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# Intracellular interaction of newly synthesized nerve growth factor and its receptors

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ABSTRACT

In autocrine cells, both a ligand and its receptors are synthesized in the same cell, but their intracellular interaction is not well known. We examined it using PC84 cells, a mutant PC12 cell line expressing nerve growth factor (NGF). We have already reported that the intracellular precursor of TrkA was phosphorylated and that MAP kinase was phosphorylated in PC84 cells. In this paper we found that the NGF receptors, TrkA and p75NTR, existed mainly as precursors, and most p75NTR localized inside PC84 cells. The phosphorylation of MAP kinase was also observed even when PC84 cells were incubated with anti-NGF antibody to block the extracellular interaction. These results suggest the possibility that newly synthesized NGF could interact intracellularly with the receptors in PC84 cells.



## 1. Introduction

Peptide hormones and growth factors are released into the extracellular space and transmit information after binding to their specific receptors on cell surface. Ligand–receptor complexes generate secondary signals at the cell surface and also inside the cell after internalization [1]. Although the binding and signal transduction of extracellular signals in paracrine and endocrine systems have been examined extensively, the story is more complicated with the autocrine system, where cells express both ligands and their receptors. The intracellular interaction between the newly synthesized ligands and receptors is not well known.

Nerve growth factor (NGF) is a well-known member of neurotrophin family proteins [2]. Neurotrophins are first observed in cells as proneurotrophins, which undergo intracellular proteolytic cleavage by proprotein convertases. The cleavage releases C-terminal peptides, which are then secreted through constitutive or regulated pathways [3,4]. Neurotrophins were initially identified for their effects on neuronal survival and growth. However, recent studies have demonstrated that proneurotrophins are also released by cells and they act as specific signaling ligands that can mediate apoptosis [5,6]. Two cell-surface receptors, TrkA and p75NTR, initiate the signal transduction pathway of NGF [7,8]. Trk receptors exert trophic functions through the intrinsic tyrosine kinase, phosphorylating and activating it and several mediators including MAP kinase and PI3 kinase. p75NTR forms not only the high-affinity receptor complex with TrkA but also the hetero receptor complex with sortilin; the latter complex preferentially interacts with proneurotrophins and regulates the induction of cell death [9,10].

Neurotrophins and their receptors are often expressed in the same cells, such as in those in cortical ventricular/subventricular zones and in cultured embryonic cortical progenitor cells [11,12]. In such autocrine cells, ligands and their receptors are synthesized in the same cells and they might interact intracellularly en route to the plasma membrane, but this kind of mechanism has not yet been substantiated.

PC12 cells, expressing both TrkA and p75NTR, are widely used in various researches [13]. These cells arrest growth and differentiate in response to NGF. The same response was observed when PC12 cells were transiently transfected with NGF cDNA [4]. We generated a mutant PC12 cell clone, PC84 cells, by cloning from transfected PC12 cells with NGF cDNA [14]. PC84 cells secreted active NGF and extended short processes but continued to proliferate. We observed that the expression level of p75NTR was low in PC84 cells compared to PC12 cells, and that NGF signaling via p75NTR was necessary to arrest PC12 cell growth, which was mediated by Akt [15]. Because PC84 cells express NGF and its receptors simultaneously, they are a good model to examine the potential intracellular interactions of the ligand and its receptors. We have previously shown that the precursor of TrkA was phosphorylated in PC84 cells [14], and MAP kinase was also phosphorylated in these cells, even after washing the cells [15]. These results suggested that NGF and TrkA could interact intracellularly and it could activate TrkA. In this paper, we further examine the occurrence of the intracellular interaction.

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## 2. Materials and methods

### 2.1. Plasmid construction

The TrkA expression plasmid was constructed by inserting rat TrkA cDNA into the mammalian expression vector pGW1 [4]. Full-length TrkA cDNA (corresponding to 249 aa) was amplified from PC12 cell cDNA using the following primers: 5′-TTTTGAATTCATGCTGCGAGGCCAGCGGCA-3′ (sense sequence, containing an *Eco*RI site at the 5′-end) and 5′-TTTTGTCGACCTAGCCCAGAACGTCCAGGT-3′ (antisense sequence, containing a *Sal*I site at the 5′-end). The amplicon was digested with *Eco*RI and *Sal*I and ligated into *Eco*RI- and *Sal*I-digested pGW1. The expression plasmid of human growth hormone was kindly supplied by Dr. Itakura (Kitasato University School of Medicine).

### 2.2. Cells and transfection

PC12 and PC84 cells were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% horse serum and 5% fetal bovine serum. In this study, the clone 6F9 was used as PC84 cells [14]. These cells were plated on collagen-coated dishes for the following experiments [4]. Transfection was performed with expression plasmids using LipofectAMINE 2000 (Invitrogen Corp.) as described previously [4].

### 2.3. NGF and anti-NGF serum

Mouse NGF was isolated from the submaxillary glands of adult male mice, and anti-NGF serum was raised in rabbits [16]. The anti-NGF serum was used to block extracellular interaction between NGF and the receptors. Normal rabbit serum was used as a control.

### 2.4. Western blotting

Cell lysis and Western blotting was conducted as reported [4]. SDS–PAGE was performed with cell lysates containing 10 μg of proteins for each lane. Antibodies used for detection were anti-Trk (C14, Santa Cruz), anti-p75NTR (Sigma–Aldrich), anti-phospho-p44/42 MAP kinase, and anti-p44/42 MAP kinase (Cell Signaling Technology Inc.). Band intensities were quantified with Scion Image software (Scion Corp.).

### 2.5. Pulse-chase study

Pulse-chase experiment was performed as previously reported [4], using PC12 or PC84 cells that were transfected with TrkA cDNA. Anti-TrkA (763, Santa Cruz) was used for immunoprecipitation.

### 2.6. Immunofluorescence

Immunofluorescence was performed with fixed and permeabilized cells as reported [4]. Some dishes were incubated with 50 nM Texas Red-labeled transferrin (Invitrogen Corp.) for 30 min or with 100 nM LysoTracker Red DND-99 (Invitrogen Corp.) for 1 h before fixation. Anti-p75NTR (AB1554, Millipore) was used to stain p75NTR.

## 3. Results

### 3.1. Molecular sizes of the receptors

Newly synthesized NGF receptors are first observed in cells as precursors (the apparent molecular sizes of the precursors for TrkA and p75NTR are 110- and 70-kD, respectively) and then processed into larger species (140- and 75-kD, respectively) [17–19]. Such change of TrkA is also shown in our pulse-chase experiment (Fig. 2A). The precursors are known to be processed into the larger mature forms by the modification of the carbohydrate moiety [17,19,20]. It has also been reported that the precursors of receptors reside inside cells and cell surface receptors are large mature type [17–20].

We examined the sizes of these receptors in PC84 cells by Western blotting. As shown in Fig. 1A, 110- and 140-kD TrkA bands were detected in PC12 cells at comparable intensities, whereas the 110-kD species was predominant in PC84 cells. In our former paper [14], we compared the levels of 110-kD of TrkA and failed to examine 140-kD species.

Similar result was obtained for p75NTR. It separated into two bands, with apparent sizes of 70- and 75-kD in PC12 cells; the 75-kD mature species was far abundant (Fig. 1B). In PC84 cells, p75NTR was observed mainly as 70-kD species, albeit at lower levels than in PC12 cells. The low expression level of p75NTR in PC84 cells was reported previously [14]. These results indicate that TrkA and p75NTR exist primarily in their precursor forms in PC84 cells.

Since PC84 cells are mutant cells, there could be defect in the protein processing or sorting process. However, we observed active NGF was secreted from these cells [14], which observation was similar to that in PC12 cells transfected with NGF [4]. Here we also examined the behavior of human growth hormone expressed by transfection. The cDNA of growth hormone was introduced in PC84 and PC12 cells, and we could not find any difference in the expression and secretion of the protein between the two cell lines (data not shown). Thus we considered that PC84 cells have no defect in the protein processing or sorting process.

The behavior of newly synthesized TrkA was examined by pulse-chase analysis (Fig. 2). The intrinsic TrkA was difficult to detect with this method, presumably due to the low expression level. So, PC12 and PC84 cells were transfected with TrkA cDNA and the behavior of the expressed proteins were examined. Two days after the transfection, the cells were incubated with $^{35}$S-Met/Cys and chased. After the pulse, the 110-kD species predominated in both cell lines, and then mature TrkA (140-kD) appeared after 1 h chase in PC12 cells, whereas the level of the 140-kD species was low in PC84 cells during the chase. This result suggests that most of the newly synthesized TrkA, though they were transiently expressed, is not processed into the mature 140-kD form in PC84 cells, which is consistent with our Western blot finding.

### 3.2. Intracellular localization of receptors

TrkA was difficult to detect with our antibodies by immunofluorescence in spite of their relatively easy detection by Western

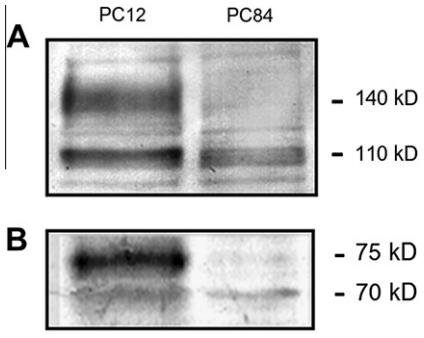


**Fig. 1.** Western blot of TrkA and p75NTR in PC12 and PC84 cells. The cell lysates of PC12 and PC84 cells (10 μg proteins for each lane) were subjected to SDS–PAGE (7.5%), and receptors were detected with anti-TrkA (A) or anti-p75NTR (B).

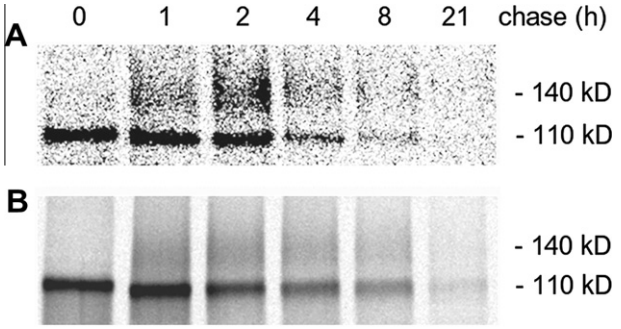


**Fig. 2.** Pulse-chase analysis of TrkA expressed in PC12 and PC84 cells. PC12 (A) and PC84 (B) cells were transfected with TrkA cDNA. Two days later, the cells were exposed to [$^{35}$S]-Met/Cys for 15 min and chased for 0, 1, 2, 4, 8, or 21 h in normal medium. Cell lysates were immunoprecipitated with anti-TrkA and electrophoresed on 7.5% SDS–PAGE gels. Dried gels were exposed to the imaging plates and analyzed on Bio-Imaging Analyzer [4].

blotting and we could not find suitable antibody. So, we focused on the localization of p75NTR. As shown in Fig. 3(A), in PC12 cells most of the p75NTR was detected at the cell surface, and was found to internalize by the addition of NGF but a considerable amount remained on the cell surface at 12 h of the incubation. In contrast, p75NTR of PC84 cells was detected mainly within cells with punctate distribution, and was minimally detected at the cell surface. To investigate the subcellular localization of p75NTR, cells were treated with Texas Red-labeled transferrin (which is largely sequestered into endosomes) for 30 min or with LysoTracker Red DND-99 (primarily taken up by acidic organelle like lysosomes) for 1 h before fixation. Cells were then stained with anti-p75NTR. Fig. 3(B) shows that lots of p75NTR colocalized with LysoTracker, and a small portion colocalized with transferrin. Thus, most of the p75NTR might localize to lysosome-related fractions in PC84 cells.

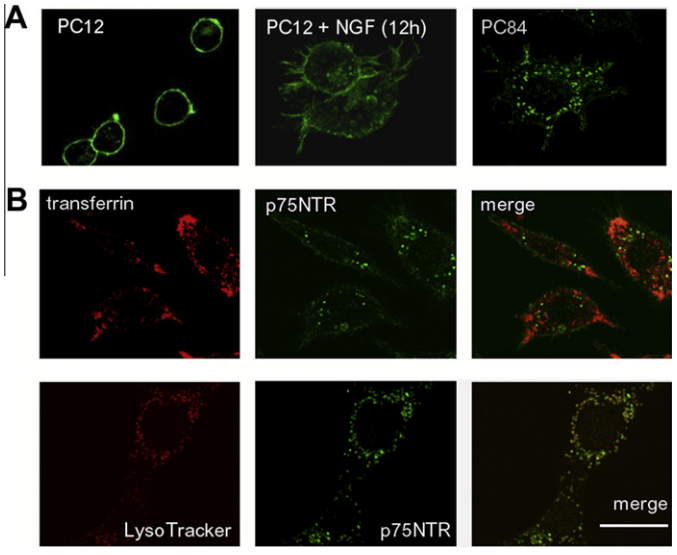


**Fig. 3.** Localization of p75NTR in PC12 and PC84 cells. (A) The localization of p75NTR was examined with PC84 and PC12 cells treated with NGF (50 ng/mL) for 0 or 12 h. The cells were fixed and permeabilized before staining. (B) PC84 cells were incubated with Texas Red-labeled transferrin or LysoTracker Red DND-99 for 30 min or 1 h, respectively. Then cells were fixed and stained using anti-p75NTR antibody. Scale bar, 20 μm.

### 3.3. Phosphorylation of MAP kinase by intracellularly activated TrkA

We have previously reported that TrkA was phosphorylated in PC84 cells, although to a lesser extent than in PC12 cells stimulated with NGF for 10 min, which results was shown in Fig. 5 in our former paper [14]. Both the 110- and 140-kD species of TrkA were observed to be phosphorylated in PC84 cells, in contrast to PC12 cells treated with NGF, wherein only the 140-kD species was strongly phosphorylated. We have also shown that MAP kinase was phosphorylated in PC84 cells [15].

To determine whether MAP kinase was phosphorylated by intracellularly activated TrkA, we tried to block extracellular interaction between NGF and TrkA with the antiserum. At first, in order to know the amount of antiserum necessary to suppress the extracellular interaction between NGF and TrkA, various amounts of anti-NGF serum were added to PC12 cells and NGF was added. MAP kinase phosphorylation was significantly inhibited by the antiserum of the concentrations greater than 5 μL/mL (Fig. 4A). In this experiment NGF of 25 ng/mL was added to 50,000 cells of

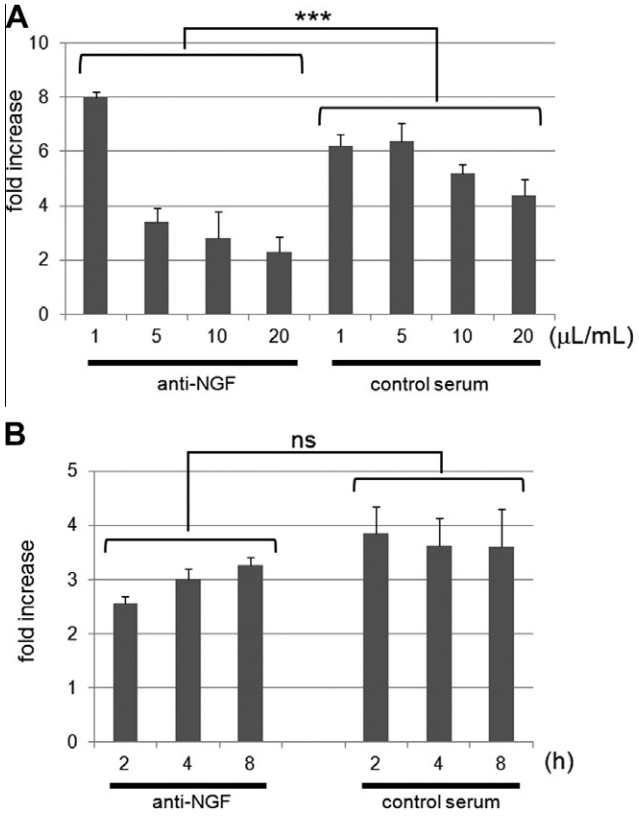


**Fig. 4.** Phosphorylation of MAP kinase in the presence of anti-NGF serum in PC12 cells treated with NGF and in PC84 cells. (A) PC12 cells (50,000 cells/well) were incubated in 1 mL of serum-free medium in 24-well plates overnight and treated with various amounts of anti-NGF antiserum or control rabbit serum for 10 min. The cells were then incubated with NGF (25 ng/mL) for 10 min, lysed, and immunoblotted with anti-MAP kinase or anti-phospho-MAP kinase as reported [15]. The experiment was repeated three times, and the intensity of the bands was quantified. The increase in relative intensity (ratio of phospho-MAP kinase to total MAP kinase) is expressed as fold-change versus the relative intensity of unstimulated PC12 cells. Significance versus control group was determined by two-way ANOVA. $^{***}p < 0.001$. (B) PC84 cells (50,000 cells) were incubated in 1 mL of serum-free medium in 24-well plates overnight and the medium was further changed to remove NGF. Then, anti-NGF antiserum or control serum (15 μL/mL each) was added and incubated for the times indicated in the figure. The cells were lysed and the phosphorylation of MAP kinase was assessed as in PC12 cells (A). The increase in intensity after three experiments is expressed as fold-change versus the intensity in unstimulated PC12 cells as in A. Significance versus control group was determined by two-way ANOVA. NS: not significant.

PC12. Since the amount of NGF secreted by $3 \times 10^5$ cells of PC84 was estimated to be 4–7 ng for 5 h [14], 15 μL/mL antiserum was supposed be sufficient to block extracellular NGF secreted by 50,000 cells of PC84. The phosphorylation of MAP kinase of PC84 cells was measured over time in the presence of the antiserum (Fig. 4B). The phosphorylation level was not altered significantly until 8 h. The extent of phosphorylation looks to decrease slightly in the presence of the antiserum compared to that in the control serum, but this change was not significant. These results suggest that MAP kinase could be phosphorylated by the intracellularly activated TrkA.

## 4. Discussion

In the autocrine mechanism, a cell synthesizes both ligands and the receptors. It is generally supposed that the ligands are secreted and bind to the receptors on the cell surface, while intracellular interactions of the newly synthesized ligands and the receptors is not well known. PC 84 cells, expressing both NGF and its receptors [14], render them a suitable model for the study of intracellular interactions between NGF and the receptors.

In our previous study [14], we noted that the 110-kD precursor form of TrkA was phosphorylated in PC84 cells, which suggested the occurrence of the intracellular interaction with NGF since 110-kD TrkA has not been detected at the cell surface [19,20]. The phosphorylation of TrkA in PC84 cells was completely blocked by K252a, an inhibitor of Trk tyrosine kinase, indicating that the phosphorylation was the consequence of the autophosphorylation [14]. In this report, we observed by Western blotting that the mature 140-kD form of TrkA was negligible and the majority of TrkA was of the 110-kD precursor species in PC84 cells. Another receptor, p75NTR, was also found to exist mainly as a 70-kD precursor. These receptor precursors are known to localize to the inside of cells [18–20]. By immunofluorescence study we found that p75NTR resided mostly inside PC84 cells. Unfortunately we could not directly determine the localization of TrkA, but its predominant expression as the low-molecular-weight precursor form suggests its intracellular localization. Our pulse-chase experiment also showed that very small amounts of TrkA were processed into mature 140-kD form. There might be another possibility such that mature 140-kD TrkA is produced and transported to the cell surface like normal PC12 cells but it preferentially binds to NGF at the cell surface and is efficiently internalized for degradation. However, this scheme could not explain the existence of the phosphorylated 110-kD TrkA in PC84 cells, which is well known as the intracellular form of TrkA [19,20].

In spite of the negligible amount of 140-kD TrkA, both 110- and 140-kD species of TrkA were found to be phosphorylated in PC84 cells as shown in Fig. 5 of our former paper [14]. Chao's group [21] has reported that the phosphorylated 110- and 140-kD TrkA were observed inside cells through transactivation and that the phosphorylated 110-kD TrkA was processed into 140-kD species without appearing on the cell surface. Such intracellular processing of phosphorylated TrkA might happen in PC84 cells, but we have to examine further to know the precise mechanism of the processing of the phosphorylated TrkA.

Finally, we showed that the level of phosphorylation of MAP kinase in PC84 cells did not significantly decrease even in the presence of sufficient amount of the anti-NGF in the medium, although there remained some ambiguity in the validity of the antibody in the medium to suppress the extracellular interaction between the just secreted ligand and the receptor. However, the existence of the phosphorylated 110-kD TrkA in PC84 cells supports the interpretation that TrkA is phosphorylated and activated intracellularly, which then phosphorylates its downstream targets. Such a mechanism might exist in physiological autocrine cells and might generate other signals different from the cell surface interactions.

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