Intracellular Dynamics of a High Affinity NGF Receptor TrkA in PC12 Cell

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A high affinity nerve growth factor (NGF) receptor, tropomyosin-receptor kinase (TrkA), is visualized by expression of TrkA conjugated with cyan fluorescent protein (CFP) in PC12 cells. TrkA was distributed on the plasma membrane of PC12 cells almost uniformly in both differentiated cells and undifferentiated cells. NGF induced differentiation of PC12 cells transfected with TrkA-CFP normally as wild cells without transfection and the expression of TrkA was observed on the entire cell membrane which surrounds the cell body, axons and growth cones. Interestingly, TrkA-CFP was also present on the membrane of filopodia sticking out from the axon and growth cone. In the axonal region, transporting vesicles of TrkA with diameters ranging from 0.5 to 1.5 μm were observed. Some of these vesicles showed net directional movement along the axon in both directions, anterograde and retrograde. The mean velocity of anterograde and retrograde transport was 0.2 ± 0.03 and 0.3 ± 0.05 $\mu\text{m/s}$ (mean $\pm$ S.E.), respectively. Some vesicles moving anterogradely changed their direction occasionally although the net transport was anterograde. On the other hand, vesicles moving retrogradely seldom switched their direction in spite of occasional stops. These results demonstrated that the behavior of TrkA transporting vesicles in axons of PC12 cells was similar to that observed in the primary culture of sympathetic or sensory neuron. Therefore, it is suggested that the PC12 cell transfected with fluorescent protein-conjugated TrkA is a useful model for studying the signal transduction of NGF.

Key words nerve growth factor; TrkA; axonal transport; PC12 cell; green fluorescent protein; confocal laser scanning microscopy

Nerve growth factor (NGF) is a neurotrophic factor which exists as a dimer of 26 kD polypeptide.¹⁾ It plays an essential role in differentiation, growth, and maintenance of sympathetic, sensory and central nervous systems.²⁾ These multiple effects of NGF are initiated by binding to the high affinity NGF receptor TrkA (tropomyosin-receptor kinase).^{3,4)} Extensive research on NGF and TrkA has been carried out using a rat pheochromocytoma cell line, PC12.^{5,6)} PC12 cell expresses TrkA on its plasma membrane, differentiates to a sympathetic neuron by NGF, and has been used as a useful model to study the effects of NGF on the differentiation, growth, and maintenance of nerve cells.

In nerve cells, TrkA is synthesized at the cell body and is transported to the nerve terminal by anterograde axonal transport. Extracellular NGF is bound to this transported TrkA at the nerve terminal and the NGF-TrkA complex is internalized and transported to the cell body by retrograde axonal transport to induce gene transcription.^{7–10)} Therefore, not only the static distribution but also the intra-axonal dynamics of TrkA is deeply involved in the signal transduction of NGF. However, the distribution and dynamics of TrkA in PC12 are lesser studied, particularly in a single living cell, although TrkA is well-characterized biochemically. Proteins and organelles such as mitochondria and synaptic vesicles are carried along axon by fast and slow axonal transport systems.¹¹⁾ Fast axonal transport is bi-directional, anterograde and retrograde. Kinesin is the motor protein responsible for the anterograde transport, and dynein is the other motor protein for the retrograde transport.¹¹⁾ These two proteins bind to microtubule and hydrolyze ATP to transport their cargo along microtubules.

In the present study, we investigated the distribution and dynamics of TrkA in a single living PC12 cell by expression of TrkA fused with a cyan fluorescent protein (CFP), a cyan

colored version of green fluorescent protein (GFP). The anterograde and retrograde fast transport were visualized in the neurites of differentiated PC12 cells and their dynamics were analyzed.

MATERIALS AND METHODS

Cell Culture PC12 cells were cultured in DMEM (Gibco BRL) supplemented with 10% fetal bovine serum (Bio Whittaker) and 5% horse serum (Gibco BRL) at 37 °C in an atmosphere of 5% CO₂.¹²⁾

Preparation of Plasmid and Transfection Poly(A)⁺ RNA was obtained with a QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech) from 1×10^7 cells of PC12 cells, and served as templates for cDNA synthesis with SuperScript II RT (Gibco BRL). The full-length of DNA sequence coding TrkA was obtained by RT-PCR with a primer pair, 5'-GAATTCTAATGCTGCGAGGCCAGCG (sense; EcoRI site is underlined)/5'-GTCCGACGCGCCCAAG-GTCCAGGTAATC (anti-sense; SalI site is underlined) using a high-fidelity Taq polymerase LA-Taq (Takara, Tokyo, Japan). PCR products were subcloned into a TA cloning vector pCRII (Invitrogen) and sequenced with a DSQ1000 DNA sequencer (Shimadzu, Kyoto, Japan) to verify the DNA sequence of TrkA. The cDNA coding TrkA was ligated with a CFP expression vector, pECFP-N1 (Clontech), at EcoRI/SalI site. PC12 cells were electroporated in cold phosphate-buffered saline with 20 μg of plasmid DNA at 270 V and 950 μF using Gene Pulser II (Bio-Rad).

Confocal Laser Scanning Microscopy Electroporated PC12 cells were plated in a ZOG-3 glass bottom chamber (Elekon; Chiba, Japan). Fluorescent images of PC12 cells transfected with TrkA-CFP were taken with a confocal laser scanning microscope (Zeiss, LSM-510) with a 63 $\times$ objective

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Fig. 1. Confocal Fluorescent Images of PC12 Cell Transfected with TrkA-CFP

(a) Fluorescent image of a PC12 cell at 24 h after transfection. TrkA-CFP resided on the plasma membrane and Golgi apparatus. (b) Fluorescent image of differentiated PC12 cell with NGF (100 ng/ml) treatment. Outgrowth of neurites were observed, the uniform distribution of TrkA-CFP on the cell membrane was similar to that of undifferentiated cells as in (a). (c) Zoomed image of the growth cone region of one of the neurites appearing in (b). Many filopodia sprouted and fluorescence was also distributed on them. Bar; 8 μ m.

lens (Plan-Apochromat 63 $\times$ /1.4 oil) at 20 h after electroporation. Samples were excited at 458 nm with an Ar laser and fluorescence of 475–575 nm was collected at every 2–3 s. Collected data were transformed to Excel files and analyzed with Excel 98 (Microsoft). The analysis of the velocity of transporting vesicles was carried out provided the movement is one-dimensional. The highest velocity of each transporting vesicle during the measurement was adopted as the value of the transport velocity.

RESULTS

Distribution of TrkA in PC12 Confocal fluorescent images of PC12 cells transfected with TrkA-CFP are shown in Fig. 1. TrkA-CFP resided on the plasma membrane and the intracellular region near the nucleus, probably the Golgi apparatus (Fig. 1a). Fluorescence on the plasma membrane was distributed almost uniformly. Treatment with NGF induced differentiation of PC12 cells transfected with TrkA-CFP normally, as observed in wild PC12 cells without transfection. In the cells transfected with pECFP vector only, fluorescence was distributed in the cytoplasm and nucleus uniformly, and neither localization nor vesicular structures were observed. Figure 1b shows the fluorescent images of a transfected cell at 30 h after NGF treatment (100 ng/ml). A few neurites extended, and growth cones were observed at their terminal. The fluorescence of TrkA-CFP was detected on the Golgi apparatus and the entire plasma membrane which surrounds the cell body, axons and growth cones. The pattern of distribution on the plasma membrane was uniform and was independent of the regions (such as cell body, axon and growth cone) of these highly polarized cells. Many filopodia stuck out from the axon and growth cone, especially in the growth cone region (Fig. 1c). They changed their shape, length and direction frequently, and TrkA-CFP was also expressed on these filopodia membrane intensely. In the case of the cell expressing CFP only, fluorescence was distributed in the cytoplasm and nucleus uniformly, including the cell body, axons and growth cones.

Dynamics of TrkA Transporting Vesicles In axons of differentiated cells, vesicles with diameters ranging from 0.5 to 1.5 μ m were observed (Fig. 2). During the measurement

Fig. 2. Visualized Axonal Transport of TrkA Transporting Vesicles

Upper side of each frame proximal to the cell body. Many filopodia sprouted from axonal membrane. Bar; 4 μ m. (a) Successive images of anterograde transport of TrkA transporting vesicle. Two vesicles indicated by arrows showed typical anterograde transport and moved toward growth cone (lower side of frames). (b) Successive images of retrograde transport of TrkA transporting vesicle. The vesicle indicated by an arrow moved toward the cell body (upper side of frames).

(ca. 5 min), about 30% of these vesicles moved along the axon while the rest of the vesicles showed Brownian motion-like movements and made no net directional transport (Fig. 3, broken lines). Both anterograde and retrograde movements were observed. Some vesicles exhibiting anterograde trans-

Fig. 3. Movements of Typical Axonal Transports of TrkA Transporting Vesicle

Position of each vesicle is plotted against the time. Initial position of each vesicle is set to be zero, and positive values represent anterograde displacement. Open symbols show the movements of vesicles which underwent anterograde transport, and closed symbols show the retrograde movements. Three representative movements towards each direction are shown. Broken lines close to zero level describe the movements of the vesicles which showed no net transport.

port made a long stretch of unidirectional motion without stopping, whereas other vesicles underwent bi-directional movement switching their direction occasionally but with net anterograde transport (Figs. 2, 3). The average velocity of anterograde transport was $0.2 \pm 0.03 \mu\text{m/s}$ (mean $\pm$ S.E.; $n=30$). The average velocity of retrograde transport was $0.3 \pm 0.05 \mu\text{m/s}$ (mean $\pm$ S.E.; $n=20$). In contrast to anterograde transport, changes in direction were seldom observed in retrograde transport, although vesicles stopped their movement occasionally (Fig. 3).

In about 10% of the fluorescent cells, differentiation was induced without NGF, and outgrowth of neurites was observed as seen in NGF treated cells. This value was significantly high, because the rate of differentiation without NGF in wild PC12 cells without transfection was less than 2% in our experimental condition.

DISCUSSION

We succeeded in visualizing TrkA and its transporting vesicles in a single living PC12 cell. There were no essential differences in distribution of TrkA between differentiated cells and undifferentiated cells. The distribution of fluorescence on the plasma membrane was almost uniform and no punctate or distinct localization was observed. The distribution of TrkA on the filopodia at axon and growth cone was demonstrated. These filopodia changed their shape and direction quite frequently, and it is interesting that TrkA exists in these remarkably mobile structure since filopodia is supposed to play an important role in finding a specific neuron in the process of neural network construction.

Both anterograde and retrograde movement of TrkA transporting vesicles were observed. Judging from their dynamics revealed in the present study, these transports were categorized as a fast axonal transport. Nakata *et al.* reported the axonal transport of TrkA in dorsal root ganglia (DRG) cells using a GFP method.¹³ As for the anterograde transport, size and bi-directional movement of TrkA transporting vesicles of PC12 are in good agreement with those in DRG cells. The

velocity of anterograde transport in DRG cells was $0.88 \mu\text{m/s}$ ¹³ and higher than that observed in PC12 by our study. The fast anterograde transport of secretory granules in differentiated PC12 was reported with GFP-conjugated plasminogen activator protein.¹⁴ According to their results, the velocities of anterograde transport of secretory granules were around $0.5 \mu\text{m/s}$ and still higher than TrkA transporting vesicles. This difference may suggest that a different member of the motor protein family (kinesin superfamily protein; KIF) is responsible for the transport of vesicles depending on the cell type, maturity of axon and the protein to be transported.¹¹

The velocity of retrograde movement of TrkA transporting vesicle in PC12 was about $0.3 \mu\text{m/s}$. This value is similar to that of the retrograde movement of vesicles visualized with GFP-conjugated GAP-43 and synaptophysin in DRG neuron.¹³ The pattern of retrograde movement is different from that of anterograde movement in PC12 cells, and switching of direction was seldom observed in retrograde transport. So far, it is impossible to conclude whether or not this difference is specific to PC12 or TrkA because no detailed description about the retrograde movement of TrkA transporting vesicles was given in DRG cells.

In some cells, transfection of TrkA induced differentiation without NGF. The mechanism of this differentiation remains to be elucidated. A trace amount of NGF existing in the culture medium might have induced differentiation in PC12 which is over-expressed. However, it might be possible to explain the mechanism that over-expressed TrkA receptors resided closely to each other and dimerization of receptors occurred without NGF resulting in differentiation.

In the present study, it was demonstrated that TrkA transporting vesicles in axons of PC12 cells are similar to that observed in the primary culture of sympathetic or sensory neuron. Therefore, PC12 cell transfected with fluorescent protein-conjugated TrkA is a useful model for the study of the signal transduction of NGF receptor.

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