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GIPC and GAIP Form a Complex with TrkA: A Putative Link between G Protein and Receptor Tyrosine Kinase Pathways

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NGF initiates the majority of its neurotrophic effects by promoting the activation of the tyrosine kinase receptor TrkA. Here we describe a novel interaction between TrkA and GIPC, a PDZ domain protein. GIPC binds to the juxtamembrane region of TrkA through its PDZ domain. The PDZ domain of GIPC also interacts with GAIP, an RGS (regulators of G protein signaling) protein. GIPC and GAIP are components of a G protein-coupled signaling complex thought to be involved in vesicular trafficking. In transfected HEK 293T cells GIPC, GAIP, and TrkA form a coprecipitable protein complex. Both TrkA and GAIP bind to the PDZ domain of GIPC, but their binding sites within the PDZ domain are different. The association of endogenous GIPC with the TrkA receptor was confirmed by coimmunoprecipitation in PC12 (615) cells stably expressing TrkA. By immunofluorescence GIPC colocalizes with phosphorylated TrkA receptors in retrograde transport vesicles located in the neurites and cell bodies of differentiated PC12 (615) cells. These results suggest that GIPC, like other PDZ domain proteins, serves to cluster transmembrane receptors with signaling molecules. When GIPC is overexpressed in PC12 (615) cells, NGF-induced phosphorylation of mitogen-activated protein (MAP) kinase (Erk1/2) decreases; however, there is no effect on phosphorylation of Akt, phospholipase C-γ1, or Shc. The association of TrkA receptors with GIPC and GAIP plus the inhibition of MAP kinase by GIPC suggests that GIPC may provide a link between TrkA and G protein signaling pathways.

INTRODUCTION

Neuronal survival and differentiation depend on trophic effects provided by neurotrophins, of which the best characterized is NGF. NGF binds to TrkA receptors, which are responsible for mediating neuronal cell survival and differentiation, axonal guidance, dendritic branching, and synaptic transmission (McAllister *et al.*, 1999). As is the case with other receptor tyrosine kinases, binding of NGF to the TrkA receptor results in ligand-induced dimerization and autophosphorylation of the receptor on tyrosine residues followed by rapid association with phospholipase C (PLC)-γ1 (Kaplan and Miller, 1997) and adaptor proteins such as Shc, FRS2 (Kouhara *et al.*, 1997), and rAPS/SH2-B (Qian *et al.*, 1998), giving rise to downstream phosphorylation cascades (York *et al.*, 1998) that lead to activation of mitogen-activated protein (MAP) kinase (ras/Erk) and phosphoinositide 3 (PI3) kinase/protein kinase B (PKB) signaling pathways. The available evidence indicates that NGF binding is followed by rapid internalization of the receptors via clathrin-coated vesicles, delivery to endosomes (Grimes *et al.*, 1996, 1997), and sorting to retrograde transport vesicles for delivery to the cell body (Riccio *et al.*, 1997; Senger and Campenot, 1997; Ure and Campenot, 1997; Tsui-Pierchala and Ginty, 1999).

Although the generation of intracellular signals by Trk tyrosine kinase receptors has been intensively investigated, how the signaling and trafficking events are regulated is still not well understood. To obtain information on these points we carried out a yeast two-hybrid screen using the juxtamembrane domain of TrkB as bait and identified GIPC as an interacting protein of the TrkB receptor in a yeast two-hybrid screen.

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Abbreviations used: GAP, GTPase-activating protein; GPCR, G protein–coupled receptors; GST, glutathione *S*-transferase; MAP, mitogen-activated protein; PLC, phospholipase C; p-TrkA, phosphorylated TrkA; RTK, receptor tyrosine kinase.

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GIPC (GAIP-interacting protein, C terminus) is a PDZ domain protein that is linked to G protein signaling pathways as it binds to GAIP, an RGS (Regulator of G protein Signaling) protein (De Vries *et al.*, 1998b). RGS proteins serve as GTPase-activating proteins (GAPs) for the $G\alpha_i$ and $G\alpha_q$ subunits of heterotrimeric G proteins and turn off $G\alpha_i$- and $G\alpha_q$-mediated signaling by increasing Gα-bound GTP hydrolysis. GAIP is localized on clathrin-coated vesicles, and there are indications that it may be involved in modulating membrane trafficking (De Vries *et al.*, 1998a; Wylie *et al.*, 1999). GIPC, which binds to the C terminus of GAIP through its PDZ domain, is also found on intracellular vesicles (De Vries *et al.*, 1998a). In addition to GAIP, GIPC has also been found to interact with several transmembrane proteins, including the Glut-1 transporter (Bunn *et al.*, 1999), semaphorin-F (Wang *et al.*, 1999), neuropilin-1 (Cai and Reed, 1999), and the TAX viral protein (Rousset *et al.*, 1998).

In this paper we describe the interaction between the juxtamembrane region of the TrkA receptor and both overexpressed and endogenous GIPC. We also demonstrate that GIPC forms a complex with GAIP and the TrkA receptor and colocalizes with phosphoryated TrkA in retrograde transport vesicles. We further show that overexpression of GIPC inhibits MAP kinase (ras/Erk) activation by NGF. Our findings suggest that GIPC may serve to cluster TrkA receptors and signaling molecules, thereby providing a putative link between NGF tyrosine kinase receptors and G protein–mediated signaling pathways.

MATERIALS AND METHODS

Cell Culture

HEK 293T cells were grown in DMEM containing 10% FBS supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine. PC12 (615) cells (Hempstead *et al.* 1992) stably overexpressing TrkA were maintained in DME containing 10% FBS and 5% heat-inactivated horse serum with 30 U/ml penicillin, 30 μg/ml streptomycin, 2 mM glutamine, and 200 μg/ml G418. For inducing neurite outgrowth, PC12 (615) cells were seeded in culture dishes or on coverslips that had been precoated with rat-tail collagen (Collaborative Biomedical, Bedford, MA). NGF (2.5 S; Boehringer Mannheim) was added to the medium (50 ng/ml) to allow neurites to grow out. For testing the interaction between TrkA and endogenous GIPC, PC12 (615) cells were cultured overnight in DME containing 1% FBS and 0.5% heat-inactivated horse serum and then treated with 100 ng/ml NGF in the same medium. For inhibition of TrkA tyrosine kinase activity, PC12 (615) cells were preincubated with 100 nM K252a (Calbiochem) for 30 min before NGF treatment. For examining Erk1/2, PKB/Akt, PLC-γ1, and Shc activation, PC12 (615) cells overexpressing GIPC or empty vector were cultured overnight in DMEM containing 1% FBS and 0.5% heat-inactivated horse serum and then were treated for 5 min with 100 ng/ml NGF in the same medium.

Antibodies

Anti-TrkA serum (RTA), which recognizes the extracellular domain of TrkA (Clary *et al.* 1994), was obtained from Dr. Louis Reichardt (University of California, San Francisco, San Francisco, CA). Anti-pan Trk rabbit serum (44) raised against the C-terminal region of the TrkA receptor was obtained from Dr. Barbara Hempstead (Cornell University, Cornell, NY). Affinity-purified mouse anti-pan Trk mouse IgG (B-3) and polyclonal rabbit anti-Trk IgG (C-14), raised against the highly conserved C-terminal region of TrkA, were purchased from Santa Cruz Biotechnology. Antibodies 44, B-3, and C-14 react broadly with TrkA, TrkB, and TrkC. Affinity-purified mouse mAb TrkA IgG1 (E-6) was purchased from Santa Cruz Biotechnology; we find it reacts primarily with Tyr-496–phosphorylated TrkA but also reacts weakly with the nonactivated form of TrkA by immunoblotting PC12 (615) cell lysates treated with or without NGF. Anti-phosphotyrosine mAbs PY99 and 4G10 were obtained from Santa Cruz Biotechnology and Dr. B. Rouot (INSERM U-431, Montpellier, France), respectively. Polyclonal anti-Erk (C-14) was purchased from Santa Cruz Biotechnology. Affinity-purified polyclonal anti-phospho-P44/42 MAP kinase (9101s), anti-phospho-Akt (Ser473), and anti-Akt (Ser473) antibodies were purchased from New England Biolabs. Affinity-purified polyclonal anti-Shc IgG and anti-PLC-γ1 IgG1 were purchased from Upstate Biotechnology. Polyclonal rabbit antiserum against full-length GIPC (De Vries *et al.*, 1998b) and the N terminus of GAIP (De Vries *et al.*, 1996) were prepared as described. Monoclonal anti-FLAG (M2) and the same antibody coupled to protein A beads were purchased from Sigma. MAb anti-lgp120 was obtained from Dr. Ira Mellman (Yale University, New Haven, CT), and affinity-purified rabbit anti-cathepsin D IgG was from Dr. Keitaro Kato (Kyushu University, Fukuoka, Japan).

Plasmid Construction

The bait plasmid pEG202-$TrkB_{458-544}$ was generated by PCR with rat TrkB cDNA as template. The mammalian expression plasmids pCMV5-rat TrkA and pCMV5-rat TrkB were generated by subcloning full-length rat TrkA and TrkB cDNAs into pCMV5 vector at the *Eco*RI site. Truncated mutants of the rat TrkA cytoplasmic domain were generated by subcloning PCR-amplified fragments into *Bam*HI- and *Sma*I-digested pCMV5-rat TrkA (Yano *et al.*, 2000). The resultant mutant constructs are pCMV5-rat-$TrkA_{1-522}$, $TrkA_{1-501}$, and $TrkA_{1-493}$. To generate pCMV5-$TrkA_{1-452}$, site-directed mutagenesis was performed by PCR with a primer containing a stop codon. $TrkA_{1-472}$, also called pcDNA3-$TrkA^{\Delta INT}$ (Gargano *et al.*, 1997), was a generous gift from Dr. Andrea Levi (Consiglio Nazionale delle Ricerche, Rome, Italy). The pGEX-4T-1-$TrkA_{448-552}$ construct encoding the 75-amino acid, juxtamembrane region of rat TrkA was generated by PCR followed by subcloning the PCR fragment into the pGEX-4T-1 vector (Pharmacia) at *Eco*RI and *Sal*I sites.

pcDNA3.1-mGIPC and pGEX-KG-mGIPC were generated by subcloning the mouse GIPC coding sequences into pCDNA3.1 or pGEX-KG (Pharmacia) at the *Bam*HI site. The C-terminal FLAG-tagged GIPC pcDNA3.1-mGIPC-FLAG was generated by PCR. The reverse primer encodes the FLAG sequences and three glycines added between GIPC and FLAG as a spacer. The pcDNA3-mGIPC(L142A/G143E)-FLAG was generated by overlapping extension PCR with the mutagenic forward and reverse primers followed by subcloning the PCR fragments into the pcDNA3 vector at *Bam*HI and *Eco*RV sites. The N-terminal FLAG-tagged GIPC vectors encoding full-length GIPC and its deletion mutants, $GIPC_{81-333}$, $GIPC_{125-333}$ (PDZ plus C terminus), $GIPC_{226-333}$ (C terminus), $GIPC_{1-124}$ (N terminus), and $GIPC_{125-225}$ (PDZ), were generated by PCR using the corresponding primer sets and mouse GIPC as a template. The forward primers contained Kozak-ATG followed by the FLAG-tag sequence. The PCR-amplified fragments were subcloned into pcDNA3 at *Bam*HI and *Xba*I sites. pCEP4-mGIPC-FLAG was generated by subcloning C terminus FLAG-tagged mouse GIPC (obtained by digestion of pcDNA3-mGIPC-FLAG with *Kpn*I and *Xho*I) into pCEP4 vector at *Kpn*I and *Xho*I sites. The PCR products were sequenced (Molecular Pathology Shared Resource, University of California, San Diego, La Jolla, CA). Primer sequences are available upon request.

Yeast Two-Hybrid Screening

Interaction screening in the yeast two-hybrid system was performed using the juxtamembrane region of rat $TrkB_{458-544}$ as bait in a rat postnatal, day-1 dorsal root ganglion cDNA library (M. Chou, un-

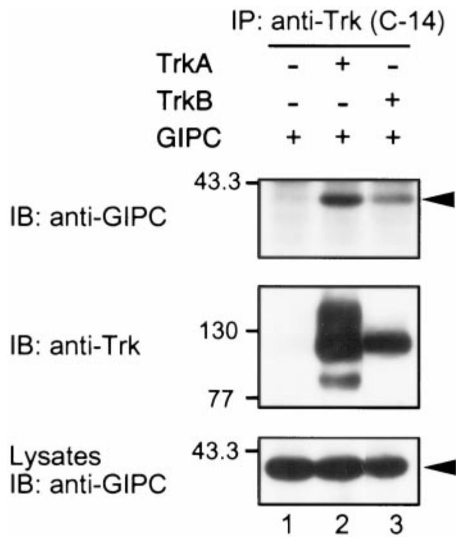

Figure 1. Coimmunoprecipitation of GIPC with TrkA and TrkB. Full-length GIPC and TrkA or TrkB were transiently coexpressed in HEK293T cells, followed by immunoprecipitation (IP) of cell lysates (50 μg) with affinity-purified anti-Trk (C-14) IgG and immunoblotting (IB) with anti-GIPC (top) and anti-Trk (44) serum (middle). GIPC coprecipitates with both TrkA (lane 2) and TrkB (lane 3). No GIPC is detected in immunoprecipitates from lysates of cells transfected with GIPC and an empty vector (lane 1). The protein expression level of GIPC is comparable in each lane (bottom).

published data) as described previously (Gyuris *et al.*, 1993). The bait plasmid and cDNA library were introduced sequentially into the yeast strain EGY48. Approximately 50 million transformants were analyzed. Selection was based on β-galactosidase activity and growth in the presence of galactose and the absence of leucine.

Preparation of Glutathione S*-Transferase (GST) Fusion Proteins and In Vitro Binding Assays*

GST-GIPC or GST-TrkA$_{448-552}$ (juxtamembrane region of rat TrkA) was prepared by transforming pGEX-KG-mGIPC or pGEX-4T-1-TrkA$_{448-552}$ into *Escherichia coli*, TOP10 strain, followed by induction with 0.5 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 37°C. GST fusion proteins were purified from bacterial lysates on glutathione-Sepharose 4B beads (Pharmacia). Full-length GIPC, GAIP, and various GIPC deletion mutants containing amino acids 81–333, 125–333 (PDZ plus C terminus), 226–333 (C terminus), 1–124 (N terminus), or 125–225 (PDZ) were in vitro transcribed/translated from the corresponding pcDNA3 constructs as described above using the TNT-coupled reticulocyte system (Promega). In vitro translated ^{35}S-labeled GIPC or GAIP proteins were incubated with GST-TrkA$_{448-552}$ or GST-GIPC immobilized on glutathione-agarose beads at 4°C for 2 h to overnight in TNE (10 mM Tris, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% NP-40). The beads were then washed extensively with TNE, and the bound proteins were separated by SDS-PAGE. The gel was soaked in Amplify (Amersham), dried, and exposed to x-ray film.

DNA Transfections and Preparation of Cell Lysates and Stable Cell Lines

HEK293T cells (1–2 × 10^6) plated in 10-cm plates were transfected by the calcium phosphate procedure. Cells were lysed 36 h after transfection by incubation for 30 min on ice in 1 ml of TNE containing protease inhibitors (0.12 mg/ml PMSF, 2 mg/ml leupeptin, 1 mg/ml aprotinin) and phosphatase inhibitors (10 mM NaF and 1 mM sodium orthovanadate). The insoluble fraction was removed by centrifugation at 1400 rpm for 20 min at 4°C. The protein concentration of the supernatant was determined by the Bio-Rad protein assay with BSA as a standard.

PC12 (615) cells overexpressing GIPC were obtained by transfection of cells with pCEP4-mGIPC-FLAG with Lipofectamin 2000 (Life Technologies-BRL). Colonies resistant to hygromycin were screened for FLAG expression by immunoblotting cell lysates with anti-FLAG antibody. Cell lines were maintained in medium containing 200 μg/ml hygromycin.

Metabolic Labeling and Immunoprecipitation

Cells were washed twice with methionine and cysteine-free DMEM, incubated for 4 h with 100 μCi/ml ^{35}S-Easy Tag Express protein-labeling mixture (>1000 Ci/mmol, DuPont-NEN) in the same medium, washed with cold PBS, and lysed as described above.

Cell lysates (3–4 mg) were incubated overnight at 4°C with primary antibody followed by incubation with protein A-Sepharose (Sigma) for an additional 2 h at 4°C, except for the anti-FLAG (M2), which was already conjugated to agarose. The beads were washed extensively with RIPA buffer and used for in vitro binding assays or washed in TNE buffer and boiled in SDS-sample buffer, and the proteins were separated by SDS-PAGE.

Immunoblotting

Cell lysates or immune complexes were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore). After the sample was blocked with TBST buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 0.05% Tween-20) containing either 5% BSA or 5–10% nonfat milk, membranes were incubated with primary antibody for 1–2 h at room temperature or overnight at 4°C, followed by incubation for 45 min with HRP-conjugated goat anti-rabbit or goat anti-mouse IgG (Jackson) and detection by enhanced chemiluminescence (ECL; Amersham).

Immunocytochemistry

For immunofluorescence, PC12 (615) cells grown on coverslips were fixed with methanol for 5 min at −20°C, incubated in 10% goat serum in PBS for 20 min, and incubated with primary antibodies for 1 h and goat anti-rabbit Alexa 594 or goat anti-mouse Alexa 488 IgG (Molecular Probes) for 1 h. Cells were examined with an MRC-1000 laser scanning confocal microscope (Bio-Rad) equipped with a krypton/argon laser. No staining was evident when primary antibodies were excluded. Images were observed with a 60× oil immersion objective on an Optiphot (Nikon) inverted microscope. The iris setting was 3.0, and the zoom setting was 2–3. Images were processed using Adobe Photoshop 6.0 (Adobe Systems, Mountain View, CA).

RESULTS

Yeast Two-Hybrid Screening

In an attempt to identify proteins involved in the regulation or signaling of Trk receptors, a yeast two-hybrid screen of a postnatal rat dorsal root ganglion cDNA library was performed with the juxtamembrane region (amino acids 458–544) of TrkB as bait. Seventy-five clones were analyzed after selection based on growth in the absence of leucine and β-galactosidase activity. Of these, seven were identified as GIPC (De Vries *et al.*, 1998b). GIPC is a 333-amino acid, PDZ protein identified by its ability to interact with the C terminus of GAIP, a GAP for Gα_i subunits of heterotrimeric G

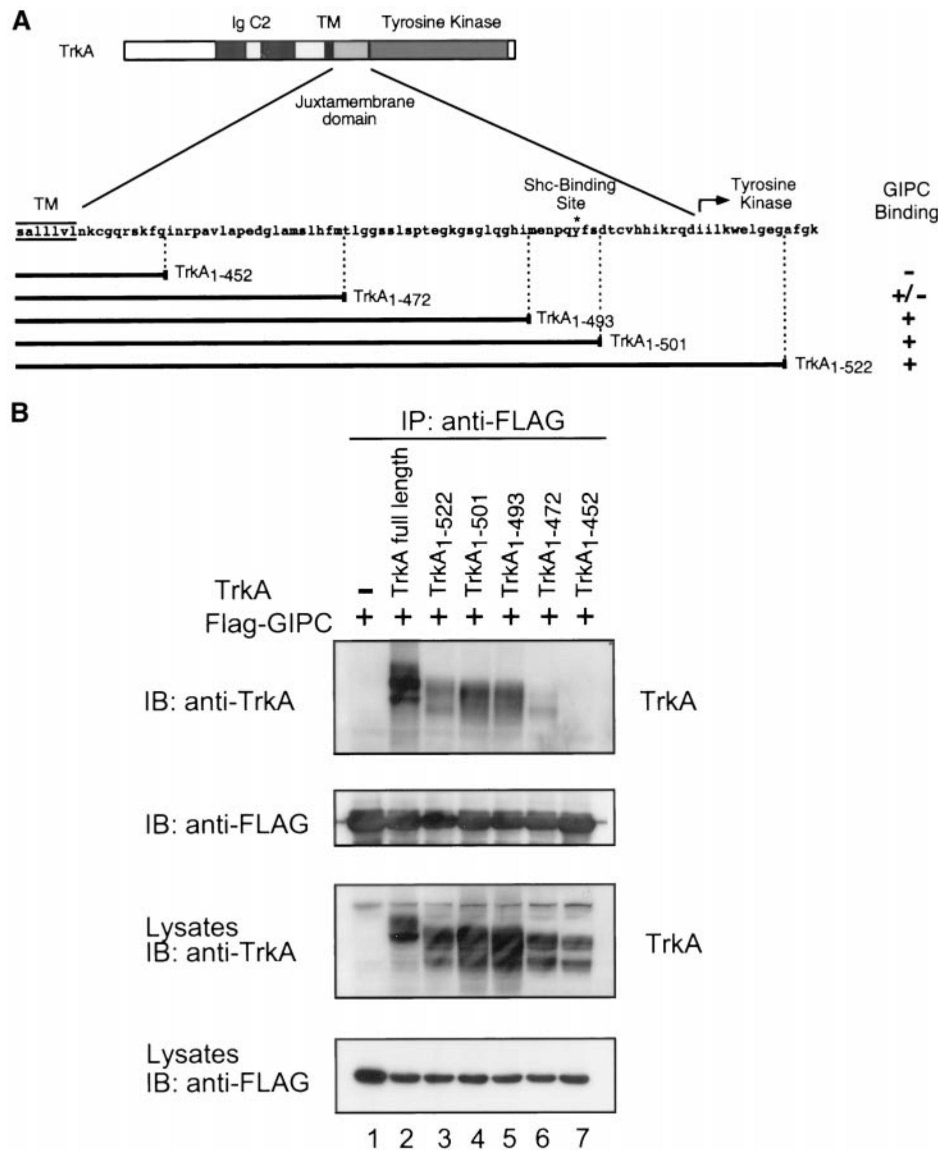

Figure 2. Mapping the GIPC-interacting region in TrkA. (A) Schematic representation of the structure of TrkA and the juxtamembrane domain deletion mutants. The interaction between GIPC and TrkA mutants shown on the right is based on data from B. (+, interaction; −, no interaction; TM, transmembrane region; IgC2, Ig-like C2-type domains.). (B) Coimmunoprecipitation of GIPC with TrkA deletion mutants. Top panel, full-length TrkA, $TrkA_{1-522}$, $TrkA_{1-501}$, and $TrkA_{1-493}$ coprecipitate with GIPC-FLAG (lanes 2–5), but no interaction is observed with $TrkA_{1-472}$ or $TrkA_{1-452}$ (lanes 6 and 7). Second panel, the amount of GIPC-FLAG precipitate is comparable in all lanes. FLAG-tagged GIPC was transiently coexpressed in HEK293T cells with full-length (lane 2) or TrkA deletion mutants (lanes 3–7) or FLAG-GIPC alone (lane 1). Cell lysates were immunoprecipitated (IP) with anti-FLAG followed by immunoblotting (IB) with anti-TrkA RTA (top panel) or anti-FLAG (second panel). Protein expression levels of TrkA mutants (third panel) and FLAG-GIPC (bottom panel) in 50 μg of cell lysate are comparable.

proteins. The shortest clone obtained from the screening encodes amino acids 81–333 of rat GIPC, indicating that $GIPC_{81-333}$ contains the binding site for the juxtamembrane region of TrkB. Specificity tests in yeast confirmed the interaction of GIPC with the juxtamembrane regions of both TrkA and TrkB but not with other proteins, such as laminin or bicoid.

Interaction of GIPC with Trk Receptors in HEK293T Cells

The ability of GIPC to interact with TrkB was also examined by immunoprecipitation after cotransfection of HEK293T cells with GIPC and TrkB. HEK293T cells do not express endogenous Trk receptors. When lysates prepared from cells expressing full-length GIPC and TrkB or GIPC alone were immunoprecipitated with anti-Trk (C-14) IgG, GIPC coprecipitated with TrkB as determined by immunoblotting (Figure 1, lane 3). Because the juxtamembrane regions of TrkA and TrkB share 56% amino acid homology, we also tested the ability of GIPC to interact with TrkA in HEK293 cells similarly cotransfected with GIPC and TrkA. We found that GIPC also coprecipitated with TrkA (Figure 1, lane 2). TrkA is seen as three bands, 140, 110, and 90 kDa, which represent differentially glycosylated forms of the protein (Watson *et al.*, 1999). These results confirm the interaction between GIPC and TrkB and suggest that GIPC can interact with both TrkA and TrkB receptors.

Mapping the GIPC-interacting Region in TrkA

To determine the GIPC-interacting site within the juxtamembrane region of TrkA, a number of deletion mutants were generated (Figure 2A), coexpressed with

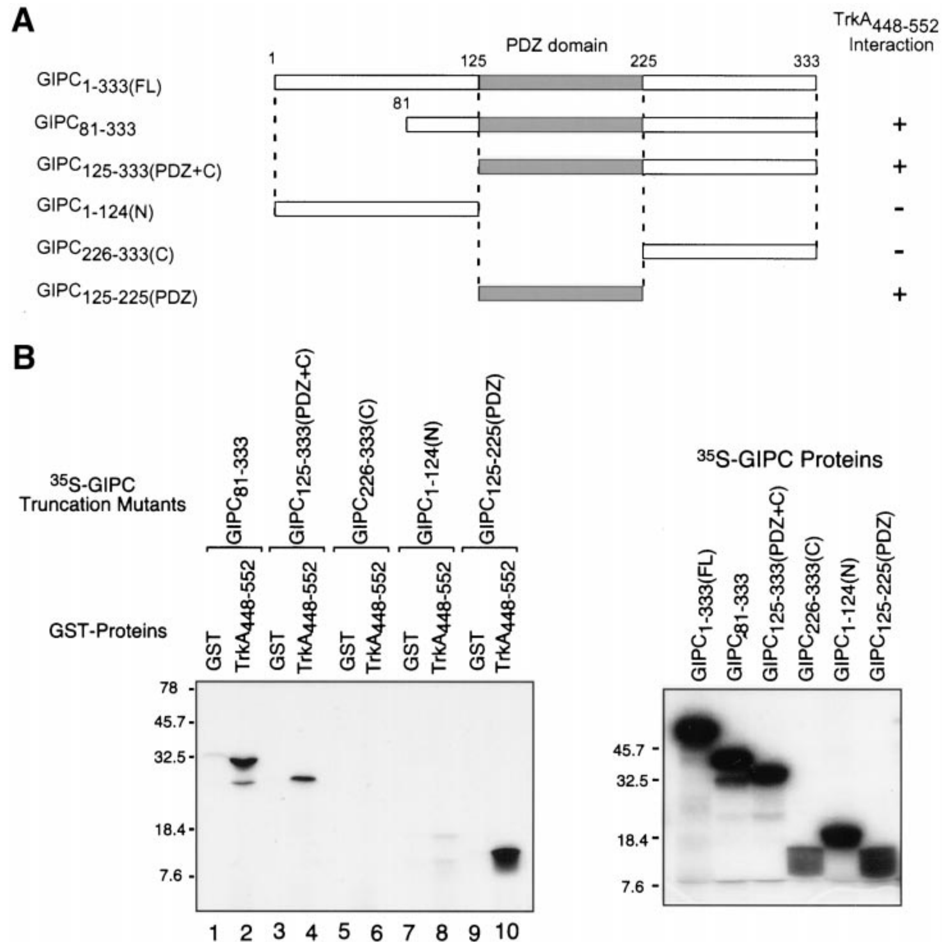

Figure 3. Mapping the interaction of TrkA with GIPC deletion mutants. (A) Schematic representation of mouse GIPC deletion mutants. The interaction between GIPC mutants and $TrkA_{448-522}$ shown on the right is based on data from B. (+, interaction; −, no interaction). (B) Left, in vitro interaction of TrkA juxtamembrane region with GIPC truncation mutants. $GIPC_{81-333}$ (lane 2), $GIPC_{125-333}$(PDZ+C) (lane 4), and $GIPC_{125-225}$(PDZ) (lane 10) bind to GST-$TrkA_{448-522}$ but not to GST alone (lanes 1, 3, and 9), whereas $GIPC_{1-124}$(N) (lane 6) or $GIPC_{226-333}$(C) (lane 8) did not bind to GST-$TrkA_{448-522}$. GST-$TrkA_{448-522}$ or GST alone immobilized on glutathione-Sepharose beads was incubated with the indicated in vitro translated, ^{35}S-labeled GIPC mutant proteins. The ^{35}S-labeled proteins bound to the beads were separated by SDS-PAGE and detected by autoradiography. Right, an aliquot of each in vitro translated product was resolved by SDS-PAGE and examined by autoradiography to confirm the protein yield and the molecular size.

FLAG-tagged GIPC in HEK293 cells, followed by immunoprecipitation with anti-FLAG IgG and immunoblotting for TrkA. Full-length TrkA, $TrkA_{1-522}$, $TrkA_{1-501}$, and $TrkA_{1-493}$ all coprecipitated with FLAG-GIPC (Figure 2B, lanes 2–5). However, little interaction was observed between $TrkA_{1-472}$ and FLAG-GIPC (Figure 2B, lane 6), and no interaction was seen with $TrkA_{1-452}$ (Figure 2B, lane 7). These experiments indicate that amino acids 472–493 in the juxtamembrane region of TrkA are required for its interaction with GIPC.

GIPC Interacts with TrkA via Its PDZ Domain

To determine which region in GIPC interacts with the juxtamembrane domain (amino acids 448–522) of TrkA, in vitro pull-down assays were performed using GST-$TrkA_{448-522}$ and several in vitro translated GIPC truncation mutants (Figure 3A). We found (Figure 3B, left) that GST-$TrkA_{448-552}$ binds $GIPC_{81-333}$ (lane 2), $GIPC_{125-333}$ (PDZ plus C terminus), and $GIPC_{125-225}$ (PDZ) but not $GIPC_{226-333}$ (C terminus) or $GIPC_{1-124}$ (N terminus). These results demonstrate that the PDZ domain of GIPC binds to the juxtamembrane region of TrkA.

TrkA Coprecipitates with Endogenous GIPC in PC12 Cells

We next tested the ability of TrkA to interact with endogenous GIPC in PC12 (615) cells stably overexpressing TrkA (Hempstead *et al.*, 1992). When immunoprecipitation was carried out with anti-GIPC antiserum followed by immunoblotting for TrkA, we found that TrkA (Figure 4A, lane 1) coprecipitated with endogenous GIPC.

Next we examined whether NGF treatment (10 or 30 min) increases the amount of endogenous GIPC that associates with TrkA in PC12 (615) cells. Activation of TrkA by NGF was verified by immunoblotting with anti-phosphotyrosine IgG (Figure 4A, bottom). The amount of TrkA that coprecipitated with GIPC from NGF-treated cells was comparable to that from nontreated cells (Figure 4A, top, lanes 1–3). Treatment of the cells with the Trk kinase inhibitor K252a (Barbacid *et al.*, 1991; Berg *et al.*, 1992) had no effect on the interaction (Figure 4A, lane 4). These results suggest that the interaction between GIPC and TrkA is independent of NGF treatment and that tyrosine phosphorylation of TrkA is not required for its interaction with GIPC.

To test whether GIPC can interact with the activated form of TrkA, GIPC was immunoprecipitated from lysates pre-

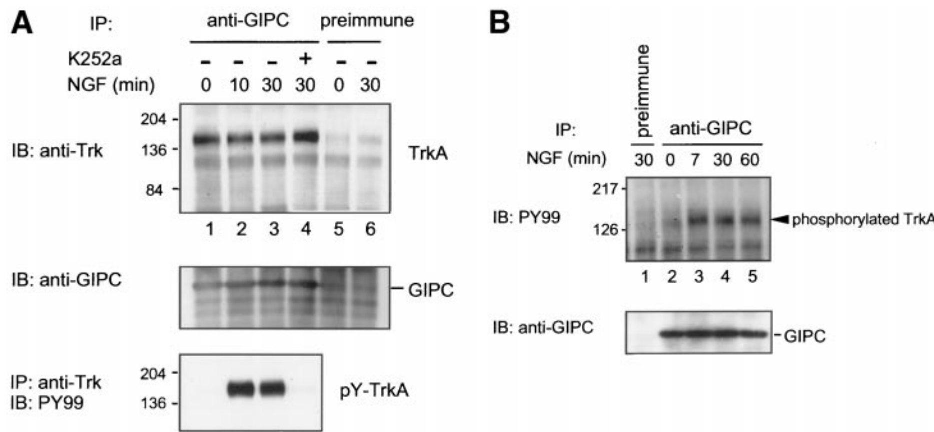

Figure 4. Association of TrkA with endogenous GIPC in PC12 (615) cells. (A) Top, TrkA coprecipitates with endogenous GIPC in both control (lane 1) and NGF-treated (lanes 2 and 3) cells. Addition of 100 nM K252a, a specific Trk kinase inhibitor, before NGF treatment had no effect on the interaction (lane 4). Little or no TrkA is seen in precipitates obtained with preimmune serum (lanes 5 and 6). Middle, the presence of GIPC in immune (lanes 1–4) but not preimmune precipitates (lanes 5, 6) was verified by immunoblotting. Bottom, an aliquot of lysate was immunoprecipitated with anti-Trk (C-14) IgG and immunoblotted with anti-phosphotyrosine (PY99) IgG to verify the activation of TrkA by NGF (lanes 2 and 3). PC12 (615) cells were cultured in low-serum–containing medium overnight and treated with 100 ng/ml NGF for 0, 10, or 30 min. Cell lysates (3.7 mg) were immunoprecipitated (IP) with either anti-GIPC or preimmune serum followed by immunoblotting (IB) with anti-Trk (B-3) (top) or anti-GIPC (middle). (B) Phosphorylated TrkA (arrowhead) coprecipitated with endogenous GIPC at all time points (7, 30, and 60 min) after NGF treatment (lanes 3–5) but not in the untreated sample (lane 2) or that precipitated with preimmune serum (lane 1). PC12 (615) cells were treated with 100 ng/ml NGF as in A. Cell lysates (3.3 mg) were immunoprecipitated with either anti-GIPC or preimmune serum followed by immunoblotting with antiphosphotyrosine PY99 (top) or anti-GIPC (bottom).

pared from PC12 (615) cells treated with NGF (0–60 min) followed by immunoblotting with anti-phosphotyrosine IgG. Tyrosine-phosphorylated TrkA coprecipitated with GIPC at all time points after NGF treatment (Figure 4B, top). Taken together, these results suggest that GIPC can interact with both phosphorylated and nonphosphorylated forms of TrkA.

TrkA, GIPC, and GAIP Form a Coprecipitable Protein Complex

GIPC has been shown to interact with GAIP in both the yeast two-hybrid system and in vitro GST pull-down assays (De Vries *et al.*, 1998b). Similarly, we found that GAIP interacts with GIPC and can be coprecipitated with GIPC from metabolically labeled HEK293T cells transiently transfected with these proteins (Figure 5, A and B).

The finding that GIPC coimmunoprecipitates with both TrkA and GAIP in HEK293T cells suggested that GIPC might mediate the formation of a signaling complex containing TrkA and GAIP. To test this possibility, we transfected HEK293T cells with TrkA, GIPC, and GAIP cDNAs, performed immunoprecipitation with anti-Trk, and tested for the presence of GIPC and GAIP by immunoblotting. We found that both GIPC and GAIP coimmunoprecipitated

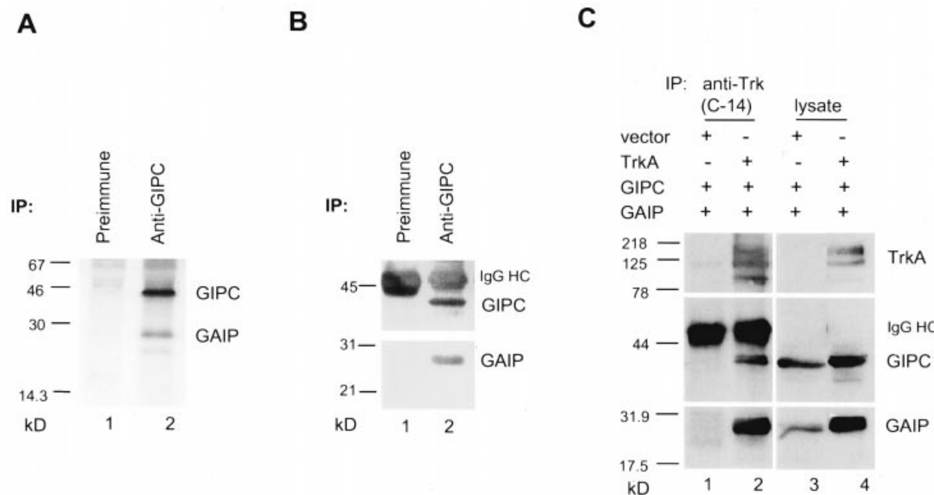

Figure 5. TrkA, GIPC, and GAIP form an immunoprecipitable protein complex. (A) GAIP (~25 kDa) coprecipitates with GIPC (~40 kDa). pcDNA3.1-mGIPC and pcDNA3-hGAIP were transiently cotransfected into HEK293 cells followed by metabolic labeling with [^{35}S]methionine. Cell lysate was immunoprecipitated (IP) with preimmune (lane 1) or anti-GIPC (lane 2) serum. (B) Duplicate coimmunoprecipitations were performed as in A followed by immunoblotting with either anti-GIPC (top) or anti-GAIP (bottom) to verify the identity of the 25 kDa band as GAIP. GAIP is detected in the immune (lane 2) but not the preimmune (lane 1) precipitate. (C) Coimmunoprecipitation of TrkA, GIPC, and GAIP. pCMV5-TrkA (lanes 2 and 4) or empty vector (lanes 1 and 3) were coexpressed with pcDNA3.1-mGIPC and pcDNA3-hGAIP in HEK293 cells. Lysates were immunoprecipitated with anti-Trk (C-14) (lanes 2 and 4) followed by immunoblotting with anti-Trk (44) (top), anti-GIPC (middle), and anti-GAIP (bottom) antisera. GIPC and GAIP coimmunoprecipitated with TrkA in cells transfected with all three proteins (lane 2) but not in the sample transfected with GIPC, GAIP, and empty vector (lane 1). The expression levels in 50 μg cell lysates are shown in lanes 3 and 4.

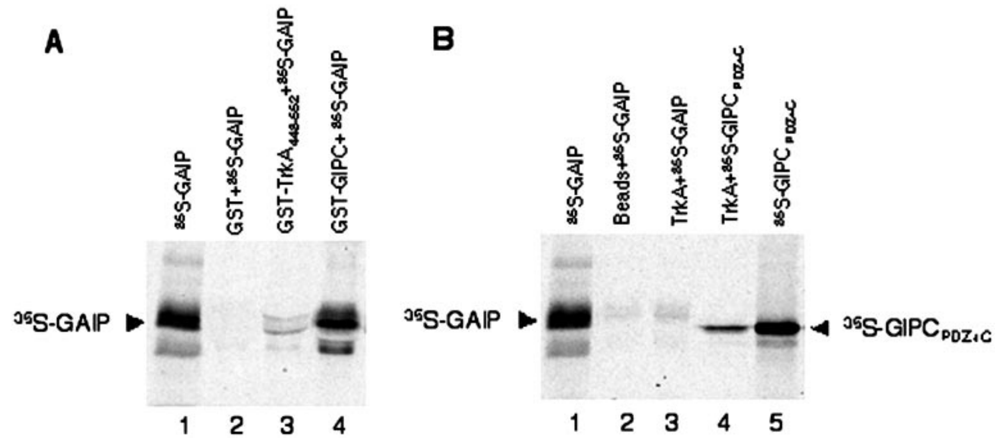

Figure 6. Lack of direct interaction between TrkA and GAIP. (A) In vitro translated ^{35}S-GAIP binds to GST-GIPC (lane 4) but does not bind to GST-TrkA$_{448-522}$ (lane 3) or GST alone (lane 2). Lane 1 shows an aliquot of in vitro translated ^{35}S-GAIP. GST-TrkA$_{448-522}$ or GST alone immobilized on glutathione-Sepharose beads was incubated with in vitro translated ^{35}S-GAIP. The ^{35}S-GAIP bound to the beads was separated by SDS-PAGE and detected by autoradiography. (B) In vitro translated ^{35}S-GAIP does not bind to TrkA (lane 3) or protein A-Sepharose beads alone (lane 2). ^{35}S-GIPC$_{PDZ+C}$ (used as a positive control) binds to TrkA (lane 4). Lanes 1 and 5 show an aliquot of each in vitro translated product. TrkA was transiently overexpressed in HEK293 cells and immunoprecipitated with anti-Trk (C-14) followed by incubation with Protein A-Sepharose beads. Beads were washed extensively with RIPA buffer and incubated with in vitro translated ^{35}S-GAIP or ^{35}S-GIPC$_{PDZ+C}$. ^{35}S-labeled proteins bound to the beads were separated by SDS-PAGE and detected by autoradiography.

with TrkA (Figure 5C, lane 2) in cells transfected with all three proteins but not in cells transfected with GIPC, GAIP, and an empty vector (Figure 5C, lane 1). These results indicate that TrkA, GIPC, and GAIP form a detergent-resistant protein complex in HEK293T cells overexpressing all three proteins. To rule out the possibility of direct interaction

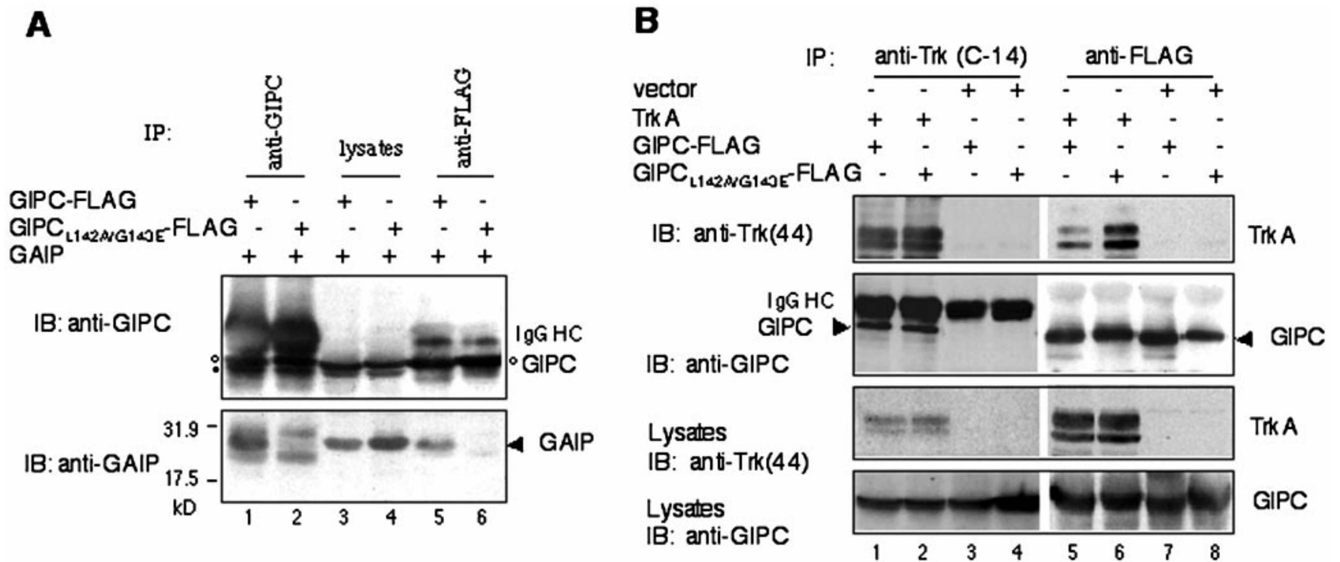

Figure 7. TrkA and GAIP bind to different sites in the PDZ domain of GIPC. (A) Lack of interaction between GIPC(L142A/G143E) and GAIP. C-terminal FLAG-tagged GIPC (lanes 1, 3, and 5) or GIPC(L142A/G143E) mutant (lanes 2, 4 and 6) were coexpressed with GAIP in HEK293 cells. GIPC was immunoprecipitated (IP) from cell lysates with anti-GIPC (lanes 1 and 2) or anti-FLAG (M2) antibody (lanes 5 and 6), and the precipitates were immunoblotted (IB) with anti-GIPC (top) and anti-GAIP (bottom). GAIP coprecipitated with wild-type GIPC (lanes 1 and 5) but not with the GIPC(L142A/G143E) mutant (lanes 2 and 6). Protein expression levels (lanes 3 and 4) were comparable. The double bands at ~40 kDa (top, lanes 1–4) represent endogenous GIPC (•) and overexpressed GIPC-FLAG (○). IgG HC, IgG heavy chain. The two bands immediately above and below GAIP (lanes 1 and 2) are IgG light chains. (B) GIPC(L142A/G143E) binds TrkA. C-terminal FLAG-tagged GIPC or GIPC(L142A/G143E) were coexpressed with TrkA (lanes 1, 2, 5, and 6) or empty vector (lanes 3, 4, 7, and 8) in HEK293 cells. Lysates were immunoprecipitated with anti-TrkA (C-14) (lanes 1–4) or anti-FLAG (lanes 5–8) IgG followed by immunoblotting with anti-TrkA (44) (top panel) or anti-GIPC (second panel). Both wild-type GIPC (lane 1) and the GIPC mutant (lane 2) coprecipitate with TrkA in cells cotransfected with TrkA. Similarly, TrkA coprecipitates with both wild-type GIPC-FLAG (lane 5) and mutant GIPC-FLAG (lane 6). No GIPC (lanes 3 and 7) or GIPC(L142A/G143E) (lanes 4 and 8) was precipitated from samples cotransfected with empty vector. Protein expression levels of TrkA (third panel) and GIPC (bottom panel) are shown.

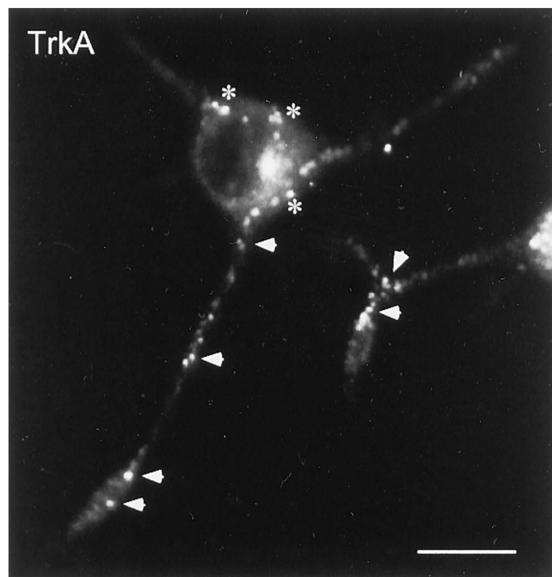

Figure 8. Distribution of TrkA by immunofluorescence. TrkA is detected within vesicular structures in the cell body (asterisks), along the neurites and in the neurite termini (arrowheads) in PC12 cells stably overexpressing TrkA. PC12 (615) cells were treated with 50 ng/ml NGF overnight to stimulate differentiation and outgrowth of neurites. Cells were fixed in methanol, labeled with polyclonal anti-TrkA (C-14; recognizes both TrkA and p-TrkA), and examined by confocal microscopy. Bar, 10 μm.

between TrkA and GAIP, we tested the ability of in vitro translated ^{35}S-GAIP to bind to GST-TrkA$_{448-552}$ (Figure 6A) or TrkA immunoprecipitated from HEK293 cells transiently transfected with TrkA (Figure 6B). The results show that GAIP does not bind to either TrkA expressed in HEK293 cells (Figure 6B, lane 3) or GST-TrkA$_{448-552}$ (Figure 6A, lane 3). We conclude that the formation of GIPC, GAIP, and TrkA complexes is through direct interaction between TrkA and GIPC and not GAIP.

TrkA and GAIP Bind to Different Sites in the PDZ Domain of GIPC

Because GIPC, GAIP, and TrkA form an immunoprecipitable complex and both GAIP and TrkA bind to the PDZ domain of GIPC, we reasoned that GAIP and TrkA must bind to different sites in the PDZ domain of GIPC. Mutation of the C-terminal oxygen-binding site (GLGF) within PDZ domains has been shown to abolish interaction of the latter with C-terminal PDZ-binding motifs (Daniels *et al.*, 1998; Edwards and Gill, 1999). Because the corresponding site in GIPC is A$_{141}$LGL$_{144}$, we generated a GIPC(L142A/G143E) mutant and tested its ability to interact with GAIP and TrkA by immunoprecipitation. The mutant failed to bind GAIP when either anti-GIPC (Figure 7A, lane 2) or anti-FLAG (Figure 7A, lane 6) were used for immunoprecipitation. By contrast, the GIPC mutant did bind to TrkA when either anti-Trk (Figure 7B, lanes 1 and 2) or anti-FLAG (Figure 7B, lanes 5 and 6) were used for immunoprecipitation. These findings indicate that Leu$_{142}$ and Gly$_{143}$ in the PDZ domain are crucial for mediating the interaction between GIPC and GAIP but are not required for the binding of GIPC to TrkA. We conclude that the TrkA-binding site within the PDZ domain of GIPC is not the same as the GAIP-binding site.

Colocalization of Endogenous GIPC with Tyr$_{499}$-phosphorylated TrkA in PC12 Cells

We have previously shown by immunofluorescence and immunoelectron microscopy that GIPC is localized on vesicles close to the plasma membrane (De Vries *et al.*, 1998b), and GAIP is localized on clathrin-coated vesicles (De Vries *et al.*, 1998a). To determine where TrkA is localized, immunofluorescence was carried out for TrkA and p-TrkA in PC12 (615) cells that had been induced to differentiate and extend neurites by NGF treatment. Punctate staining was seen both in the cell body and along the neurites of PC12 (615) cells using either a polyclonal antibody that recognizes both phosphorylated and nonphosphorylated forms of TrkA (Figure 8) or an anti-TrkA mAb that recognizes primarily p-TrkA (Figure 9B). We next investigated by double labeling whether there is overlap in the distribution of endogenous GIPC and GAIP with p-TrkA in NGF-stimulated PC12 (615) cells. Endogenous GIPC (Figure 9A) showed punctate staining in the cell body and along the neurites where its distribution partially overlapped with p-TrkA (Figure 9, B and C). GIPC also had a diffuse cytoplasmic distribution, in keeping with the existence of both cytosolic and membrane-associated pools (De Vries *et al.*, 1998b). Endogenous GAIP also showed punctate cytoplasmic staining that was finer than the staining for TrkA and GIPC (Figure 9D), but overlap between endogenous GAIP and the p-TrkA was minimal (Figure 9, E and F) under these conditions. To examine whether the vesicular structures in which p-TrkA and GIPC colocalized are lysosomes, double labeling was carried out with the lysosomal markers cathepsin D and lgp120. There was little overlap in the distribution of GIPC (Figure 10A) with that of lgp120 (Figure 10B). Similarly, there was little overlap in the distribution of p-TrkA (Figure 10D) and cathepsin D (Figure 10E). These experiments demonstrate that p-TrkA and GIPC but not GAIP colocalize in vesicles distinct from lysosomes in the cell bodies and along the neurites of PC12 (615) cells. These vesicles presumably correspond to retrograde transport vesicles because p-TrkA can be considered a potential marker for retrograde transport vesicles (Tsui-Pierchala and Ginty, 1999).

Overexpression of GIPC Inhibits NGF-induced Phosphorylation of MAP kinase (Erk1/2)

Binding of neurotrophins to Trk receptors is known to initiate down-stream signaling cascades that result in phosphorylation and activation of MAP kinase (Erk1/2), PI-3 kinase, and PLC-γ1 (Kaplan and Miller, 1997; Klesse and Parada, 1999). We therefore tested the effect of overexpression of GIPC on these pathways. For this purpose we prepared PC12 (615) cells stably overexpressing GIPC, stimulated them with NGF for 5 min, and determined the effect of overexpressing GIPC on phosphorylation of Erk1/2, PKB (Akt), which is down-stream of PI-3 kinase, and PLC-γ1 (Figure 11). We found that phosphorylation of Erks is greatly reduced in NGF-stimulated cells stably overexpressing GIPC (Figure 11A) compared with controls, whereas phosphorylation of Akt and PLC-γ1 was not significantly

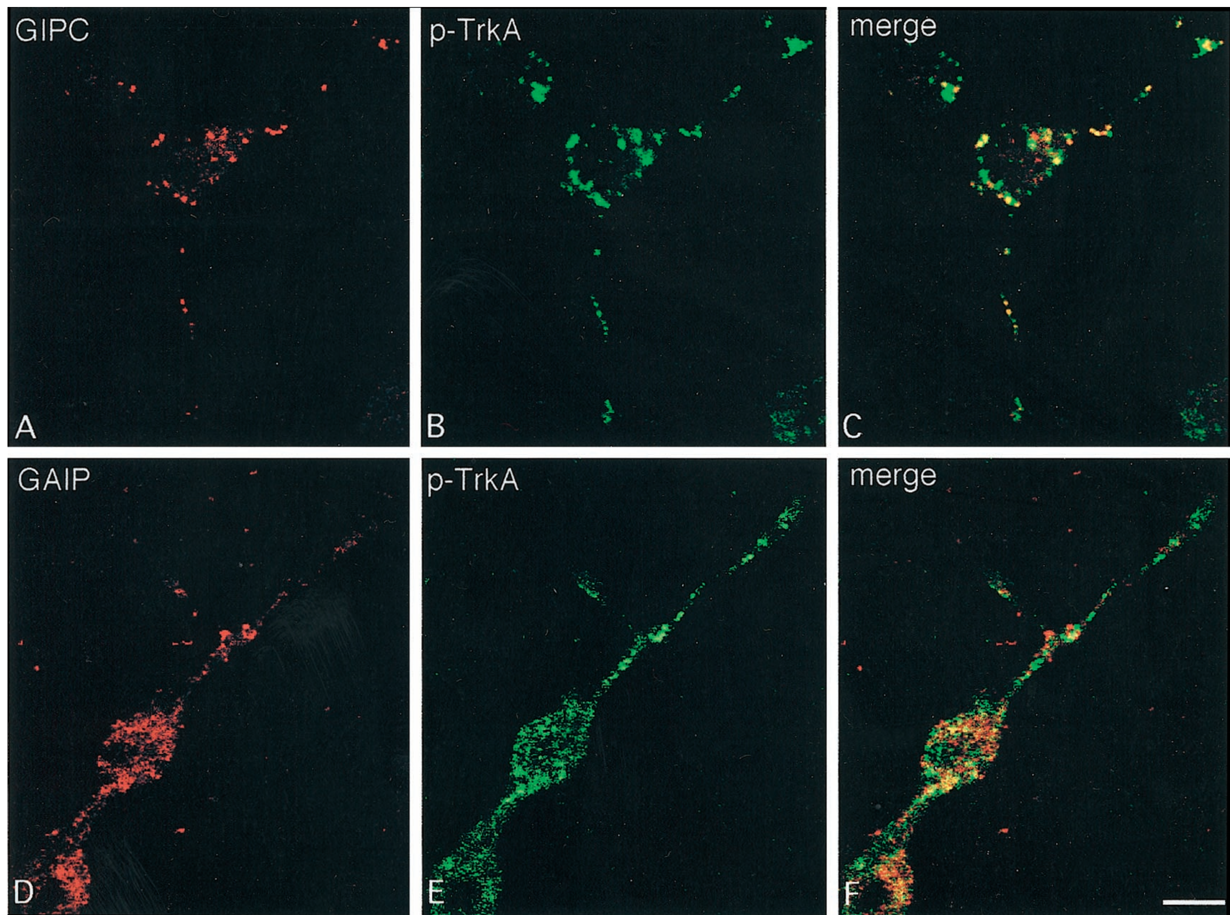

Figure 9. Codistribution of Tyr496–phosphorylated TrkA (p-TrkA) and endogenous GIPC or endogenous GAIP by immunofluorescence. Endogenous GIPC (A) and p-TrkA (B) are widely distributed within vesicles in the cell bodies, neurites, and neurite termini of PC12 (615) cells. The yellow signals in the merged image (C) demonstrate regions where GIPC and p-TrkA overlap. Endogenous GAIP also shows a vesicular distribution (D), that is finer than that for GIPC. There is less overlap (F) in staining for GAIP (D) and TrkA (E). PC12 (615) cells were treated with 50 ng/ml NGF overnight to allow outgrowth of neurites. Cells were fixed, double labeled with anti-TrkA mAB E-6 (recognizes only p-TrkA) and polyclonal anti-GIPC or anti-GAIP, and examined by confocal microscopy. Bar, 10 μm.

changed (Figure 11B). These results indicate that NGF-induced activation of Erk1/2, but not Akt or PLC-γ1, is inhibited by overexpression of GIPC.

To rule out that inhibition of Erk1/2 could be caused by inhibition of the activation of the Shc adaptor protein by GIPC, we examined the activation of Shc (Figure 11C) in response to NGF stimulation. We found that the phosphorylation of Shc is unchanged in NGF-stimulated cells stably overexpressing GIPC (Figure 11C, top, lanes 4 and 6) compared with controls (Figure 11C, top, lane 2). These results indicate that the inhibition of NGF-induced MAP kinase (Erk1/2) activation by overexpression of GIPC is not due to decreased Shc phosphorylation.

DISCUSSION

PDZ proteins (PSD-95/Dig/ZO-1) play important roles in organizing and assembling protein complexes by spatially clustering cytosolic proteins, which usually are components of signal transduction pathways to transmembrane receptors or channels. Thus, PDZ proteins provide a mechanism for assembling signaling molecules into macromolecular signaling complexes, thereby generating specificity from the use of common signaling components (Fanning and Anderson, 1999). The protein-protein interactions mediated by PDZ-containing proteins lead to diverse biological outcomes, such as phototransduction (Tsunoda *et al.*, 1997), synapse formation (Craven and Bredt, 1998; Kennedy, 1998), tight junction formation (Anderson *et al.*, 1995; Anderson and Van Itallie, 1995), and muscle contraction (Brenman *et al.*, 1996).

We have shown here that the PDZ domain protein GIPC similarly forms a protein complex with the Trk receptor, a transmembrane receptor for NGF, and GAIP, a signaling molecule that serves as a GAP for $G\alpha_i$ subunits of heterotrimeric G proteins. We further demonstrated by immunoflu-

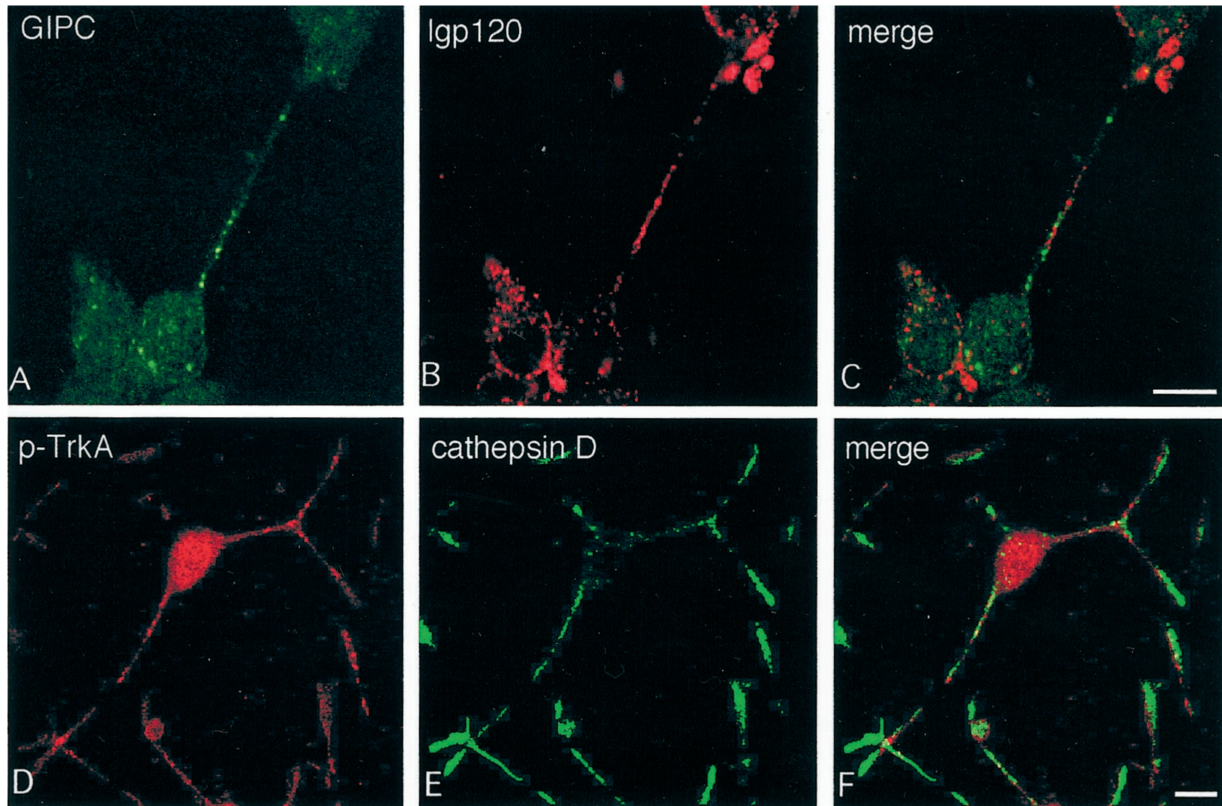

Figure 10. Endogenous GIPC and TrkA do not colocalize with lysosomal markers. Immunofluorescence staining for endogenous GIPC (A) shows little overlap with that of lgp120 (B), because virtually no overlapping yellow signal is seen in the merged image (C). Similarly, TrkA (D) does not codistribute with cathepsin D (E). Double labeling was performed in NGF-induced, differentiated PC12 (615) as in Figure 8 with polyclonal anti-GIPC and mAb anti-gp120 or anti-TrkA (E-6) and polyclonal anti-cathepsin D. Bar, 10 μm.

orescence the presence of both GIPC and TrkA receptors in the cell bodies and along the cell processes or so-called neurites of differentiated PC12 cells. Using a mAb directed against the activated (phosphorylated) form of the receptor, we colocalized GIPC and TrkA in vesicles in both the cell body and cell processes of PC12 (615) cells. According to the information available, binding of NGF to its receptor initiates dimerization of TrkA and TrkB (Levi *et al.*, 1980) and their internalization by receptor-mediated endocytosis, as in the case of other receptor tyrosine kinases (RTKs). In the case of Trk receptors, it is generally believed that, after NGF binding, the receptors are phosphorylated and retrogradely transported in the axon or neurite to the cell body (Ehlers *et al.*, 1995; Senger and Campenot, 1997; Tsui-Pierchala and Ginty, 1999) and that signaling, e.g., phosphorylation of CREB, can occur during retrograde transport of NGF-TrkA complexes (Riccio *et al.*, 1997). In keeping with this scenario, it was recently shown that NGF–p-TrkA complexes formed in distal axons are retrogradely transported to the cell bodies of sympathetic neurons (Tsui-Pierchala and Ginty, 1999). Also, a number of signaling components, such as PLC-γ, are associated with isolated vesicles containing TrkA receptors (Grimes *et al.*, 1997).

The finding that NGF–p-TrkA complexes are retrogradely transported up axons indicates that phosphorylated TrkA can be considered a potential marker for retrograde transport vesicles. We found that GIPC colocalizes with p-TrkA in the neurites and cell bodies of PC12 (615) cells. Our collective findings are compatible with a model in which TrkA, GIPC, and GAIP form a macromolecular complex on the plasma membrane at the neurite terminus, and after TrkA activation by NGF binding GIPC, but not GAIP, is transported together with TrkA along the neurite to the cell body via retrograde transport vesicles. GAIP, which has previously been localized to clathrin-coated vesicles in other cell types (De Vries *et al.*, 1998a), may remain associated with clathrin-coated vesicles. The finding that GIPC is associated with retrogradely transported TrkA receptors in these vesicles suggests that the effects of GIPC on signaling could occur during axonal transport. That GIPC is involved directly or indirectly in signaling was demonstrated by our finding that NGF-induced phosphorylation of MAP kinase (Erk1/2) was reduced by overexpression of GIPC. The precise mechanisms involved remain to be elucidated.

The fact that GIPC can bind to both TrkA and GAIP suggests that GIPC may link TrkA receptors to G protein signal transduction pathways. In this regard, it is notable that neurotrophins have been shown to elevate cAMP levels in neuronal cells, and induction of cAMP was reduced by

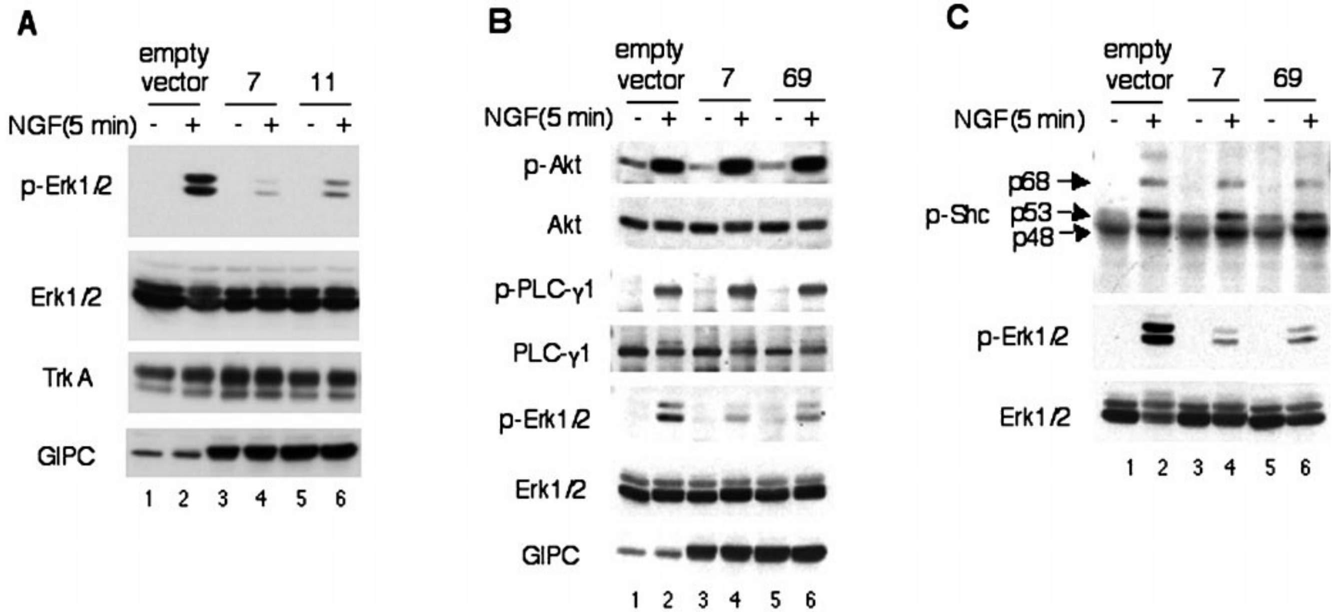

Figure 11. Overexpression of GIPC inhibits NGF-induced phosphorylation of MAP kinases (Erk1 and 2) but has no effect on phosphorylation of Akt, PLC-γ1, or Shc. (A) PC12 (615) cells (clones 7 and 11) stably overexpressing GIPC (lanes 3–6) or empty vector (lanes 1 and 2) were grown in medium containing 1.5% serum overnight. Cells were treated with 100 ng/ml NGF for 5 min, after which cell lysates (50 μg) were immunoblotted with anti-p-Erk1/2 (top panel) or anti-Erk1/2 (second panel). Phosphorylation of Erks (p-Erk1/2) is reduced in NGF-stimulated cells stably overexpressing GIPC (lanes 4 and 6) compared with controls with empty vector (lane 2). No p-Erk1/2 is detected in the absence of NGF stimulation (lanes 1, 3, and 5). The total amount of Erk1/2 expressed (second panel) is comparable under all conditions. Amounts of TrkA (third panel) and GIPC (bottom panel) expressed in 50 μg of cell lysate. (B) PC12 (615) cells (clones 7 and 69) stably overexpressing GIPC (lanes 3–6) or those expressing empty vector (lanes 1 and 2) were grown and treated as in A. Cell lysate (50 μg) was immunoblotted with anti-p-Akt, anti-Akt, anti-p-Erk1/2, or anti-Erk1/2. The same lysates (700 μg) were immunoprecipitated with anti-PLC-γ1 IgG followed by immunoblotting with anti-phosphotyrosine 4G10 (p-PLC-γ1). The same membrane was stripped and reblotted with anti-PLC-γ1 (PLCγ1). Phosphorylation of Akt (p-Akt) and PLC-γ1 (p-PLC-γ1) in NGF-stimulated cells stably overexpressing GIPC (lanes 4 and 6) was unchanged compared with controls transfected with empty vector (lane 2), whereas the phosphorylation of Erks (p-Erk1/2) is reduced (lanes 4 and 6) compared with controls transfected with empty vector (lane 2). Note that the amounts of Akt, PLC-γ1, and Erk1/2 expressed are comparable under all conditions. Bottom, amounts of GIPC in 50 μg of cell lysate. (C) PC12 (615) cells (clones 7 and 69) stably overexpressing GIPC (lanes 3–6) or those expressing empty vector (lanes 1 and 2) were grown and treated as in A. Lysates (700 μg) were immunoprecipitated with anti-Shc IgG followed by immunoblotting with anti-phosphotyrosine 4G10 (p-Shc, top panel). The same lysates (50 μg) were immunoblotted with anti-p-Erk1/2 (second panel) or anti-Erk1/2 (third panel). Phosphorylation of all three forms of Shc (48, 53, and 68 kDa) in NGF-stimulated cells stably overexpressing GIPC (lanes 4 and 6) is similar to controls transfected with empty vector (lane 2), whereas phosphorylation of Erks (p-Erk1/2) is reduced (lanes 4 and 6) compared with controls transfected with empty vector (lane 2). Lanes 1, 3, and 5 show the basal level of p-Shc. The total amount of Erk (Erk1/2) expressed is comparable under all conditions.

GDP-β-S and pertussis toxin (Knipper *et al.*, 1993; Cai *et al.*, 1999), suggesting cross-talk between NGF signaling and G protein signaling. Although the intracellular mechanism by which cAMP levels are regulated by neurotrophins is not well understood, it is plausible that GAIP, a GAP for Gαi family members, might be involved.

Previously, cross-talk between GPCRs and RTKs has been demonstrated for at least three RTKs (Luttrell *et al.*, 1999), the EGF (Daub *et al.*, 1996; Prenzel *et al.*, 1999), PDGF (Linseman *et al.*, 1995), and insulin-like growth factor (Rao *et al.*, 1995) receptors, which become tyrosine phosphorylated after GPCR activation, although little is known about the mechanisms whereby GPCRs regulate RTK activity.

Binding of neurotrophins to Trk receptors in PC12 cells is known to stimulate three main signaling pathways, i.e., MAP kinase (Erk1/2), PI3-kinase/Akt, and PLC-γ1, connected with cell differentiation and survival (Klesse and Parada, 1999; Kaplan and Miller, 2000). We found that overexpression of GIPC inhibits the NGF-induced activation of MAP kinases (Erk1/2) but has no effect on the activation of Akt or PLC-γ1. We further demonstrated that inhibition of the phosphorylation of MAP kinase is not due to its blocking the accessibility of Shc adaptor protein to TrkA. The precise mechanism is not known, but one possible explanation for this effect is that GIPC and its associated protein, GAIP, play a role in Ras-MAP kinase activation by decreasing free G$\beta\gamma$. Alternatively, GIPC may affect Ras-independent pathways, such as activation of Rap1, which has been shown to be required for sustained activation of MAP kinase by NGF (York *et al.*, 1998).

Most PDZ-mediated interactions are through recognition of a short PDZ-binding motif or consensus sequence (T/SXV) at the COOH terminus. The interaction between the PDZ domain of GIPC and the C-terminal, PDZ-binding motif (SEA) of GAIP is an example of such an interaction (De Vries *et al.*, 1998b). By contrast, we found that GIPC binds

through its PDZ domain to an internal sequence, i.e., the juxtamembrane region of TrkA near the tyrosine kinase domain. This interaction was observed not only for GIPC overexpressed in HEK293T cells but also for endogenous GIPC in PC12 (615) cells. Although binding of a PDZ domain to an internal sequence is more unusual, there are a few other examples, such as those between the PDZ domains of the protein tyrosine phosphatase PTP-BL and an internal (LIM) domain in RIL (Cuppen *et al.*, 1998) and between the fifth PDZ domain of InaD and an internal region overlapping a putative G protein–interacting site in PLC-β (van Huizen *et al.*, 1998).

Our finding that the PDZ domain of GIPC interacts with both GAIP and TrkA through different binding sites increases the versatility of PDZ domains in clustering and assembling protein networks. Other examples of the presence of multiprotein-interacting sites in PDZ domains include the third PDZ domain of InaD, which can form a homodimer without affecting its interaction with the C terminus of PKC (Xu *et al.*, 1998). Also, the nNos PDZ domain has been shown by three-dimensional crystallographic analysis to have two interaction surfaces and to be capable of participating in diverse interactions (Hillier *et al.*, 1999).

GIPC has also been shown to associate with several other transmembrane proteins, i.e., the Glut-1 transporter (Bunn *et al.*, 1999), M-SemF (Wang *et al.*, 1999), and the semaphorin III receptor, neuropilin-1 (Cai and Reed, 1999), and with the Tax oncoprotein (Rousset *et al.*, 1998). The interaction between GIPC and all these proteins is through a C-terminal, PDZ-binding motif. This raises the question of whether GIPC mediates formation of multiprotein complexes and provides a link between semaphorin and neurotrophin receptor signaling. Further studies to determine the outcome of these PDZ-mediated interactions should provide insight into the association of GIPC with receptor trafficking and downstream signaling events mediated by neurotrophins and semaphorins.

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