Nerve growth factor (NGF) binding to its receptors TrkA and p75NTR enhances the survival, differentiation, and maintenance of neurons. Recent studies have suggested that NGF receptor activation may occur in caveolae or caveolae-like membranes (CLM). This is an intriguing possibility because caveolae have been shown to contain many of the signaling intermediates in the TrkA signaling cascade. To examine the membrane localization of TrkA and p75NTR, we isolated caveolae from 3T3-TrkA-p75 cells and CLM from PC12 cells. Immunoblot analysis showed that TrkA and p75NTR were enriched about 13- and 25-fold, respectively, in caveolae and CLM. Binding and cross-linking studies demonstrated that the NGF binding to both TrkA and p75NTR was considerably enriched in CLM and that about 90% of high affinity binding to TrkA was present in CLM. When PC12 cells were treated with NGF, virtually all activated (i.e. tyrosine phosphorylated) TrkA was found in the CLM. Remarkably, in NGF-treated cells, it was only in CLM that activated TrkA was coimmunoprecipitated with phosphorylated Shc and PLCγ. These results document a signaling role for TrkA in CLM and suggest that both TrkA and p75NTR signaling are initiated from these membranes.

The neurotrophins make up a family of structurally related polypeptide neurotrophic factors that bind to specific receptors to enhance the survival, differentiation, and maintenance of neurons in both the central and peripheral nervous system (1–4). Nerve growth factor (NGF)1 is the best characterized neurotrophin, and much is known about NGF structure and actions (4–7). However, important questions remain with respect to the signaling properties and membrane trafficking of NGF receptors.

Two distinct classes of binding sites differ in their affinity for NGF by 2 orders of magnitude (8, 9). Low affinity (10⁻⁹ M) and high affinity (10⁻¹¹ M) NGF receptors are distinguished kinetically by a much slower rate of dissociation from the high affinity sites (9–12). Many NGF actions in neurons appear to be mediated by high affinity receptors (9). NGF binds to two receptor proteins, TrkA and p75NTR (13). TrkA is a receptor tyrosine kinase whose activation accounts for many of the classical neurotrophic properties of NGF. NGF binding induces TrkA dimerization, kinase activation, and receptor tyrosine autophosphorylation (14–18). Dissociation of NGF from TrkA is slow (19), suggesting that TrkA contributes to the formation of high affinity receptors. However, TrkA alone is unlikely to account for high affinity NGF binding because most binding to TrkA in cells expressing only this receptor is of low affinity (20). p75NTR is a single-transmembrane glycoprotein; it is a member of the TNF receptor superfamily (21). p75NTR binds with low affinity to NGF and other members of the neurotrophin family (22). p75NTR appears to signal independently of TrkA as well as to regulate TrkA signaling (21, 23–27). The latter may be due, in part, to direct interaction of p75NTR with TrkA. p75NTR has been shown to increase the rate of association of NGF with TrkA, thereby increasing TrkA activation and the number of high affinity binding sites (20, 28). Moreover, there is increasing evidence from photobleaching studies (29), copatching experiments (30), cross-linking (31), and co-immunoprecipitation studies (32–34) to suggest that p75NTR and TrkA interact directly in surface membranes.

Caveolae are specialized membrane microdomains that in many cells exist as vesicular invaginations of the plasma membrane (35–37). Enriched in cholesterol, sphingolipids, and the ganglioside GM1, caveolae contain structural proteins of the caveolin family (36, 38). In cell homogenates, caveolae sediment with a low buoyant density (36, 39). Membranes of similar composition and properties have been isolated from cells that do not contain caveolin; in these cells these membranes have been referred to as CLMs (40). Wu et al. (40) showed that TrkB and p75NTR were highly enriched in membranes of low buoyant density prepared from the synaptic plasma membranes of rat forebrain. p75NTR was also enriched in caveolae in p75NTR-transfected 3T3 cells and in CLMs in PC12 cells (41). In addition, many of the intermediates in the TrkA signaling cascade are also present in caveolae or CLMs, including phosphatidylinositol 3-kinase, phospholipase C-γ1, MAPK, mitogen-activated protein kinase; CLM, caveolae-like membranes; NCM, noncaveolae-like membranes; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; PDGF, platelet-derived growth factor; IL, interleukin.

© 1999 by The American Society for Biochemistry and Molecular Biology, Inc.

From the Departments of Neurology and Neurological Sciences and Pediatrics and the Program in Neuroscience, Stanford University, Stanford, California 94305, the Department of Pathology and the Program in Neuroscience, University of California, San Francisco, California 94143, and the University of Utrecht Medical School, 3584 CX Utrecht, The Netherlands

Chin-shiou Huang‡§‡, Jie Zhou‡§, Andrew K. Feng‡, Casey C. Lynch‡ ¶, Judith Klumperman‡‡, Stephen J. DeArmond‡, and William C. Mobley‡

(Received for publication, February 17, 1999, and in revised form, September 17, 1999)
receptors in CLMs, and that TrkA activation and signaling are initiated in these membranes.

**EXPERIMENTAL PROCEDURES**

**Materials**

RTA antibody, a polyclonal antibody raised against the extracellular domain of rat TrkA, was a gift of Dr. L. F. Reichardt (University of California, San Francisco) (30). Anti-phosphotyrosine, anti-phospholipase C-γ1, anti-EGFR, anti-TrkA ED, and anti-Shc antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). Anti-TRC (C terminus), anti-Ras, and anti-caveolin-1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The rabbit polyclonal antibody to caveolin-1 used for ultrastructural studies was obtained from Transduction Laboratories (Lexington, KY). Mouse NGF was prepared by ion-exchange chromatography as indicated (46). Unless specified, all other reagents were purchased from Sigma.

**Methods**

**Cell Lines and Cell Culture—**PC12 cells were maintained in Dulbecco's modified Eagle's medium (DME H-21) supplemented with 10% heat-inactivated horse serum and 5% fetal calf serum. 3T3-TrkA cells were established by transfecting NIH-3T3 cells with a rat trkA expression vector, pDM115 (a gift of Dr. M. V. Chao, New York University Medical Center) (46). To establish 3T3-p75 cells, the plasmid NGFR1 (a gift of Dr. E. M. Shooter, Stanford University) was cut with NcoI, blunt-ended, and digested with BglII. The resulting 1.5-kilobase pair DNA fragment, which contains the full-length open reading frame for p75NTR expression plasmid (pMHZ100) was used to transfect NIH-3T3 cells. 3T3 cells that co-express TrkA and p75NTR (3T3-TrkA-p75) were established by co-transfecting the cells with both pDM115 and pMHZ100. The parental 3T3 cells and their derivatives were grown in DME H-21 medium containing 10% bovine calf serum and appropriate antibiotics (500 μg/ml G418 or 200 μg/ml hygromycin or both). The cells were treated with NGF, or with vehicle alone, at 37°C for the time indicated. The treated cells were washed with ice-cold PBS.

**Immunoelectron Microscopy—**Cells were fixed for immunoelectron microscopy by adding to the culture medium an equal volume of freshly prepared 4% formaldehyde in 0.1 M phosphate buffer, pH 7.4. After 30 min at room temperature, the medium plus fixative was replaced by 4% formaldehyde, and cells were post-fixed for at least 24 h at 4°C. Fixed cells were washed three times with 0.15 M glucose in phosphate buffer, and then the cell monolayer was gently scraped from the dish in a small volume of 1% gelatin. Cells were established by transfecting NIH-3T3 cells with a rat p75NTR expression plasmid (pMHZ100) was used to transfect NIH-3T3 cells. 3T3 cells that co-express TrkA and p75NTR (3T3-TrkA-p75) were established by co-transfecting the cells with both pDM115 and pMHZ100. The parental 3T3 cells and their derivatives were grown in DME H-21 medium containing 10% bovine calf serum and appropriate antibiotics (500 μg/ml G418 or 200 μg/ml hygromycin or both). The cells were treated with NGF, or with vehicle alone, at 37°C for the time indicated. The treated cells were washed with ice-cold PBS.

**NGF Signaling**

**Caveolae and CLM Fraction—**Caveolae and CLM were prepared using a nondetergent extraction method essentially as described (41, 44). PC12 cells or 3T3 cells and their derivatives were grown to confluence in 15-cm tissue culture dishes; three dishes of cells were prepared using a nondetergent extraction method essentially as described (50). PC12 cells were incubated with 0.5 mg/ml of NHS-SS-Biotin (Pierce) in PBS at 4°C for 30 min. The reaction was terminated by washing with ice-cold PBS, followed by washing with ice-cold PBS, then incubated with 2 ml of binding buffer containing 1 mg/ml each of glucose and bovine serum albumin at 4°C for 30 min, followed by 1 ml of PBS. The bound proteins were solubilized in SDS-PAGE sample buffer.

**NGF Cross-linking—**NGF binding and cross-linking was carried out essentially as described (46). PC12 cells (105/15-cm dish) were washed with binding buffer (PBS containing 1 mg/ml each of glucose and bovine serum albumin) at 4°C for 10 min and then incubated with 2 ml of binding buffer containing 2 nM 125I-NGF for 1 h at 4°C. To measure binding to high affinity (slowly dissociating) receptors, cells were washed with ligand-free binding buffer for 1 h at 4°C before cross-linking. To correct for nonspecific binding and cross-linking, unlabeled NGF (1 μM) was included during binding and cross-linking. To cross-link NGF to TrkA, bis-sulfosuccinimidyl suberate was added to a final concentration of 0.8 mM in binding buffer. For cross-linking NGF to p75NTR, EDAC was used at a final concentration of 20 mM. Reaction mixtures were incubated at 4°C for 30 min, followed by washing cells with binding buffer. The cross-linking reaction was stopped by adding 10 mM of glutathione to the samples. The samples were then subjected to membrane fractionation as described above. The resulting fractions were treated as described, and the proteins were separated using 4–15% SDS-PAGE. Gels were then fixed, dried, and exposed to x-ray film XAR-5 (Eastman Kodak Co.). The film was scanned by a Umax super-Vista S-12 scanner, and the intensity of each protein band was measured using NIH image software.

**Surface Biotinylation—**Biotinylation of surface receptors was carried out as described (50). PC12 cells were treated with 0.5 mg/ml of NHS-SS-Biotin (Pierce) in PBS at 4°C for 30 min. The reaction was terminated by washing with ice-cold PBS, followed by washing with ice-cold PBS, then incubated with 2 ml of binding buffer containing 1 mg/ml each of glucose and bovine serum albumin at 4°C for 30 min, followed by 1 ml of PBS. The bound proteins were solubilized in SDS-PAGE sample buffer.

**RESULTS**

**Evidence for localization of p75NTR and TrkA in Caveolae in 3T3-TrkA-p75 Cells—**To show whether or not p75NTR and TrkA are present in caveolae, we used nondetergent extraction to isolate caveolae from 3T3-TrkA-p75 cells. Fourteen fractions (0.85 ml each) from the discontinuous sucrose gradient were collected and analyzed for distribution of p75NTR, TrkA, and caveolar markers including the proteins caveolin-1, Ras and the ganglioside GM-1. As expected from earlier studies, caveolin-1, Ras, and GM-1 were found almost exclusively in fractions 4–6 (Fig. 1). Immunoblot analysis showed that about 40% of TrkA and 60% of p75NTR were also present in fractions 4–6. The ratio of TrkA in caveolar to noncaveolar fractions was 0.6; the caveolar to noncaveolar ratio for p75NTR was 1.5. Because the amount of protein in these fractions accounts for only about 5% of total cellular protein, TrkA was enriched 13-fold in these membranes, whereas p75NTR was enriched 25-fold. The distribution of TrkA was similar in 3T3-TrkA cells (data not shown).

Using immunoprecipitation, some receptors and signaling proteins localized to caveolae can be shown to interact with caveolin-1. In agreement with an earlier study (37), p75NTR was found in anti-caveolin-1 immunoprecipitates from 3T3-TrkA-p75 cells (Fig. 2A). NGF treatment had no effect on the ability to immunoprecipitate p75NTR with caveolin, suggesting that NGF binding to p75NTR has no effect on the interaction of p75NTR with caveolin-1. In the same experiments, we found no evidence for immunoprecipitation of TrkA with anti-caveo-
immunoblot analysis was performed with antibodies to p75NTR. The lysate was immunoprecipitated with an antibody to caveolin-1, and the immunoprecipitate was mixed with SDS sample buffer in the presence or absence of 20% glycerol. SDS-PAGE, and autoradiographed. The gel for lanes 1 and 2, B, TrkA is palmitoylated on cysteine. 3T3-TrkA cells were incubated with [3H]palmitic acid for 4 h. The cell lysate was immunoprecipitated with an antibody to caveolin-1, and immunoblot analysis was performed with antibodies to p75NTR (lanes 1 and 2) or TrkA (lanes 3 and 4). The gel for lanes 3 and 4 was exposed 60 times longer than the gel for lanes 1 and 2. B, TrkA is palmitoylated on cysteine. 3T3-TrkA cells were incubated with [3H]palmitic acid for 4 h. The cells were lysed and immunoprecipitated using antibody to TrkA. The immunoprecipitate was mixed with SDS sample buffer in the presence or absence of 20% β-mercaptoethanol (BME), separated by SDS-PAGE, and autoradiographed. C, TrkA phosphorylation was inhibited by filipin. 3T3-TrkA cells were pretreated with filipin (10 µg/ml) for 30 min and then treated with NGF (5 ng/ml) for 5 min. The cells were lysed, immunoprecipitated with an antibody to TrkA, and subjected to immunoblot analysis with an antibody to phosphotyrosine. D, treatment of filipin altered the distribution of TrkA. 3T3-TrkA cells that were untreated or treated with filipin as above, were extracted and fractionated as described under "Experimental Procedures." Blots were probed with an antibody to TrkA. Note the increased staining for TrkA in fractions 6–11.

Fig. 1. TrkA and p75NTR co-fractionate with proteins that are found in caveolae. 3T3-TrkA-p75 cells were extracted and fractionated as described under "Experimental Procedures." Fractions were collected and subjected to immunoblot analysis using the antibodies against the proteins indicated. GM1 was probed with a cholera toxin B-chain-peroxodase conjugate.

Fig. 2. Studies characterizing TrkA. A, TrkA does not coimmunoprecipitate with caveolin-1. 3T3-TrkA-p75 cells were either untreated (lane 1 and 3) or treated with NGF (50 ng/ml) (lanes 2 and 4). The cell lysate was immunoprecipitated with an antibody to caveolin-1, and immunoblot analysis was performed with antibodies to p75NTR (lanes 1 and 2) or TrkA (lanes 3 and 4). The gel for lanes 3 and 4 was exposed 60 times longer than the gel for lanes 1 and 2. B, TrkA is palmitoylated on cysteine. 3T3-TrkA cells were incubated with [3H]palmitic acid for 4 h. The cells were lysed and immunoprecipitated using antibody to TrkA. The immunoprecipitate was mixed with SDS sample buffer in the presence or absence of 20% β-mercaptoethanol (BME), separated by SDS-PAGE, and autoradiographed. C, TrkA phosphorylation was inhibited by filipin. 3T3-TrkA cells were pretreated with filipin (10 µg/ml) for 30 min and then treated with NGF (5 ng/ml) for 5 min. The cells were lysed, immunoprecipitated with an antibody to TrkA, and subjected to immunoblot analysis with an antibody to phosphotyrosine. D, treatment of filipin altered the distribution of TrkA. 3T3-TrkA cells that were untreated or treated with filipin as above, were extracted and fractionated as described under "Experimental Procedures." Blots were probed with an antibody to TrkA. Note the increased staining for TrkA in fractions 6–11.

lin-1 antibodies (Fig. 2A). This suggests that TrkA either fails to interact with caveolin-1 or does so with lesser affinity than p75NTR.

Palmitoylation marks many proteins present in caveolae (51, 52), and for some proteins this modification enhances their targeting to this locus (53). p75NTR was shown to be palmitoylated in an earlier study (51). To ask whether TrkA was also palmitoylated, we incubated 3T3-TrkA cells with [3H]palmitic acid, and, after lysis, determined whether the receptor was acylated. SDS-PAGE analysis of TrkA immunoprecipitates showed that TrkA was covalently linked to the fatty acid (Fig. 2B). This covalent bond was sensitive to 2-mercaptoethanol, indicating that like other palmitoylated proteins found in caveolae (51–53), palmitic acid is linked through a thioester bond to a cysteine residue(s) in TrkA. Palmitoylation of TrkA and p75NTR is consistent with their presence in caveolae.

Filipin binds cholesterol and disrupts caveolae (54). Filipin treatment has been shown to decrease PDGF-mediated activation of PDGF receptor, a receptor tyrosine kinase localized in caveolae, and to decrease signaling downstream from PDGF receptor (41). To show whether or not filipin would also reduce TrkA activation, 3T3-TrkA cells were treated with filipin (10 µg/ml) for 30 min prior to adding NGF (5 ng/ml). TrkA phosphorylation was decreased to about 63% (±6%) of the nonfilipin treated control (n = 5) (Fig. 2C). To ask whether altered TrkA signaling following filipin treatment was associated with a change in the distribution of the receptor, we examined gradients prepared from 3T3-TrkA cells treated with filipin under conditions identical to those used to examine signaling. Compared with untreated cells, TrkA was more widely distributed in filipin treated cells. There was an increase in TrkA in the heaviest CLM fraction (i.e. fraction number 6) and in fractions 7–11 (Fig. 2D). These results show that the distribution and signaling of TrkA receptors is influenced by a treatment that disrupts caveolae. They provide further support for the localization of TrkA in caveolae.

Isolation of Caveolae-like Membrane Domains in PC12 Cells;
Presence of TrkA and p75NTR.—Both differentiated and undifferentiated PC12 cells have been reported to express caveolin-1 (55, 59). Using a fractionation protocol that enriches for CLM, a small amount of caveolin was present in immunoprecipitates from undifferentiated PC 12 cells (59). We found very little evidence for caveolin-1 in the PC12 cells used for our experiments. Whereas by immunoprecipitation followed by Western blot we readily detected caveolin-1 in 3T3 cells, we were unable to do so in untreated PC12 cells or in PC12 cells treated with NGF for 4 days (Fig. 3D). We also carried out immunoelectron microscopy analysis. Membrane invaginations characteristic of caveolae were seen in both 3T3-TrkA and 3T3 cells (Fig. 3A and B). In both, we found immunolabeling of caveolin-1 in these structures. However, we could not show that caveolin-1 was present in PC12 cells (Fig. 3C) using conditions under which the protein was readily seen in 3T3 cells. We conclude that there is little, if any, caveolin-1 in the PC12 cells used in our experiments.

To determine whether or not the membrane localization of p75NTR and TrkA in PC12 cells is similar to that in 3T3 cells, PC12 cells were subjected to extraction and fractionation as described above. Aliquots (15 µl) of fractions were analyzed by SDS-PAGE and the proteins were visualized using Coomassie Blue staining. About 95% of cellular protein was found in immunoprecipitates from undifferentiated PC 12 cells (59). We found very little evidence for caveolin-1 in the PC12 cells used for our experiments. Whereas by immunoprecipitation followed by Western blot we readily detected caveolin-1 in 3T3 cells, we were unable to do so in untreated PC12 cells or in PC12 cells treated with NGF for 4 days (Fig. 3D). We also carried out immunoelectron microscopy analysis. Membrane invaginations characteristic of caveolae were seen in both 3T3-TrkA and 3T3 cells (Fig. 3A and B). In both, we found immunolabeling of caveolin-1 in these structures. However, we could not show that caveolin-1 was present in PC12 cells (Fig. 3C) using conditions under which the protein was readily seen in 3T3 cells. We conclude that there is little, if any, caveolin-1 in the PC12 cells used in our experiments.

To determine whether or not the membrane localization of p75NTR and TrkA in PC12 cells is similar to that in 3T3 cells, PC12 cells were subjected to extraction and fractionation as described above. Aliquots (15 µl) of fractions were analyzed by SDS-PAGE and the proteins were visualized using Coomassie Blue staining. About 95% of cellular protein was found in fractions 10–14 (Fig. 4A). Only 5% was present in fractions 4–6. Immunoblot analysis showed that most GM1 was in fractions 5 and 6.}

To show whether or not TrkA and p75NTR were present in CLM, an aliquot of each gradient fraction was analyzed using
antibodies to TrkA or p75\textsuperscript{NTR}. Bands of the correct molecular weight were detected for each receptor. Similar to 3T3-TrkA-p75 cells, about 48% of TrkA was present in CLM (Fig. 5A), giving a CLM to NCM TrkA ratio of \(-1.0\). TrkA was thus enriched 17-fold in these membranes. Also consistent with the findings in 3T3 cells, immunoblots showed that about 55% of total cellular p75\textsuperscript{NTR} was present in CLM (Fig. 5A), a CLM to NCM ratio of \(-1.0\). Because the preparation used to isolate CLMs yields both surface and internal cell membranes, we used biotinylation to mark surface TrkA and p75\textsuperscript{NTR} receptors. About 70% of cell surface TrkA was found in CLM (73% \(\pm\) 11%; \(n = 3\)); about 70% of surface p75\textsuperscript{NTR} was found in these fractions (66% \(\pm\) 7%; \(n = 2\)) (Fig. 5B). Thus, for both receptors, there was significant enrichment in CLM at the cell surface.

Binding of NGF to TrkA and p75\textsuperscript{NTR} in CLM—The enrichment of TrkA and p75\textsuperscript{NTR} in CLM suggested that NGF binding would also be enriched in this fraction. To test this, we examined NGF binding and cross-linking to surface receptors. PC12 cells were incubated with \(125^I\)-NGF (2 nm) at 0 °C for 1 h; this NGF concentration is sufficient to label both low affinity and high affinity receptors (46). After cross-linking, we prepared CLM and NCM. TrkA and p75\textsuperscript{NTR} were immunoprecipitated and analyzed by SDS-PAGE. A radioactive band consistent with the molecular weight of the TrkA-NGF complex was found in both CLM (Fig. 6A, lanes 1 and 2) and NCM (lanes 4 and 5). The band was not present when binding was carried out in the presence of excess unlabeled NGF (lanes 3 and 6), indicating that NGF bound specifically to TrkA in both membrane fractions. The TrkA-NGF band in CLM was about 3.6-fold more intense than that found in the NCM (Fig. 6, lanes 1 and 4). In some binding experiments, cells were washed with binding buffer for 1 h prior to cross-linking. Because the half-life for NGF dissociation from low affinity receptors on PC12 cells at
NGF Signaling

4 °C is about 5 min (56), only binding to high affinity receptors (i.e. slow dissociating) would be expected to remain. After washing, the intensity of the TrkA-NGF band in NCM decreased markedly (70%), whereas the intensity of the band in CLM decreased only slightly (8%). Accordingly, the ratio of binding in CLM versus NCM increased to about 10:1.

As for TrkA, the NGF-p75NTR band representing surface NGF binding to this receptor was more intense in CLM than in NCM (Fig. 6B, lanes 1 and 4); however, for p75NTR the ratio was only 2:1. Remarkably, NGF was found cross-linked to p75NTR under conditions that enriched for high affinity binding and, as for TrkA, there was more binding in CLM than NCM. The ratio of binding in CLM to NCM in washed cells was 2.6:1. These data show that NGF binding to p75NTR was increased in CLM. In summary, binding and cross-linking studies showed that NGF was bound to TrkA and p75NTR in CLM, that this binding consisted of both high and low affinity components, and that both high and low affinity receptors were enriched in CLM relative to NCM.

NGF-induced TrkA Phosphorylation in CLM—NGF receptor binding studies suggested that most NGF-induced TrkA activation would occur in CLM. To test this, CLM and NCM were isolated from NGF-treated and untreated PC12 cells. When probed with an antibody to phosphotyrosine, the immunoblot showed a prominent band of phosphorylated TrkA in the CLM at all time points following NGF treatment (Fig. 7, panel A; lanes 1–4). The level of TrkA phosphorylation was maximal at 5 min and decreased from the maximal by 15 min (decrease = 73%; n = 2). In contrast, there was little activated TrkA in NCM at any time point following treatment of NGF (Fig. 7, lanes 5–8). These data show that TrkA activation was markedly enhanced in CLM.

Persistence of TrkA and p75NTR in CLM Following NGF Treatment—We noted that tyrosine phosphorylation of TrkA persisted in CLM following NGF treatment. This pattern differed from that seen in earlier studies of EGFR following EGF treatment (42). Like TrkA, EGFR is concentrated in caveolae. However, in Rat-1 cells, activation of EGFR was shown to result in rapid movement of activated receptors out of these membranes. To demonstrate whether or not EGFR behaved similarly in PC12 cells, we treated PC12 cells with EGF (50 ng/ml). Following EGF addition for only 1 min, virtually all EGFR disappeared from CLMs; this was also true at 5 and 15 min (Fig. 8A). To ask whether or not TrkA and p75NTR levels changed with NGF treatment, CLM from NGF-treated and untreated PC12 cells were prepared and probed with antibodies to TrkA and p75NTR (Fig. 8B). The amount of p75NTR varied little (<15%, n = 3), indicating that the level of this receptor in CLM was not markedly changed by NGF treatment. TrkA levels in the CLM of the NGF treated cells were unchanged at 1 and 5 min. At 15 min TrkA decreased slightly (decrease = 30%; n = 3). The results for TrkA and p75NTR point to a clear difference between the trafficking of these receptors and EGFR following ligand addition.

Downstream Signaling from TrkA in CLM—Activation of TrkA results in recruitment and phosphorylation of the signaling intermediates Shc and PLCγ (50, 57). TrkA localization and activation in CLM suggested that Shc and PLCγ may be recruited to CLM in NGF-treated PC12 cells. To test this, CLM was isolated from NGF-treated and untreated PC12 cells. The CLM fractions were immunoprecipitated with anti-Shc or anti-PLCγ antibodies. Immunoblot analysis of Shc immunoprecipitates revealed a similar level of the 46- and 52-kDa forms of Shc in CLM and NCM. Increased phosphorylation of all Shc isoforms was present in both CLM and NCM fractions after NGF treatment (Fig. 9A). However, only in CLM did we find that tyrosine phosphorylated TrkA was present in Shc immunoprecipitates as determined by probing with antibodies against phosphotyrosine (Fig. 9A) and TrkA (data not shown). Also, TrkA protein was detected in Shc immunoprecipitates only from CLM and only from NGF-treated cells (data not shown). These data suggest that Shc was recruited to activated TrkA receptors located in CLM. They show that activated TrkA in CLM is directly linked to activated Shc.

The results for experiments examining anti-PLCγ immunoprecipitates are shown in Fig. 9B. PLCγ was found in both CLM and NCM of PC12 cells; most was located in NCM. This was true for both untreated and treated cells. Treatment with NGF resulted in an increase in tyrosine phosphorylated PLCγ in both the CLM and NCM. The diffuse band in lane 2 of the top panel of Fig. 9B (anti-pTyr) is actually a doublet and reflects the presence of both PLCγ and TrkA. Activated TrkA was present in PLCγ immunoprecipitates only following NGF treatment and only in CLM. Some nonphosphorylated TrkA was present in PLCγ immunoprecipitates of the CLM of untreated cells, but the amount increased with NGF treatment. Little if any TrkA was present in immunoprecipitates prepared from NCM. These results show that activated PLCγ and activated TrkA are found together in CLM.

DISCUSSION

We examined the plasma membrane locus of NGF receptors and of NGF signaling. Our findings showed that TrkA and p75NTR were enriched in caveolae and in caveolar-like plasma membranes. Significantly, NGF binding and signaling were highly concentrated in these membranes, and downstream signaling was initiated from them. NGF binding to receptors in surface membranes specialized for signal transduction may play an important role in NGF signaling.
antibodies to caveolin-1, a finding that points to an interaction between p75NTR and caveolin-1. TrkA co-sedimented in sucrose gradients with p75NTR and other proteins contained in caveolae and CLMs. Moreover, we demonstrated that filipin, which binds to cholesterol and disrupts caveolae, altered the distribution of TrkA and inhibited NGF signaling through TrkA in 3T3-TrkA cells. Finally, TrkA was shown to be palmitoylated, a feature that favors localization of some proteins in caveolae. That we did not find TrkA in caveolin-1 immunoprecipitates from 3T3-TrkA-p75 cells suggests either that TrkA fails to bind to caveolin-1 or does so with a lower affinity than does p75NTR. Perhaps favoring the latter interpretation, it is noteworthy that the proposed consensus sequence for caveolin binding (38, 58) is found in the intracellular domain for TrkA (human TrkA residues 705–715) and that similar sequences are found in TrkB and TrkC. Moreover, others have recently provided evidence to suggest that TrkA can interact with caveolin under certain conditions (59). Taken together, these findings give strong support to the view that TrkA and p75NTR are both present in caveolae and in CLMs.

Many proteins localized to caveolae have been found to interact with caveolin (38). Using both Western blotting and immunoelectron microscopy, we found that in comparison with 3T3 cells, PC12 cells contained little if any caveolin-1. Nevertheless, the distributions of TrkA, p75NTR, and other caveole markers in these two cell types were very similar. These observations raise the possibility that caveolin-1 plays no role in the localization of TrkA and p75NTR in caveolae and CLM. However, the possibility exists that even very low levels of caveolin-1 may be sufficient to cluster these proteins in caveole-like membranes. Two additional possibilities can be suggested to explain the localization of TrkA and p75NTR in caveolae and CLM. One is that both TrkA and p75NTR may bind to a caveolin-related protein that fails to interact with the antibodies to caveolin-1 that we used in these experiments. Another is that palmitoylation of these receptors could play a decisive role, as it does for other proteins (49). One example of such a protein is endothelial nitric-oxide synthetase. Palmitoylation of endothelial nitric-oxide synthetase is required for its targeting to caveolae (60). For Fyn, a member of the Src family, palmitoylation of a cysteine residue in the N terminus appears to be both necessary and sufficient for targeting to plasma membranes with certain of the characteristics of caveolae (61). It will be important to further define the determinants of caveolar trafficking for TrkA and p75NTR because they may have an important influence on NGF signaling. For example, in PC12 cells overexpressing caveolin-1, others have shown that caveolin binding to TrkA-inhibited NGF-mediated activation of TrkA (59). In another example, a p75NTR mutant in which the deletion of intracellular domain residues included C279, which is palmitoylated (51), resulted in elimination of high affinity NGF binding and failure of NGF cross-linking to TrkA (62). Moreover, Hantzopoulos and colleagues (63) presented evidence that the p75NTR juxtamembrane region, which includes C279, regulates TrkA activation. These findings raise the possibility that palmitoylation of p75NTR contributes to NGF binding and signaling. Whether palmitoylation is important for the trafficking and function of TrkA receptors must be addressed.

Our findings showed that p75NTR and TrkA initiate NGF signaling from caveolae and CLM membranes at the cell surface. This report and earlier studies show that p75NTR is enriched in caveolae. Importantly, in cells that contain true caveolae, p75NTR was immunoprecipitated with antibodies to caveolin-1, a finding that points to an interaction between p75NTR and caveolin-1. TrkA also appears to be present in caveolae. TrkA co-sedimented in sucrose gradients with p75NTR and other proteins contained in caveolae and CLMs. Moreover, we demonstrated that filipin, which binds to cholesterol and disrupts caveolae, altered the distribution of TrkA and inhibited NGF signaling through TrkA in 3T3-TrkA cells. Finally, TrkA was shown to be palmitoylated, a feature that favors localization of some proteins in caveolae. That we did not find TrkA in caveolin-1 immunoprecipitates from 3T3-TrkA-p75 cells suggests either that TrkA fails to bind to caveolin-1 or does so with a lower affinity than does p75NTR. Perhaps favoring the latter interpretation, it is noteworthy that the proposed consensus sequence for caveolin binding (38, 58) is found in the intracellular domain for TrkA (human TrkA residues 705–715) and that similar sequences are found in TrkB and TrkC. Moreover, others have recently provided evidence to suggest that TrkA can interact with caveolin under certain conditions (59). Taken together, these findings give strong support to the view that TrkA and p75NTR are both present in caveolae and in CLMs.

Our findings are consistent with the view that p75NTR and TrkA initiate NGF signaling from caveolae and CLM membranes at the cell surface. This report and earlier studies show that p75NTR is enriched in caveolae. Importantly, in cells that contain true caveolae, p75NTR was immunoprecipitated with antibodies to caveolin-1, a finding that points to an interaction between p75NTR and caveolin-1. TrkA also appears to be present in caveolae. TrkA co-sedimented in sucrose gradients with p75NTR and other proteins contained in caveolae and CLMs. Moreover, we demonstrated that filipin, which binds to cholesterol and disrupts caveolae, altered the distribution of TrkA and inhibited NGF signaling through TrkA in 3T3-TrkA cells. Finally, TrkA was shown to be palmitoylated, a feature that favors localization of some proteins in caveolae. That we did not find TrkA in caveolin-1 immunoprecipitates from 3T3-TrkA-p75 cells suggests either that TrkA fails to bind to caveolin-1 or does so with a lower affinity than does p75NTR. Perhaps favoring the latter interpretation, it is noteworthy that the proposed consensus sequence for caveolin binding (38, 58) is found in the intracellular domain for TrkA (human TrkA residues 705–715) and that similar sequences are found in TrkB and TrkC. Moreover, others have recently provided evidence to suggest that TrkA can interact with caveolin under certain conditions (59). Taken together, these findings give strong support to the view that TrkA and p75NTR are both present in caveolae and in CLMs.
It has also been suggested that p75NTR acts to concentrate NGF locally in the microenvironment of TrkA surface receptors (64). The increased concentration of p75NTR and TrkA receptors in CLM would facilitate this aspect of p75NTR function. The molecular basis for increased high affinity binding in CLM is yet to be defined. The most obvious possibility is that through increased concentration, TrkA and p75NTR bind NGF in dimeric or oligomeric complexes. Recent findings indicate that the interaction of p75NTR with TrkA results in enhanced affinity of NGF binding to TrkA (31). Increased interaction of p75NTR with TrkA in CLM may have contributed to increased high affinity binding to TrkA and to p75NTR in these membranes. TrkA-p75NTR hetero-oligomers may also have contributed to high affinity binding in the NCM. Interestingly, however, the ratio of high affinity to total binding in the NCM was greater for p75NTR than for TrkA. This result contrasts with that for CLM and is as yet unexplained. However, it does suggest that slowly dissociating receptors in NCM differed from those in CLM. Finally, it is possible that other molecules concentrated in CLM contribute to high affinity NGF binding. Such accessory molecules could either contribute directly to NGF binding or interact with TrkA and p75NTR to influence their binding affinity. For example, the interleukin-1 receptor accessory protein (IL-1R AcP) has been shown to exert its biological function by interacting with the IL-1 receptor 1 (IL-1R1) to stabilize the active IL-1 receptor-ligand complex (65, 66). In other studies, the IL-1R AcP was shown to increase the binding affinity of IL-1R1 for IL-1β (67). It is noteworthy that IL-1β has been shown to stimulate conversion of sphingomyelin to ceramide in membranes with the characteristics of caveolae (68). It will be important to examine further the possibility that p75NTR and TrkA interact with proteins whose functions resemble those of IL-1R AcP.

Consistent with the data for NGF binding and cross-linking, we found that NGF activated TrkA in CLM. It was to these membranes that Shc and PLCγ were apparently recruited for activation. Indeed, we found no evidence that Shc and PLCγ were bound to TrkA in NCM. Of note, the methods for producing CLM and NCM include the use of a high pH buffer during membrane preparation, a treatment that could disrupt interaction of TrkA and signaling intermediates. Thus, although we could demonstrate recruitment and binding of Shc and PLCγ to TrkA in CLM, we cannot rule out the possibility that our methods disrupted the interaction of these proteins with TrkA in NCM. Nevertheless, our data for NGF binding and TrkA signaling together with data for p75NTR signaling from earlier studies (41) points to CLM as an important and perhaps predominant locus for initiating NGF receptor activation.

A number of functions have been suggested for caveolae and CLMs. Considerable recent interest focuses on a role in signal transduction (35, 36). A number of signaling receptors and associated signaling intermediates are concentrated in these membranes. Indeed, caveolae have been shown to contain most of the intrinsic tyrosine kinase activity of the plasma membrane (45). EGFR, PDGF receptor, insulin receptor, and other receptors are localized in CLM (38). The enrichment in caveolae and CLM of these receptors together with proteins important for downstream signaling (i.e. Src family kinases, Ras, and the components of the MAPK pathway, phosphatidylinositol 3-kinase, PLCγ, and PKC) suggests the existence of clustered and preassembled signaling complexes (38). As for TrkA, ligand-mediated signaling through EGFR and PDGF receptor was shown to be highly enriched in caveolae (42, 43, 45). However, although for each of these receptor tyrosine kinase signaling is initially concentrated in these membranes, differences are apparent in their trafficking. For example, EGFR was depleted from CLM within 1 min of EGF treatment in our study, a result consistent with earlier studies in fibroblasts (43). Conversely, the levels of TrkA and p75NTR in CLM changed relatively little during NGF treatment for 15 min. At 5 min treatment, at which time there was no decrease in TrkA levels, one would expect there to have been internalization of an appreciable number of activated TrkA receptors (50); thus, it can be speculated that such receptors are either internalized in membranes derived from CLM or are sorted into membranes with similar physical properties. However, additional experiments are needed to address these possibilities. In preliminary studies, we have shown that a significant fraction of internalized radiolabeled NGF is present in membranes of light buoyant density.²

It is tempting to speculate that the difference in trafficking of EGFR and TrkA has physiological significance. Although these receptors engage many of the same downstream signaling pathways, they induce very different biological responses in PC12 cells (69, 70). Treatment with NGF leads to neurite outgrowth, whereas treatment of EGF leads to cell proliferation. One quantitative difference in signaling is that NGF stimulation results in more prolonged MAPK activation than EGF (71, 72). This finding has led to the hypothesis that sustained activation of this pathway contributes to differentiation (70). It is possible that activated receptors in CLM have a unique influence on the extent and time course of MAPK activation. If so, our results showing that NGF produces prolonged TrkA phosphorylation in CLM may suggest how NGF increases the duration of MAPK activation with resulting neuronal differentiation. Consistent with the idea, overexpression of EGFR in PC12 cells resulted in EGF-dependent neurite outgrowth with sustained MAPK activation (73). It will be interesting to test the speculation that differences in EGFR and TrkA trafficking are important to the different actions of EGF and NGF and to ask whether p75NTR contributes to the persistence of TrkA signaling in CLM.

Acknowledgments—We thank Dr. Chengbiao Wu, Chuck L. Howe, and Janice S. Valletta for constructive comments. We acknowledge Viola Oorschot for preparing ultrathin cryosections.

REFERENCES
1. Levi-Montalcini, R. (1987) Science 237, 1154–1162
2. Thoenen, H. (1991) Trends Neurosci. 14, 165–170
3. Zang, L., Schmidt, R. E., Yan, Q., and Snider, W. D. (1994) J. Neurosci. 14, 5187–5201
4. Yuen, E. C., and Mobley, W. C. (1996) Ann. Neurol. 40, 346–354
5. Orlinick, J. R., and Chao, M. V. (1998) Cell Signal. 10, 543–551
6. Connor, B., and Dragunow, M. (1998) Brain Res. Brain Res. Rev. 27, 1–39
7. Longo, P. M., Holtzman, D. M., Grimes, M. L., and Mobley, W. C. (1993) in Neurotrophic Factors (Fallon, J., and Loughlin, S., eds) pp. 209–256, Academic Press, New York
8. Sutter, A., Riopelle, R. J., Harris-Warrick, R. M., and Shooter, E. M. (1979) J. Biol. Chem. 254, 5972–5982
9. Meakin, S. O., and Shooter, E. M. (1992) Trends Neurosci. 15, 323–331
10. Landreth, G. E., and Shooter, E. M. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 4751–4755
11. Schechter, A. L., and Bothwell, M. A. (1981) Cell 24, 867–874
12. Woodruff, N. R., and Neet, K. E. (1986) Biochemistry 25, 7956–7966
13. Kaplan, D. R., and Miller, F. D. (1997) Curr. Opin. Cell Biol. 9, 213–221
14. Kaplan, D. R., Martin-Zanca, D., and Parada, L. F. (1991) Nature 350, 158–160
15. Kaplan, D. R., Hempstead, B. L., Martin-Zanca, D., Chao, M. V., and Parada, L. F. (1991) Science 253, 554–558
16. Klein, R., Jing, S. Q., Nanduri, V., O’Rourke, E., and Barbacid, M. (1991) Cell 65, 189–197
17. Meakin, S. O., and Shooter, E. M. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 5862–5866
18. Jing, S., Tapley, P., and Barbacid, M. (1992) Neuron 9, 1067–1079
19. Meakin, S. O., Stutes, U., Drinkwater, C. C., Welch, A. A., and Shooter, E. M. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 2374–2378
20. Mahadeo, D., Kaplan, L., Chao, M. V., and Hempstead, B. L. (1994) J. Biol. Chem. 269, 6884–6891
21. Carter, B. D., and Lewin, G. R. (1997) Neuron 18, 187–190

² C. S. Huang, J. Valletta, C. Howe, and W. Mobley, unpublished observations.
