Neurochemical Research, Vol. 30, Nos. 6/7, June/July 2005 (© 2005), pp. 767–777
DOI: 10.1007/s11064-005-6870-z

Biochemical Characterization of Intracellular Membranes Bearing Trk Neurotrophin Receptors

Hiroko Yano[1,2] and Moses V. Chao[1]

(Accepted May 18, 2005)

Neurotrophin receptor trafficking plays an important role in directing cellular communication in developing as well as mature neurons. However, little is known about the requirements for intracellular localization of the neurotrophin receptors in neurons. To isolate the subcellular membrane compartments containing the Trk neurotrophin receptor, we performed biochemical subcellular fractionation experiments using primary cortical neurons and rat PC12 pheochromocytoma cells. By differential centrifugation and density gradient centrifugation, we have isolated Trk-bearing compartments, suggesting distinct membranous localization of Trk receptors. A number of Trk-interacting proteins, such as GIPC and dynein light chain Tctex-1 were found in these fractions. Additionally, membranes enriched in phosphorylated activated forms of Trk receptors were found upon ligand treatment in primary neurons and PC12 cells. Interestingly, density gradient centrifugation experiments showed that Trk receptors from PC12 cells are present in heavy membrane fractions, while Trk from primary neurons are fractionated in lighter membrane fractions. These results suggest that the intracellular membrane localization of Trk can differ according to cell type. Taken together, these biochemical approaches allowed separation of distinct Trk-bearing membrane pools, which may be involved in different functions of neurotrophin receptor signaling and trafficking.

KEY WORDS: Endocytosis; neurotrophin receptors; subcellular fractionation; trafficking; Trk.

INTRODUCTION

Neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and neurotrophin (NT)-3, exert a variety of functions including survival, differentiation, and synaptic plasticity throughout the nervous system. Each neurotrophin is capable of binding to a specific Trk receptor tyrosine kinase (TrkA, TrkB, and TrkC) and the p75 neurotrophin receptor (1,2).

Considerable evidence has indicated that Trk receptor trafficking and localization are important for determining survival and modulation of neuronal activity and synaptic functions (3–6). A number of Trk trafficking events occur in neurons. In CNS neurons, the BDNF receptor TrkB has been shown to be present in dendritic spines, axon initial segments, axon terminals, dendritic shafts, and cell bodies (7–9). Except for dendritic spines, TrkB receptors in these cellular regions are often found in intracellular membranes, suggesting that extensive trafficking of this receptor occurs in neurons (8). Trk in specific neuronal subdomains has been suggested to be important for specific

[1] Molecular Neurobiology Program, Skirball Institute of Biomolecular Medicine, Departments of Cell Biology; Physiology and Neuroscience, New York University School of Medicine, New York, NY 10016, USA.
[2] Address reprint requests to: Hiroko Yano, Molecular Neurobiology Program, Skirball Institute of Biomolecular Medicine, Departments of Cell Biology; Physiology and Neuroscience, New York University School of Medicine, 540 First Avenue, New York, NY 10016, USA; E-mail: yano@saturn.med.nyu.edu

function. For example, a number of studies have indicated that presynaptic TrkB may mediate enhancement of glutamate release, while postsynaptic TrkB may enhance NMDA receptor function in central neurons (3,6,10). Synaptic TrkB receptors are heavily involved in determining changes in synaptic transmission and LTP that can modulate synaptic plasticity (3,10). Thus, the targeting and localization of Trk receptors must be precisely regulated to exert appropriate neurotrophin responses (11).

In the PNS, retrograde transport of endocytosed neurotrophin–Trk complex at the axon terminal has been demonstrated to be important for mediating the retrograde survival signaling from the axon terminal to the cell body (5,12–16). Thus, there is intimate relationship between Trk signaling and trafficking.

Engagement of Trk receptors results in the interaction with several cytoplasmic proteins involved in signaling and trafficking. Upon ligand binding to Trk, several adaptor proteins, including Shc, are recruited to the receptor and link to the activation of downstream kinases, such as Erk1/2 (extracellular signal-regulated kinases 1 and 2), PI3-kinase, and Akt. Erk1/2 are key kinases to mediate neurotrophin-induced neuronal survival and differentiation (17). The phosphatidylinositol 3-kinase (PI3-K) pathway plays important roles in retrograde survival signaling (18). In addition to shc, the juxtamembrane region of Trk has been shown to interact with a dynein light chain, Tctex-1 (19), and the PDZ-containing GIPC protein (20), cellular proteins that have been implicated in trafficking of Trk receptors.

Upon neurotrophin binding, Trk receptors undergo endocytosis that requires dynamin GTPase activity (21). Ligand-dependent Trk endocytosis is mediated by both clathrin-dependent and-independent mechanisms, including pinocytotic pathways (22). The internalized neurotrophin–Trk complex is proposed to be sorted to an endosomal compartment and then enter recycling, retrograde transport, or degradation pathways (23). Clearly, identification of membrane compartments carrying Trk is important to understand the regulation and the components of Trk trafficking vesicles. Although several studies have analyzed intracellular localization of the Trk receptors by immunohistochemical or immunoelectron microscopic analysis, there have been few comparative studies using biochemical approaches. In particular, studies of Trk vesicular trafficking in CNS neurons have lagged behind PNS neurons. Here, we report biochemical fractionation of different Trk-containing membrane compartments from primary CNS neurons and PC12 cells using differential and density gradient centrifugation. Neuronal membrane fractions enriched with activated Trk receptors after BDNF treatment were also isolated. These biochemical approaches have been able to define Trk-containing intracellular membranes that may represent different neurotrophin receptor signaling modules.

EXPERIMENTAL PROCEDURE

Materials. NGF and BDNF were obtained from Harlan (Indianapolis, IN) and Peprotech (Rocky Hill, NJ), respectively. Polyclonal anti-pan Trk (C-14), polyclonal anti-Erk1, and polyclonal anti-Erk2 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against EEA1 (early endosomal antigen 1), clathrin heavy chain, dynamin, and Akt1/3 were purchased from BD Biosciences (San Diego, CA). Monoclonal anti-phospho-Erk1/2, polyclonal anti-phospho-Akt (pSer473), and polyclonal anti-phospho-Trk (pY490) were purchased from Cell Signaling Technology (Beverly, MA). Polyclonal anti-GIPC (981) was generated by immunization with a GST-fused mouse GIPC C-terminal polypeptide (amino acids 226–333) and was immunoaffinity purified using the antigen-binding column. Monoclonal anti-PSD-95 was from Upstate Biotechnology (Lake Placid, NY). Monoclonal anti-synaptophysin (clone SVP-38) was purchased from Sigma-Aldrich. Monoclonal anti-dynein 74 kD intermediate chain was from Chemicon International Inc (Temecula, CA). Polyclonal anti-ribophorin was obtained from Gert Kreibich (New York University School of Medicine, New York, NY). Polyclonal anti-ARMS (ankyrin repeat-rich membrane spanning) (892) against the C-terminus of this protein was generated and described before (24). Polyclonal antibody against 14-kDa dynein light chain (Tctex-1), R5205, was obtained from S. King (University of Connecticut Health Center, Farmington, CT).

Cell Cultures. PC12 (615) cells stably overexpressing TrkA (25) are maintained in DMEM with GlutaMAX (Invitrogen-Gibco) containing 10% heat-inactivated horse serum, 5% fetal bovine serum with 30 U/ml penicillin, 30 μg/ml streptomycin, and 200 μg/ml G418. Rat primary cortical neuron cultures were prepared from embryonic day 18 (E18) Spraque-Dawley fetuses and maintained in serum-free Neurobasal medium plus B27 supplement (Invitrogen-Gibco) as previously described (26).

Subcellular Fractionation. Fractionation of PC12 (615) cells was performed with modification of methods described previously (27). PC12 (615) cells were serum-starved for one day and treated with or without 50 ng/ml NGF for 30 min. The cells were then homogenized using a Dounce homogenizer and passed through a 27G needle in Buffer H (250 mM sucrose, 20 mM Tricine–NaOH [pH 7.8], 1 mM EDTA, and 2 mM $MgCl_2$ containing protease and phosphatase inhibitors). The homogenate was subjected to sequential centrifugation at 800 × *g*, 20,000 × *g*, and 200,000 × *g*, as shown in the diagram in Fig. 4a. The resultant membrane fractions, P2 and P3, were suspended respectively in Buffer H with 0% iodixanol (OptiPrep™), 5,5′-[(2-hydroxy-1–3 propanediyl)-bis(acetylamino)] bis[*N*,*N*′-bis(2,3dihydroxypropyl-2,4,6-triiodo-1,3-benzenecarboxamide], (Accurate, Westbury, NY), and were loaded on the top of the discontinuous iodixanol gradient

(5, 10, 15, 20, 25% in Buffer H). Gradients were spun at 4°C either in an SW40Ti rotor (Beckman, Fullerton, CA) at 27,000 rpm (approximately 100,000 × *g*) for 18 h or in a TLS55 rotor (Beckman) at 38,000 rpm (approximately 100,000 × *g*) for 5 h depending on the scale of experiments. After the gradient centrifugation, membrane fractions were collected from the top of the gradient, and equal volumes of the fractions were analyzed by SDS-PAGE and immunoblotting. Immunoreactive proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

For the subcellular fractionation of neurons, primary cultured cortical neurons (DIV10–11) were homogenized with a Dounce homogenizer in Buffer H. Membrane fractions were prepared by differential centrifugation, 800 × *g*, 16,000 × *g*, and 200,000 × *g*, as described previously (11). The fractionation scheme is depicted in Fig 1a. The resultant fractions, P2, P2 + P3, and S1, were then subjected to flotation analysis on iodixanol step gradients. Each fraction was adjusted to 25% iodixanol respectively and overlaid with 20%, 15%, 10%, and 5% iodixanol in Buffer H. Gradients were centrifuged with the same condition as described above for PC12 (615) cells. After the gradient centrifugation, membrane fractions were collected from the top of the gradient, and equal volumes of the fractions were analyzed by SDS-PAGE and immunoblotting. In the case of limiting quantities of neuronal membranes, the TLS55 rotor was used. Homogenates from cortical primary neurons from one 10-cm dish (approximately 5×10^6 cells) were required to detect proteins by western blotting after density gradient separation of P2 with the TLS55 rotor.

RESULTS

Subcellular Fractionation of Trk Receptors from Primary Neurons

To characterize the Trk-containing membranes in primary cultured neurons, we performed biochemical subcellular fractionation, which allowed for separation of Trk receptors from different membranes. Rat primary cortical neurons were grown for 10 days *in vitro* (DIV) and then subjected to homogenization followed by differential centrifugation (Fig. 1a). The pellet from the first centrifugation (800 × *g*), P1, contained unbroken cells, nucleus, and large plasma membrane sheets (28). All other intracellular membrane compartments, including organelles, vesicles, and cytosolic proteins, were present in the S1 supernatant or the postnuclear fraction. The S1 fraction was further separated into P2 and S2 by the centrifugation at 16,000 × *g* (Fig. 1a). The resulting P2 pellet represented organelle-enriched components and also contained some vesicles and possibly synaptosomes. The P3 pellet contained the majority of vesicles including synaptic vesicles and microsomes.

Each fraction from differential centrifugation was subjected to immunoblotting with anti-pan Trk antibody (Fig. 1b). As expected for integral membrane proteins, Trk receptors were present exclusively in pellet fractions (P2 and P3), not in the S3 cytosolic fraction, and were especially abundant in P2 over P3 fractions (lanes 4 and 6 in Fig. 1b). The profile of the endoplasmic reticulum (ER) resident protein, ribophorin, is shown as a control (Fig. 1b). To separate Trk-enriched membranes further from the P2 membrane fraction, discontinuous density gradient centrifugation using iodixanol (Optiprep) was utilized (Fig. 1c). The fractionation patterns of Trk receptors and other proteins including Trk-interacting proteins and organelle marker proteins were analyzed by western blotting (Fig. 1c). Trk were enriched in I_1 (5–10% iodixanol) interface and also present weakly in I_2 (10–15% iodixanol) and I_3 (15–20% iodixanol) interfaces.

A Trk-interacting protein, GIPC (20), was followed during the fractionation. GIPC was previously found to localize in vesicular and tubular membranous structures as well as clathrin-coated pits in non-neuronal cells (29). Here we found that GIPC was present in membrane as well as cytosolic fractions of primary neurons (Fig. 1c and data not shown). The fractionation profile of GIPC was very similar to that of Trk, suggesting that these two proteins are present in the similar membrane fraction. The GIPC protein does not posses any known membrane-binding motifs. Hence, GIPC may associate with membranes by interactions with another membrane-associated proteins, such as Trk receptors and other membrane proteins.

Another Trk-interacting protein, the Tctex-1 dynein light chain protein (19), partially co-fractionated with Trk. Tctex-1 is a component of the cytoplasmic dynein, the major microtubule-based motor. In neurons, the dynein motor is required for the vesicular transport of cargoes towards the minus-end of microtubules and accounts for the retrograde axonal transport of Trk receptors (30). Tctex-1 has been suggested to serve as an adaptor to link Trk receptors to the dynein motor machinery (19). An intermediate chain of dynein which binds directly to Tctex-1 (31,32), also showed a similar fractionation profile to Trk and Tctex-1 proteins.

Interestingly, Akt, one of downstream Ser/Thr kinases for Trk receptors, was also found in the P2 membrane fraction and was enriched in the fractions similar to Trk (I_1 interface) after the gradient centrifugation, suggesting that Akt is available for the Trk-mediated signaling in these membranes.

The I_1 interface also contains synaptic membrane compartments since synaptophysin, a presynaptic

vesicle protein, and a postsynaptic protein, PSD-95, were also recovered in this interface. Dynamin, an endocytic protein, was present abundantly in the I_1 interface. Clathrin showed a broad fractionation profile with an enrichment in the I_1 interface. In contrast, an ER resident protein, ribophorin, migrated to heavier density fractions where only low amounts of Trk were present, suggesting that Trk receptors are not highly enriched in ER membranes from primary neurons. The fractionation profile indicated that effective separation was achieved by this procedure. An early endosomal marker protein, EEA1, and a *cis*-Golgi marker protein, GM-130, were enriched in $I_1 + I_2$ and $I_2 + I_3$ fractions, respectively (data not shown; (11)). Taken together, these results showed that Trk receptors in different neuronal locations can be biochemically separated. In addition, the data indicated that Trk receptors and components of dynein were in the similar membrane compartments, together with GIPC, Akt, endocytic and synaptic marker proteins.

Neuronal Endocytic Compartments Containing Activated TrkB

Upon ligand binding, Trk undergoes dimerization, activation, and autophosphorylation on specific tyrosine residues. The ligand–Trk receptor complex is then internalized and sorted into intracellular compartments. The endocytotic mechanisms and fate of

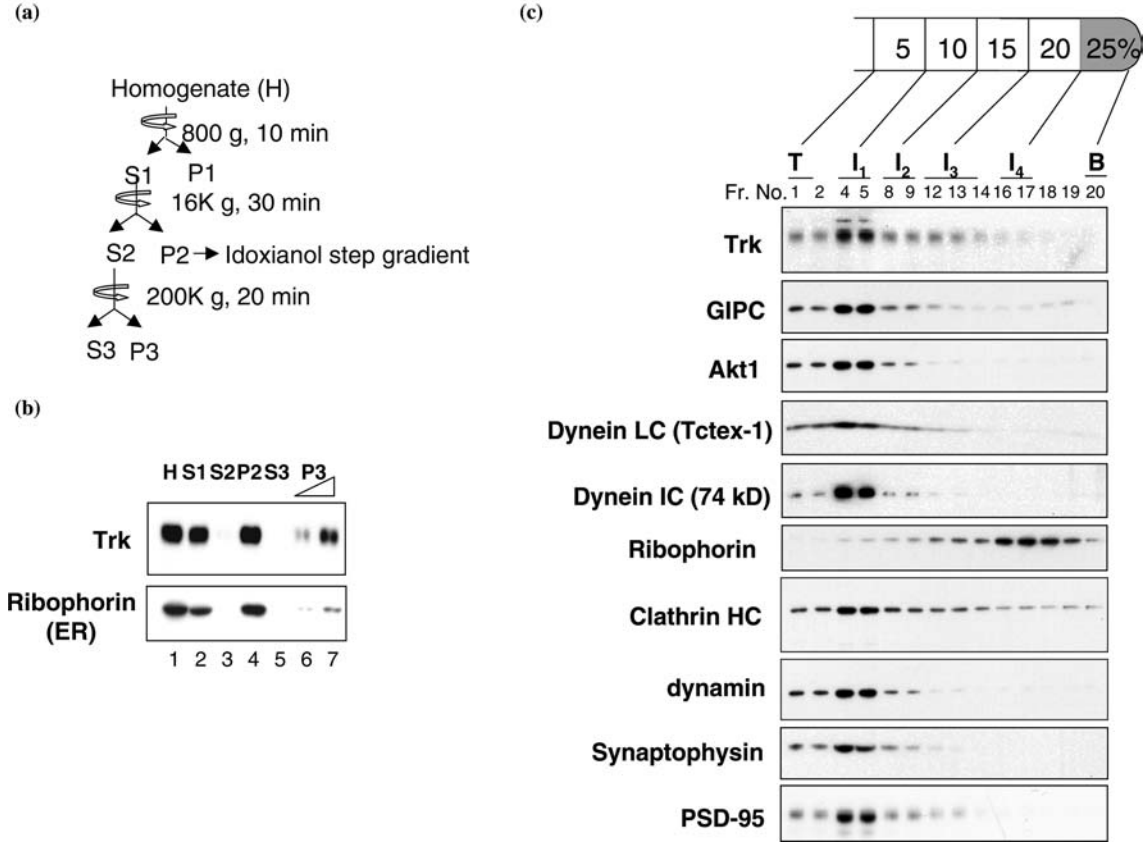

Fig. 1. Subcellular fractionation of the Trk neurotrophin receptors, associated proteins, and a downstream kinase in primary neurons. (a) Subcellular fractionation of primary cortical neurons (DIV 10) was performed using iodixanol density gradient centrifugation. Membrane (P2 and P3) and cytosolic (S3) fractions were prepared from the neuron homogenate by differential centrifugation. (b) Each fraction from approximately equal number of neurons (lanes 1–6), except for P3 in lane 7, was loaded on SDS-PAGE and immunoblotted for Trk and an ER resident protein, ribophorin. Compared to lane 6, approximately 3-fold more volume of P3 was loaded in lane 7 to visualize proteins in this fraction. H, homogenate; S, supernatant; P, pellet. (c) Trk-enriched P2 fraction was adjusted to 25% iodixanol and overlaid with 20%, 15%, 10%, and 5% iodixanol. After the flotation gradient centrifugation, total 20 fractions were collected from the top of the gradient. Equal volumes of indicated fractions including interfaces were analyzed by SDS-PAGE and sequential immunoblotting for the indicated proteins. Dynein LC, dynein light chain; dynein IC, dynein intermediate chain; clathrin HC, clathrin heavy chain; PSD-95, postsynaptic density 95. I_1–I_4, interfaces 5–10% (I_1), 10–15% (I_2), 15–20% (I_3), 20–25% (I_4); T, top; B, bottom; Fr. No., fraction number.

internalized Trk receptors in central neurons are not completely understood. To isolate membrane compartments responsible for the endocytic trafficking of activated Trk in neurons, we performed subcellular fractionation analysis. Cortical neurons, which express TrkB and TrkC, but not TrkA, were treated with BDNF, a specific ligand for TrkB, for various times as indicated in Fig. 2. The neurons were then homogenized and subjected to differential centrifugation ($800 \times g$ and $200{,}000 \times g$) to obtain all intracellular membrane pools (P2 + P3). The membrane fraction was further separated by iodixanol density gradient centrifugation and analyzed by sequential western blotting with anti-Trk and anti-phospho-Trk antibodies (Fig. 2).

Trk receptors were abundant in the I_1 interface and they became enriched in I_2 and I_3 interface after BDNF treatment for 30 min (left panel, Fig. 2). The ligand-dependent increase of Trk in these fractions peaked at 30 min after BDNF treatment and was back to nearly basal level in 4 h. Correspondingly, phosphorylated Trk was increased in the I_2 and I_3 interfaces, and peaked at 30 min. Interestingly, Trk in the I_1 interface was weakly phosphorylated after BDNF treatment (30 min–4 h). After 4 h of BDNF treatment, phospho-Trk was still found in I_2 and I_3 interfaces in spite of no obvious increase in total amount of Trk in these fractions.

These results indicated that I_2 and I_3 interfaces contain endocytic membrane compartments where Trk is directed after BDNF treatment. This fractionation protocol provides the first biochemical isolation of endocytic compartments from primary central neurons that contain phosphorylated, activated Trk. Further studies, including ultrastructural analysis of these fractions and immuno-isolation of phospho-Trk containing membrane compartments, will be required for the identification of intracellular membrane compartments containing active Trk receptors.

Neuronal Membranes Enriched with Erk1/2 and Akt

The Akt/protein kinase B family of serine/threonine kinases is a critical mediator of cell survival in response to trophic factor stimulation and Ca^{2+}-influx (33). Our data indicated that Akt was present in intracellular membrane fractions in which Trk were also present (Fig. 1c). We asked if Akt was activated upon BDNF binding to Trk in these membrane fractions. We treated primary cortical neurons (DIV 10) with BDNF for 40 min and prepared the postnuclear fraction (S1) from the homogenate by low-speed centrifugation ($800 \times g$). The S1 fraction was further separated by iodixanol flotation gradient centrifugation and was subjected to sequential immunoblotting with antibodies against total Akt and active Akt phosphorylated at Ser473 (Fig. 3a). Consistent with the results in Fig. 1c, Akt was present in the I_1 membrane compartments from both non-treated and BDNF-treated cortical neurons (Fig. 3a). Phosphorylated Akt was detected in I_1 membrane fractions after BDNF treatment and also in the cytosolic pool at the bottom of the gradient. Similar results were obtained for Erk1/2 (Fig. 3a). These results confirm that Akt and Erk1/2 phosphorylated upon TrkB activation were associated with membranes in the I_1 fractions. Phosphorylated forms of Akt and Erk1/2 were also in the bottom cytosolic pool (Fig. 3a).

To confirm the results in Fig. 3a, we performed immunoblotting analysis for each fraction prepared from differential centrifugation with anti-Akt and also anti-phospho-Akt antibodies. The ratio of phospho-Akt to total Akt was higher in the S3 cytosolic fraction than in the P2 plus P3 membrane fractions (see lanes 8 and 10 in Fig. 3b). These results suggest that phosphorylation of Akt and Erk are differentially regulated in membrane bound forms of the enzyme compared to the bulk found in the cytosol. Taken together, BDNF treatment results in the localization of activated Akt and Erk enzymes with Trk receptors in intracellular membrane pools.

Subcellular Fractionation of TrkA Receptors in PC12 Cells

To extend the fractionation analysis, we studied the behavior of TrkA in PC12 (615) cells, a transfected PC12 cell that stably overexpressed TrkA NGF receptors (25). PC12 cells have been widely used for NGF signaling studies (34). Both PC12 and PC12 (615) cells exhibit pronounced neurite outgrowth in response to NGF. Following a biochemical procedure shown in Fig. 4a, membrane fractions from the PC12 (615) cells treated with or without NGF for 30 min were first separated by differential centrifugation. As before, the P1 pellet contained unbroken cells, nucleus, and large plasma membrane sheets, whereas P2 and P3 represented intracellular membrane-enriched fractions (28).

The fractionation patterns of Trk receptors were followed along with organelle and vesicles marker proteins (Fig. 4b). PC12 (615) cells express two

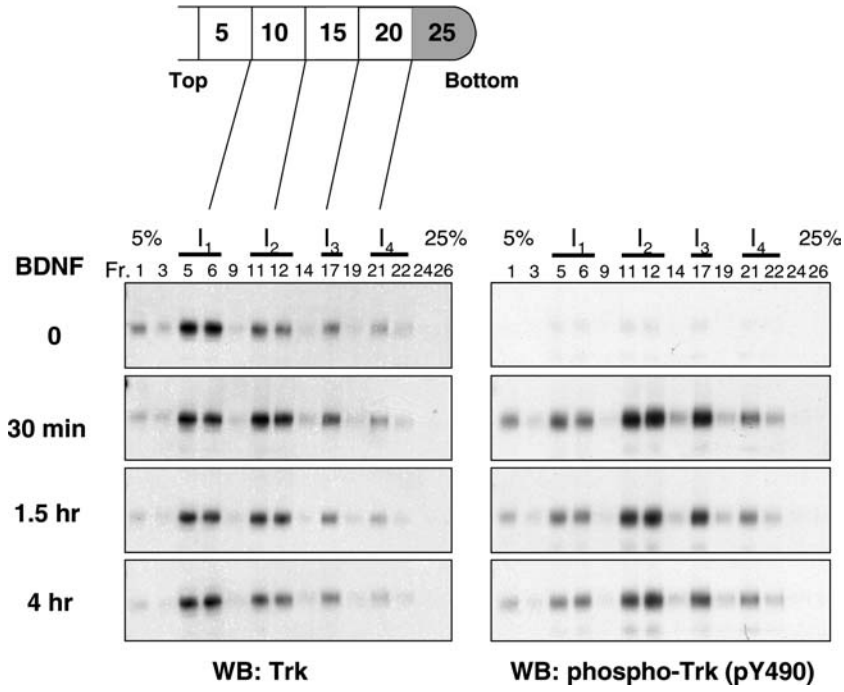

Fig. 2. Isolation of intracellular membrane compartments containing activated TrkB in primary neurons. Primary cortical neurons (DIV 11) were treated with 50 ng/ ml BDNF for indicated times. The cells from each condition were then homogenized and subjected to low-speed centrifugation. The resultant postnuclear fractions were centrifuged at 200,000 × *g* for 20 min to obtain total membrane fraction (P2 + P3). The membrane pellets were adjusted to 25% iodixanol and placed at the bottom of the iodixanol step gradient (5%, 10%, 15%, 20%, and 25%). After the flotation gradient centrifugation, a total of 26 fractions were collected from the top of the gradient. Equal volumes of the indicated fractions including interfaces were analyzed by SDS-PAGE and immunoblotting with anti-panTrk (C14) (left panels). The blots were then stripped and reprobed with anti-phospho-Trk (pY490) for activated TrkB (right panels). I_1–I_4, interfaces 5–10% (I_1), 10–15% (I_2), 15–20% (I_3), 20–25% (I_4); Fr., fraction. Upon BDNF treatment, augmentation of Trk in I_2 and I_3 fractions was observed with a peak at 30 min (left panels). These Trk receptors in I_2 and I_3 were highly tyrosine-phosphorylated upon BDNF treatment (right panels). By contrast, much weaker tyrosine phosphorylation was observed in Trk in the I_1 interface.

different TrkA species of 110 and 140 kDa which reflected immature and fully glycosylated forms of the receptor, respectively (35). TrkA existed exclusively in the membrane fractions and was found to be abundant in the P2 fraction (Fig. 4b), similar to the results obtained from primary neurons (Fig. 1b). Phosphorylated 140-kDa TrkA receptors were found in the membrane fractions after NGF treatment (Fig 4b).

Two k-interacting proteins, GIPC and ARMS (Kidins220), an ankyrin repeat-rich membrane spanning protein (24,36), were followed. ARMS represents an integral membrane protein which has been shown to associate with Trk receptors and to be a downstream substrate for the Trk receptor tyrosine kinase (24,37). Similar to TrkA, ARMS protein was enriched in the P2 fraction. Synaptophysin, a marker protein for synaptic-like vesicles, showed different profile from Trk and was enriched in P3, suggesting that effective separation of membrane compartments by this procedure. GIPC and endocytic marker proteins, clathrin and dynamin, were also present in membrane and cytosolic fractions.

The P2 and P3 fractions from NGF-treated and untreated PC12 (615) cells were further separated by iodixanol density-gradient centrifugation as described above. Each fraction from P2 and P3 gradient centrifugation were analyzed by immunoblotting for Trk as well as its associated proteins, organelle, endocytic, and secretory vesicle marker proteins (Fig. 4c). TrkA receptors in P2 were well separated after the gradient centrifugation. The 140-kDa form of TrkA was abundant in the I_3 interface. In contrast, the immature form of 110-kDa TrkA was enriched in the I_4 interface. Upon NGF treatment for 30 min, TrkA in internal membrane fractions (P2) was increased (Figs. 4b and c). The 140-kDa TrkA species, but not the 110-kDa spices, was preferentially

phosphorylated after NGF treatment (Figs 4b and c), indicating that the 140-kDa TrkA receptors are internalized from the cell surface upon ligand binding. Interestingly, the TrkA fractionation pattern was very different from that of primary cortical neurons since Trk receptors from neuronal membranes were highly enriched in the I_1 interface (Fig. 1c). Moreover, the Trk-interacting molecules, GIPC and ARMS, were also present in I_3 and I_4 fractions of P2 density gradient together with TrkA (Fig. 4c), suggesting that these proteins are localized in similar membrane compartments in PC12 (615) cells.

Besides TrkA, almost all proteins examined here, except ribophorin, showed a different fractionation pattern than cortical neurons (Fig. 1c). In contrast to the gradient profile for cortical neurons, many PC12 proteins were found in the heavier fractions I_3 and I_4, where an ER marker protein, ribophorin, was also present. Although TrkA in the P3 fraction was particularly enriched in the I_4 interface (20–25% iodixanol), GIPC and ARMS, together with endocytic proteins such as EEA1 and dynamin, also were found in the same fractions. The tyrosine-phosphorylated 140 kDa TrkA species in P3 was also detected in these fractions (Figs. 4b and c). PC12 cells differ from primary neurons in the distribution of axons and dendrites. Taken together, these results suggest that TrkA and its associated proteins are present in different types of membrane compartments in PC12 cells. Finally, these comparative fractionation results indicate PC12 cells display a different intracellular membrane organization than primary neurons.

DISCUSSION

Using a subcellular fractionation approach, we investigated the intracellular membrane compartments containing Trk receptors in primary cortical neurons as well as in the PC12 cell line. By this biochemical approach, we found that a large portion of Trk receptors was present in intracellular membrane compartments. This is consistent with the localization of intracellular Trk receptors in neurons in the central

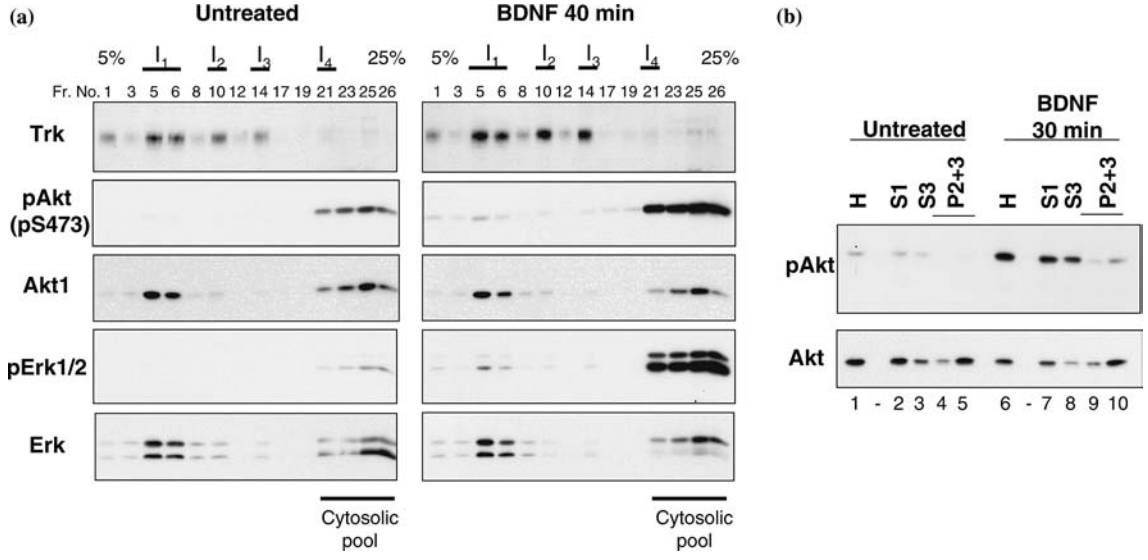

Fig. 3. Subcellular fractionation of Trk and its downstream kinases, Akt and Erk1/2. (a) Cortical neurons (DIV 10) were treated with or without 50 ng/ml BDNF for 40 min, homogenized, and centrifuged (800 × *g*, 10 min). The resultant S1 pellets (postnuclear fractions) were adjusted to 25% iodixanol and placed at the bottom of the iodixanol discontinuous gradient (5%, 10%, 15%, 20%, and 25%). After the density gradient centrifugation, total 26 factions were collected from the top of the gradient. Equal volumes of the indicated fractions including interfaces were analyzed by SDS-PAGE and sequential immunoblotting for Trk, phospho-Akt (phospho-Ser473), Akt, phospho-Erk1/2, and Erk1/2. I_1–I_4, interfaces 5–10% (I_1), 10–15% (I_2), 15–20% (I_3), 20–25% (I_4); T, top; B, bottom. (b) Cortical neurons (DIV 11) were treated with or without 50 ng/ml BDNF for 30 min and then homogenized. Indicated subcellular fractions were prepared from the homogenate by differential centrifugation as described in material and methods and in the diagram in Fig 1a. Each fraction from approximately equal number of neurons (lanes 1–4 and 6–9) was analyzed by immunoblotting with anti-phospho-Akt (pSer473). Compared to lanes 4 and 9, approximately 3-fold more volume of P2 + P3 was loaded in lanes 5 and 10, respectively, to visualize proteins in these fractions. The same blot was stripped and reprobed with anti-Akt to detect total Akt. After BDNF treatment, activated phospho-Akt was detected in the homogenate (H), postnuclear fraction (S1), soluble fraction (S3), and pellet (P2 + P3). Although Akt was present in both cytosolic and membrane fractions, phospho-Akt/total Akt ratio was higher in the cytosolic pool (S3) than in total membrane pools (P2 + P3).

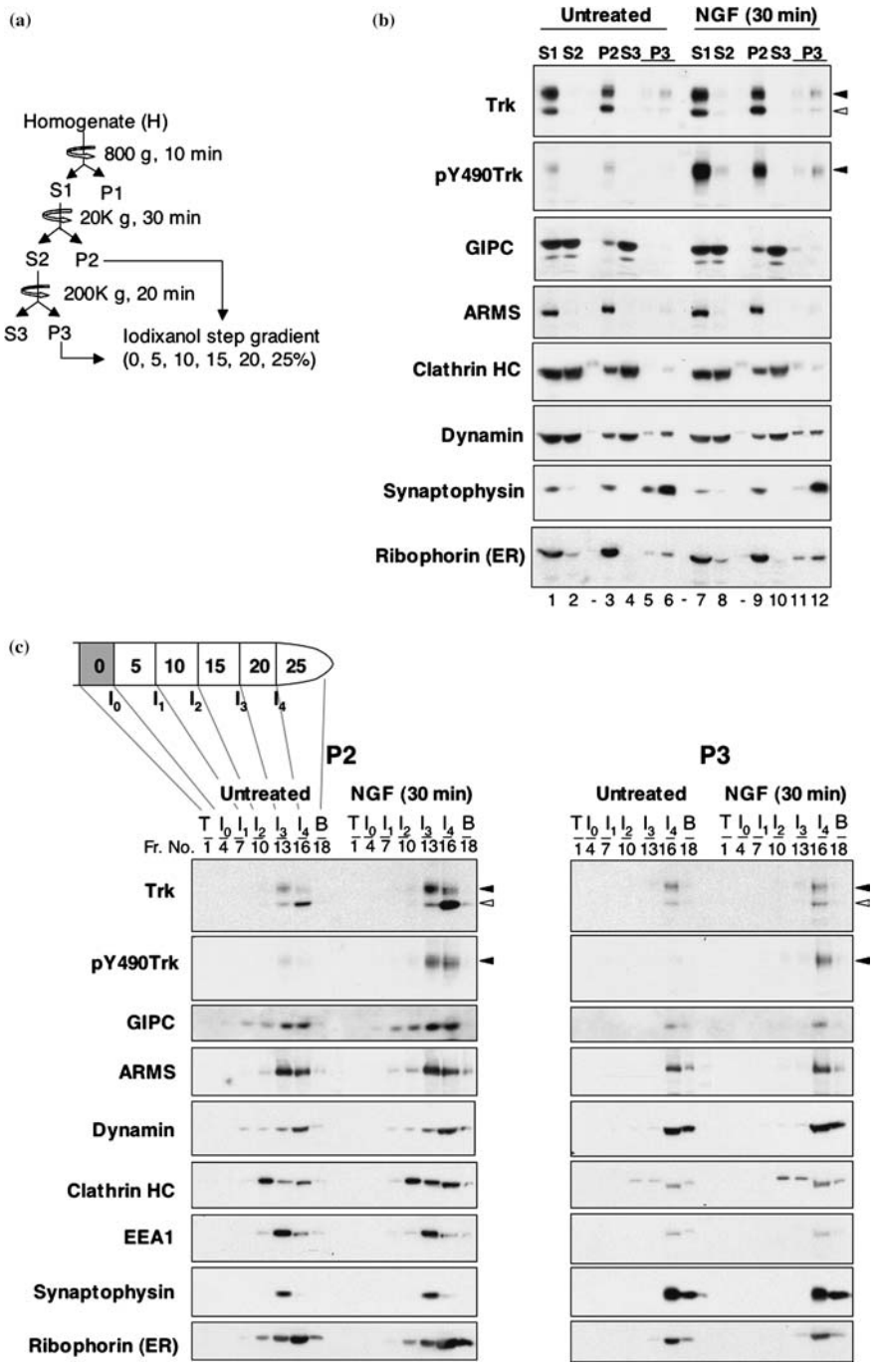

Fig. 4. Subcellular fractionation of the Trk neurotrophin receptors and associated proteins in PC12 (615) cells. PC12 cells stably overexpressing TrkA, were serum-starved for one day and treated with or without 50 ng/ml NGF for 30 min. (a) Cells were then homogenized and subjected to differential centrifugation. (b) Each fraction from approximately equal number of cells, except for P3 in lanes 6 and 12, was loaded on SDS-PAGE and immunoblotted for Trk, Trk-interacting proteins, GIPC and ARMS, and the indicated proteins including organelle marker proteins. Compared to lanes 5 and 11, 3-fold more volume of P3 was loaded in lanes 6 and 12, respectively, to visualize proteins in these fractions. H, homogenate; S, supernatant; P, pellet. Closed and open arrowheads refer to the 140- and 110-kDa TrkA, respectively. (c) P2 and P3 fractions were further separated by loading on the top of the discontinuous iodixanol gradient (5%, 10%, 15%, 20%, and 25%). After gradient centrifugation, equal volumes of total 18 fractions were collected from the top of the gradient, and equal volumes of indicated interface fractions were analyzed by SDS-PAGE and immunoblotting for the indicated proteins. EEA1, early endosomal antigen 1; clathrin HC, clathrin heavy chain; ribophorin, an ER resident protein. I_0–I_4, interfaces 0–5% (I_0), 5–10% (I_1), 10–15% (I_2), 15–20% (I_3), 20–25% (I_4); T, top; B, bottom; Fr. No., fraction number. Closed and open arrowheads refer to the 140- and 110-kDa TrkA, respectively. Approximately 4-fold more cells were used for the gradient centrifugation of P3 than that of P2.

nervous system (8). In peripheral neurons such as sympathetic neurons, it is well established that Trk receptors undergo transport and are found in discrete neuronal locations (13,14). In our experiments, Trk receptors in different membranes could be separated and co-fractionated with other proteins including known Trk-interacting proteins and downstream signaling molecules. These results suggest that molecules involved in Trk receptor signaling or trafficking are localized in similar membrane compartments and reside in the vicinity of Trk receptors.

The intracellular membranous localization of phosphorylated Trk receptors in primary CNS neurons has been poorly understood because of a lack of reliable anti-phosphospecific TrkB and TrkC antibodies for immunocytochemistry. Biochemical fractionation has allowed for the detection of membranes enriched in BDNF-stimulated TrkB receptors that are phosphorylated and activated in primary cortical neurons. This represents the first biochemical isolation of BDNF-responsive TrkB receptors in intracellular membranes from CNS neurons. Biochemical separation of these membrane fractions by immuno-membrane isolation followed by ultrastructural analysis will lead to more precise identification of active Trk-bearing membrane compartments. Also, the combination of these techniques will reveal additional proteins that act functionally during Trk transport.

For these subcellular fractionation experiments, iodixanol was used due to its properties of isotonicity and low viscosity. These features are ideal to separate vesicles and organelles that are osmotically sensitive. Iodixanol also permits the quick separation of membranes with different densities. Colocalization of regulatory and signaling proteins by fractionation has been previously documented for membrane proteins, such as the amyloid precursor protein (38). The localization of Trk receptors in early endosome membranes from sciatic nerve has been verified by membrane fractionation (16). In this study, retrogradely transported Trk was found in heavier fractions in Optiprep gradients than we found with TrkB from cortical neurons. This implies that retrograde axonal transport involves a discrete set of membranes and proteins. Another conclusion from these studies is the capability of internalized Trk receptors to signal in a productive manner in segregated compartments. Activation of intracellular Trk receptors is associated with the recruitment of many adaptor proteins and substrates for phosphorylation (16,18,27).

Activation of Trk receptor tyrosine kinases has also been observed by transactivation through G-protein-coupled receptor (GPCR) in intracellular locations in the absence of cognate neurotrophins (39,40). This is exemplified by the recent findings that GPCR ligand adenosine is capable of activating Trk receptors in intracellular membranes, such as Golgi structures, and not at the cell surface (41). These observations confirm that intracellular Trk receptors are found in discrete organelles (42–44), where they are fully active and promote survival and differentiation signals, such as those transmitted through activated Akt or Erk enzymes. These combined observations indicate that Trk receptors function not only at the cell surface but can be found to discrete intracellular locations. Trafficking of neurotrophin receptors in different membrane compartments, therefore, plays a decisive role in regulating a variety of fundamental signaling pathways in neurons.

ACKNOWLEDGMENTS

We thank S. King (University of Connecticut Health Center, Farmington, CT) and G. Kreibich (New York University School of Medicine, New York, NY) for providing antibodies against dynein light chain (Tctex-1) and ribophorin, respectively. This research was supported by grants the NIH.

REFERENCES

1. Huang, E. J. and Reichardt, L. F. 2001. Neurotrophins: Roles in neuronal development and function. Annu. Rev. Neurosci. 24:677–736.
2. Chao, M. V. 2003. Neurotrophins and their receptors: A convergence point for many signaling pathways. Nat. Rev. Neurosci. 4:299–309.
3. Poo, M.-M. 2001. Neurotrophins as synaptic modulators. Nat. Rev. Neurosci. 2:24–32.
4. Heerssen, H. M. and Segal, R. A. 2002. Location, location, location: A spatial view of neurotrophin signal transduction. Trends Neurosci. 25:160–165.
5. Ginty, D. D. and Segal, R. A. 2002. Retrograde neurotrophin signaling: Trk-ing along the axon. Curr. Opin. Neurobiol. 12:268–274.
6. Lu, B. 2003. BDNF and activity-dependent synaptic modulation. Learn. Mem. 10:86–98.
7. Fryer, R. H., Kaplan, D. R., Feinstein, S. C., Radeke, M. J., Grayson, D. R., and Kromer, L. F. 1996. Developmental and mature expression of full-length and truncated TrkB receptors in the rat forebrain. J. Comp. Neurol. 374:21–40.
8. Drake, C. T., Milner, T. A., and Patterson, S. L. 1999. Ultrastructural localization of full-length trkB immunoreac-

tivity in rat hippocampus suggests multiple roles in modulating activity-dependent synaptic plasticity. J. Neurosci. 19:8009–8026.
9. Yan, Q., Radeke, M. J., Matheson, C. R., Talvenheimo, J., Welcher, A. A., and Feinstein, S. C. 1997. Immunocytochemical localization of TrkB in the central nervous system of the adult rat. J. Comp. Neurol. 378:135–157.
10. Lessmann, V. 1998. Neurotrophin-dependent modulation of glutamatergic synaptic transmission in the mammalian CNS. Gen. Pharmacol. 31:667–674.
11. Yano, H. and Chao, M. V. 2004. Mechanisms of neurotrophin receptor vesicular transport. J. Neurobiol. 58:244–257.
12. Watson, F. L., Heerssen, H. M., Bhattacharyya, A., Klesse, L., Lin, M. Z., and Segal, R. A. 2001. Neurotrophins use the Erk5 pathway to mediate a retrograde survival response. Nat. Neurosci. 4:981–988.
13. Tsui-Pierchala, B. A. and Ginty, D. D. 1999. Characterization of an NGF-P-TrkA retrograde-signaling complex and age-dependent regulation of TrkA phosphorylation in sympathetic neurons. J. Neurosci. 19:8207–8218.
14. Riccio, A., Pierchala, B. A., Ciarallo, C. L., and Ginty, D. D. 1997. An NGF-TrkA-mediated retrograde signal to transcription factor CREB in sympathetic neurons. Science 277:1097–1100.
15. Ye, H., Kuruvilla, R., Zweifel, L. S., and Ginty, D. D. 2003. Evidence in support of signaling endosome-based retrograde survival of sympathetic neurons. Neuron 39:57–68.
16. Delcroix, J.-D., Valletta, J. S., Wu, C., Hunt, S. J., Kowal, A. S., and Mobley, W. C. 2003. NGF signaling in sensory neurons: Evidence that early endosomes carry NGF retrograde signals. Neuron 39:69–84.
17. Huang, E. J. and Reichardt, L. F. 2003. Trk receptors: Roles in neuronal signal transduction. Annu. Rev. Biochem. 72:609–642.
18. Kuruvilla, R., Ye, H., and Ginty, D. D. 2000. Spatially and functionally distinct roles of PI3-K effector pathway during NGF signaling in sympathetic neurons. Neuron 27:499–512.
19. Yano, H., Lee, F. S., Kong, H., Chuang, J.-Z., Arevalo, J. C., Perez, P., Sung, C.-H., and Chao, M. V. 2001. Association of Trk neurotrophin receptors with components of the cytoplasmic dynein motor. J. Neurosci. 21:RC125 .
20. Lou, X., Yano, H., Lee, F., Chao, M. V., and Farquhar, M. G. 2001. GIPC and GAIP form a complex with TrkA: A putative link between G protein and receptor tyrosine kinase pathways. Mol. Biol. Cell 12:615–627.
21. Zhang, Y., Moheban, D. B., Conway, B. R., Bhattacharyya, A., and Segal, R. A. 2000. Cell surface Trk receptors mediate NGF-induced survival while internalized receptors regulate NGF-induced differentiation. J. Neurosci. 20:5671–5678.
22. Shao, Y., Akmentin, W., Toledo-Aral, J. J., Rosenbaum, J., Valdez, G., Cabot, J. B., Hilbush, B. S., and Halegoua, S. 2002. Pincher, a pinocytic chaperone for nerve growth factor/TrkA signaling endosomes. J. Cell. Biol. 157:679–691.
23. Howe, C. L. and Mobley, W. C. 2004. Signaling endosome hypothesis: A cellular mechanism for long distance communication. J. Neurobiol. 58:207–216.
24. Kong, H., Boulter, J., Weber, J. L., Lai, C., and Chao, M. V. 2001. An evolutionarily conserved transmembrane protein that is a novel downstream target of neurotrophin and ephrin receptors. J. Neurosci. 21:176–185.
25. Hempstead, B. L., Rabin, S. J., Kaplan, L., Reid, S., Parada, L. F., and Kaplan, D. R. 1992. Overexpression of the trk tyrosine kinase rapidly accelerates nerve growth factor-induced differentiation. Neuron 9:883–896.
26. Kim, A. H., Yano, H., Cho, H., Meyer, D., Monks, B., Margolis, B., Birnbaum, M. J., and Chao, M. V. 2002. Akt1 regulates a JNK scaffold during excitotoxic apoptosis. Neuron 35:697–709.
27. Wu, C., Lai, C. F., and Mobley, W. C. 2001. Nerve growth factor activates persistent Rap1 signaling in endosomes. J. Neurosci. 21:5406–5416.
28. Spector D. L., Goldman R. D., and Leinwand L. A. 1998. A laboratory manual, culture and biochemical analysis of cells. Pp. 34.1–34.9. Cold Spring Harbor Laboratory Press.
29. Lou, X., McQuistan, T., Orlando, R. A., and Farquhar, M. G. 2002. GAIP, GIPC and Gαi3 are concentrated in endocytic compartments of proximal tubule cells: Putative role in regulating megalin's function. J. Am. Soc. Nephrol. 13:918 927.
30. Heerssen, H. M., Pazyra, M. F., and Segal, R. A. 2004. Dynein motors transport activated Trks to promote survival of target-dependent neurons. Nat. Neurosci. 7:596–604.
31. Mok, Y. K., Lo, K. W., and Zhang, M. 2001. Structure of Tctex-1 and its interaction with cytoplasmic dynein intermediate chain. J. Biol. Chem. 276:14067–14074.
32. Tai, A. W., Chuang, J.-Z., and Sung, C.-H. 2001. Cytoplasmic dynein regulation by subunit heterogeneity and its role in apical transport. J. Cell. Biol. 153:1499–1509.
33. Datta, S. R., Brunet, A., and Greenberg, M. E. 1999. Cellular survival: A play in three Akts. Genes Dev. 13:2905–2927.
34. Kaplan, D. R. and Miller, F. D. 1997. Signal transduction by the neurotrophin receptors. Curr. Opin. Cell Biol. 9:213–221.
35. Jullien, J., Guili, V., Reichardt, L. F., and Rudkin, B. B. 2002. Molecular kinetics of nerve growth factor receptor trafficking and activation. J. Biol. Chem. 277:38700–38708.
36. Iglesias, T., Cabrera-Poch, N., Mitchell, M. P., Naven, T. J., Rozengurt, E., and Schiavo, G. 2000. Identification and cloning of Kidins220, a novel neuronal substrate of protein kinase D. J. Biol. Chem. 275:40048–40056.
37. Arevalo, J. C., Yano, H., Teng, K. K., and Chao, M. V. 2004. A unique pathway for sustained neurotrophin signaling through an ankyrin-rich membrane-spanning protein. EMBO J. 23:2358–2368.
38. Kamal, A., Almenar-Queralt, A., LeBlanc, J. F., Roberts, E. A., and Goldstein, L. S. 2001. Kinesin-mediated axonal transport of a membrane compartment containing beta-secretase and presenilin-1 requires APP. Nature 414:643–648.
39. Lee, F. S. and Chao, M. V. 2001. Activation of Trk neurotrophin receptors in the absence of neurotrophins. Proc. Natl. Acad. Sci. USA 98:3555–3560.
40. Lee, F. S., Rajagopal, R., and Chao, M. V. 2002. Distinctive features of Trk neurotrophin receptor transactivation by G protein-coupled receptors. Cytokine Growth Factor Rev. 13:11–17.
41. Rajagopal, R., Chen, Z. Y., Lee, F. S., and Chao, M. V. 2004. Transactivation of Trk neurotrophin receptors by G-protein coupled receptor ligands occurs on intracellular membranes. J. Neurosci. 24:6650–6658.
42. Grimes, M. L., Zhou, J., Beattie, E. C., Yuen, E. C., Hall, D. E., Valletta, J. S., Topp, K. S., LaVail, J. H., Bunnett, N. W.,

and Mobley, W. C. 1996. Endocytosis of activated TrkA: Evidence that nerve growth factor induces formation of signaling endosomes. J. Neurosci. 16:7950–7964.

43. Jullien, J., Guili, V., Derrington, E. A., Darlix, J. L., Reichardt, L. F., and Rudkin, B. B. 2003. Trafficking of TrkA-green fluorescent protein chimerae during nerve growth factor-induced differentiation. J. Biol. Chem 278:8706–8716.
44. Weible M. W. 2nd and Hendry I. A. 2004. What is the importance of multivesicular bodies in retrograde axonal transport *in vivo*? J. Neurobiol. 58:230–243.