has been reported [23]. EGFR aggregation was considered to be an important step for receptor activation and autophosphorylation [23]. Conditions that optimally stimulated kinase activity led to the formation of large aggregates *in vitro* [23]. Thus, these findings suggest that NOK is involved in endocytosis and that its aggregation may be a critical characteristic of this functional involvement.

NOK could be mainly colocalised with EGFR in endosomal vesicles, but not clearly in the membrane. Typically, EGFR is primarily localised in the cell membrane. The binding of EGF to EGFR results in the polymerisation and internalisation of EGFR [24]. The accumulation of EGF and EGFR can be detected in early endosomes at 1–5 min, in late endosomes at 10–20 min and in mature lysosomes at 40–60 min after incubation with EGF at 37 °C [25,26]. Once EGFRs are internalised, they will be delivered to the endosomal system, from which they will traffic through different pathways, either being recycled to the cell surface or sorted to lysosomes for degradation [20]. The recycling to the membrane can be achieved either through direct sorting by an endosome or through the Golgi network back to the cell surface [20]. Our results indicated that the colocalisation of NOK with EGFR could be observed at all of the tested times (from 5 to 60 min), but NOK and EGFR progressively deviated from each other as the stimulation time increased, especially after 5 min, suggesting that NOK is mainly involved in the trafficking from early endosomes to late endosomes after EGFR internalisation. Western Blot analysis of the difference in the protein levels of NOK and EGFR over time indicated that EGFR began to traffic through the degradation pathway after treatment with EGF for 20 min whereas NOK did not, indicating that NOK and EGFR have different fates or destinations at later stages. Most EGFR proteins might dissociate from the early endosomes and traffic to lysosomes for degradation, whereas most NOK proteins might be retained in early endosomes or recycled to other organelles, such as the Golgi apparatus. Taken together, NOK plays a potential role at the early endosome to late endosome/lysosome sorting step of EGFR.

Thus, this report described the cellular distribution of NOK and identified its localisation in endosomes and its potential role at a post-internalisation step of EGFR. The potential role of NOK in assisting in the interaction of EGFR with the endosomal sorting complex will shed new light on understanding the molecular mechanisms regulating the intracellular trafficking of both NOK and EGFR.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.016.

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