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Ganglioside GM1 binds to the Trk protein and regulates receptor function

(*trk* protooncogene product/PC12 cells/B subunit of cholera toxin)

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ABSTRACT **Several lines of evidence have suggested that ganglioside GM1 stimulates neuronal sprouting and enhances the action of nerve growth factor (NGF), but its precise mechanism is yet to be elucidated. We report here that GM1 directly and tightly associates with Trk, the high-affinity tyrosine kinase-type receptor for NGF, and strongly enhances neurite outgrowth and neurofilament expression in rat PC12 cells elicited by a low dose of NGF that alone is insufficient to induce neuronal differentiation. The potentiation of NGF activity by GM1 appears to involve tyrosine-autophosphorylation of Trk, which contains intrinsic tyrosine kinase activity that has been localized to the cytoplasmic domain. In the presence of GM1 in culture medium, there is a >3-fold increase in NGF-induced autophosphorylation of Trk as compared with NGF alone. We also found that GM1 could directly enhance NGF-activated autophosphorylation of immunoprecipitated Trk *in vitro*. Monosialoganglioside GM1, but not polysialogangliosides, is tightly associated with immunoprecipitated Trk. Furthermore, such tight association of GM1 with Trk appears to be specific, since a similar association was not observed with other growth factor receptors, such as low-affinity NGF receptor ($p75^{NGFR}$) and epidermal growth factor receptor (EGFR). Thus, these results strongly suggest that GM1 functions as a specific endogenous activator of NGF receptor function, and these enhanced effects appear to be due, at least in part, to tight association of GM1 with Trk.**

Monosialoganglioside GM1 is found largely in synaptosomal plasma membranes of the brain. GM1 enhances the activity of nerve growth factor (NGF) in NGF-responsive cells and stimulates neuronal sprouting both *in vitro* and *in vivo* (1–6). However, the precise mechanism of this GM1 function has not been elucidated yet. NGF was the first identified member of a family of neurotrophic factors that function both *in vitro* and *in vivo* to promote neuronal survival and differentiation (7). NGF induces differentiation of the rat pheochromocytoma cell line PC12 into cells resembling sympathetic neurons (8), thus providing a well-characterized model for the investigation of the mechanism of action of NGF. Following the application of NGF to PC12 cells, long-term transcription-mediated events occur. These include the extension of neurites and the acquisition of a differentiated phenotype that is characterized by the development of electrical excitability and the biosynthesis of neurotransmitters (8, 9). The biological effects of NGF are mediated by high-affinity binding to cell-surface glycoprotein receptors called Trk (for tyrosine kinase receptor) encoded by the *trk* protooncogene [now designated *Ntrk* (*NTRK*) for neurotrophic tyrosine kinase receptor in the mouse (human) genome data base] (10, 11). Trk contains intrinsic tyrosine kinase activity that has been localized to the cytoplasmic domain, and it is thought that activation of this kinase represents the initial step in the intracellular signal transduction pathway of NGF (10–13).

We recently reported that the B subunit of cholera toxin (CTB) enhances the action of NGF (14). The effect of CTB on the action of NGF appeared to be dependent on the stability of cytoskeletal proteins and to be mediated by Trk. Because CTB specifically binds to GM1, these data suggested that there may be a functional interaction of GM1 with Trk.

In this study, we examined the direct interaction of GM1 with Trk. In a manner that is SDS resistant, GM1 exhibits tight binding to Trk and activates Trk receptor function. This demonstration of the binding of an acidic glycosphingolipid to a glycoprotein provides insight into the mechanism of GM1 potentiation of NGF action.

MATERIALS AND METHODS

Cell Cultures. PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) as described (15). Cells were preincubated in serum-free medium for 1 hr at 37°C and treated with NGF at a concentration of 50 ng/ml for immunoprecipitation and immunocomplex kinase assay of Trk. For morphological studies, the cells were cultured on collagen/poly(L-lysine) (25:1)-coated 12-well plates with serum-free DMEM and pretreated, when indicated, with 50 μM GM1 for 12 hr at 37°C. After exposure to GM1, the cells were then treated with NGF at either 1 ng/ml or 50 ng/ml for 36 hr at 37°C. In addition, the expression of neurofilament in these cells was also examined by Western blot (immunoblot) with an anti-neurofilament M antibody (Affiniti Research Products, Nottingham, U.K.) as a probe in an enhanced chemiluminescence (ECL) detection system (Amersham).

Immunoprecipitation and Immunocomplex Kinase Assay. Control and NGF-treated PC12 cells cultured in the presence or absence of 50 μM GM1 were lysed in lysis buffer (20 mM Hepes, pH 7.2/1% Nonidet P-40/10% (vol/vol) glycerol/50 mM NaF/1 mM phenylmethylsulfonyl fluoride (PMSF)/1 mM Na_3VO_4/10 μg of leupeptin per ml). Cell-free lysates were normalized for proteins and immunoprecipitated with an anti-Trk antibody as well as antibodies against epidermal growth factor (EGF) receptor (EGFR) and low-affinity NGFR ($p75^{NGFR}$) (14). The immunocomplex kinase assay was performed in the presence or absence of GM1 as described (14). In some cases, phosphoamino acid analysis of Trk was performed. Trk was immunoprecipitated with an anti-Trk antibody and subjected to electrophoresis on sodium dodecyl

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Abbreviations: NGF, nerve growth factor; EGF, epidermal growth factor; EGFR, EGF receptor; $p75^{NGFR}$, low-affinity nerve growth factor receptor; CTB, B subunit of cholera toxin; Trk, tyrosine kinase receptor product of protooncogene *Trk*; HRP, horseradish peroxidase; Tyr(*P*), phosphotyrosine; ECL, enhanced chemiluminescence.

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sulfate (SDS)/7.5% polyacrylamide gels. We excised the band corresponding to Trk from gels and tryptic digestions, and subsequent hydrolysis of Trk with constant-boiling-point 6 M HCl was performed. The resultant hydrolysates were analyzed by electrophoresis on cellulose thin-layer plates (16). The phosphoamino acids were visualized with ninhydrin and analyzed by autoradiography. Anti-Trk antibody was obtained from albino rabbits immunized with an HPLC-purified synthetic peptide representing a 14-amino acid residue derived from the carboxyl terminus of Trk as described by Martin-Zanca *et al.* (17). This antibody has been shown to be satisfactory for both immunohistochemistry (18) and immunoblotting (19).

Lipid Extraction. Total lipids were extracted from Trk, $p75^{NGFR}$, and EGFR immunoprecipitates with chloroform methanol, 2:1 (vol/vol), and further fractionated by using Sep-Pak C_{18} gel as reported (20). The samples obtained were subjected to ascending thin-layer chromatography developed in chloroform/methanol/0.02% $CaCl_2$, 55:45:10 (vol/vol) on TLC plates (Polygram siL N-HR, Macherey & Nagel). The identification of GM1 was accomplished by using an immunostaining method with horseradish peroxidase (HRP)-conjugated CTB as described (21) with minor modifications (omission of neuraminidase treatment of TLC plates to avoid the loss of sensitivity).

Cellular lipids were labeled by incubating PC12 cells in serum-free DMEM containing 10 μCi (370 kBq) of [^{3}H]-palmitic acid that had been sonicated for 30 min at 37°C. After stimulation with NGF at 50 ng/ml for 5 min, the cells were collected by low-speed centrifugation, washed with chilled phosphate-buffered saline, and lysed with lysis buffer. Cell lysates were immunoprecipitated with an anti-Trk antibody. A portion of the immunoprecipitates was subjected to receptor tyrosine kinase assay to assure the immunoprecipitation of Trk. An additional portion was used for lipid extraction as described above.

Immunoblot Analyses. Trk, $p75^{NGFR}$, and EGFR immunoprecipitates were obtained by immunoprecipitation of equal amounts of protein (usually 1 mg/ml) prepared from PC12 cell-free lysates as described above. The resultant immunoprecipitates were electrophoretically transferred to polyvinylidene difluoride membranes after SDS/PAGE with 7.5% gels and were probed with an anti-GM1 antibody or an HRP-conjugated CTB. Detection was performed according to the manufacturer's directions (ECL, Amersham; and Western-light, Tropix, Bedford, MA). Manufacturer-specified protocols were used to strip an anti-GM1 antibody from the blots (ECL manual, Amersham) and to reprobe the membranes with antibodies to Trk, $p75^{NGFR}$, and EGFR. In selected cases, an anti-phosphotyrosine [anti-Tyr(*P*)] antibody (4G10 from Upstate Biotechnology) was used to determine tyrosine phosphorylation.

RESULTS

Morphology. We examined the effect of GM1 on NGF-induced neurite outgrowth in PC12 cells. After 36 hr in culture, NGF at 1 ng/ml did not induce neurite outgrowth in PC12 cells (as reported in refs. 14 and 22), whereas combined treatment with NGF at 1 ng/ml and 50 μM GM1 resulted in a clear induction of neurite outgrowth (Fig. 1*D*). The addition of NGF at 50 ng/ml and 50 μM GM1 resulted in an enhanced effect, shown by an induction of more highly branched neurites and the presence of varicosity-like structures (Fig. 1*E*) as compared with NGF alone (Fig. 1*C*). We further quantified the expression of neurofilament in these cells. Both low and high concentrations of NGF in combination with 50 μM GM1 induced obvious neurofilament expression in PC12 cells (Fig. 1 *D* and *E Right*). GM1 alone had no effect on the morphological differentiation and neurofilament expression of PC12 cells (Fig. 1*F*), and in concentrations exceeding 50 μM, it was found to be toxic to PC12 cells under the present conditions.

Effect of GM1 on Trk-Associated Tyrosine Kinase Activity. The effect of GM1 on receptor tyrosine kinase activity was examined by using both *in vitro* and whole-cell assays. The addition of GM1 to the *in vitro* assay mixture enhanced autophosphorylation of NGF-activated Trk kinase (Fig. 2 *Upper C*). The EC_{50} of GM1 on NGF-activated Trk receptor tyrosine kinase activity was found to be 1 μM. This augmentation of receptor autophosphorylation of tyrosine residues

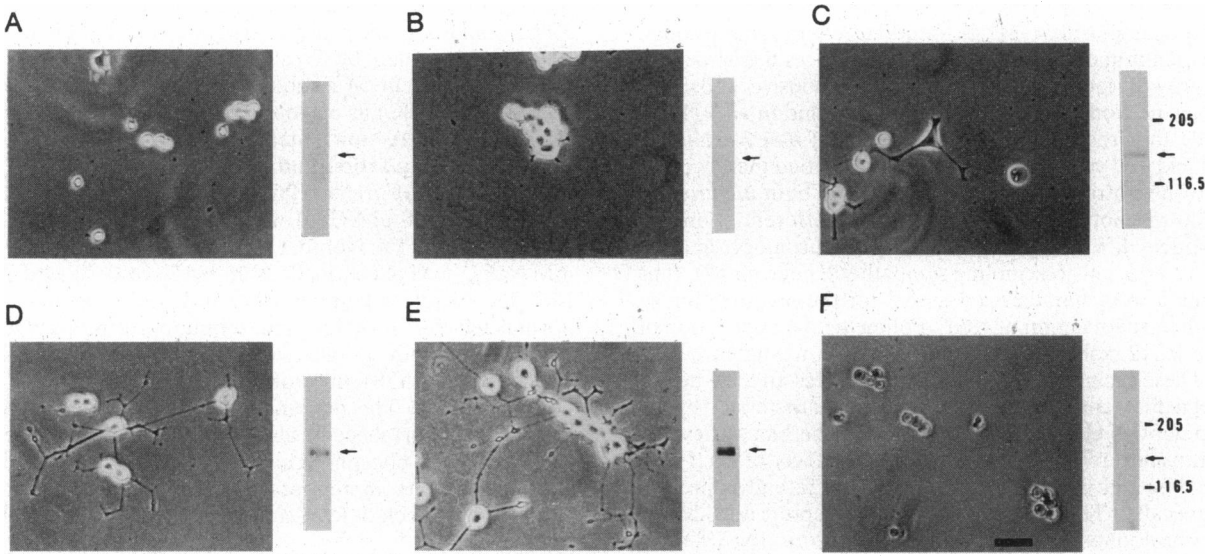

FIG. 1. Effects of GM1 on NGF-induced morphological differentiation and neurofilament expression in PC12 cells. PC12 cells were cultured on 25:1 collagen/poly(L-lysine)-coated 12-well plates with serum-free DMEM and were incubated with (*D–F*) or without (*A–C*) 50 μM GM1 for 12 hr at 37°C. Then cells were treated with 1 ng (*B* and *D*) or 50 ng (*C* and *E*) of NGF per ml for an additional 36 hr at 37°C. (*Left*) Phase-contrast micrographs made from typical areas of these cultures. (*Right*) Neurofilament expressions determined by Western blot probed with an antineurofilament M antibody. One hundred micrograms of protein from total cell lysates was loaded to each lane. For quantification, 100 cells from each of three dishes were evaluated. The numbers of cells exhibiting induction and formation of neurites as long as the cell body are as follows: control, 0; NGF (1 ng/ml), 1 ± 0.3; NGF (50 ng/ml), 33 ± 2.7; NGF (1 ng/ml) + GM1, 46 ± 3.1; NGF (50 ng/ml) + GM1, 78 ± 4.3; and GM1 alone, 0 (mean ± SD). (Bar = 20 μm.)

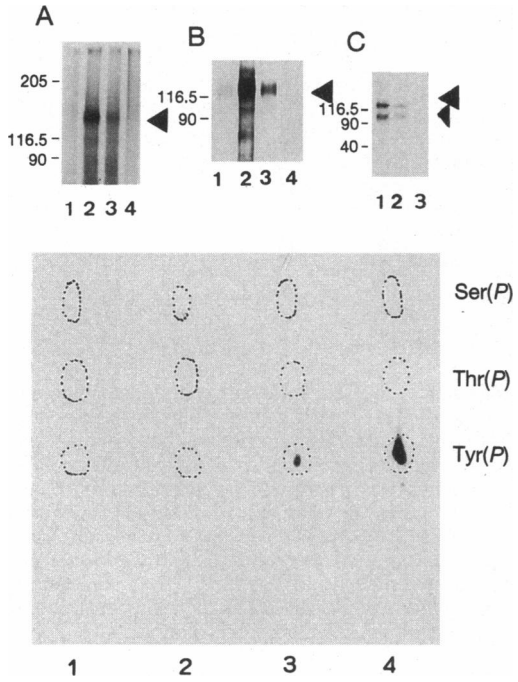

FIG. 2. GM1 enhances NGF-induced Trk autophosphorylation both *in vitro* and in whole cells. (*Upper A*) PC12 cells were pretreated with (lanes 1 and 2) or without (lanes 3 and 4) 50 μM GM1 for 12 hr at 37°C in serum-free DMEM. Upon completion, NGF (50 ng/ml) was added to two samples for the final 5 min (lanes 2 and 3). Trk was immunoprecipitated with an anti-Trk antibody, and an immunocomplex kinase assay was performed as described in text. GM1 clearly enhanced NGF-induced Trk autophosphorylation. The arrowhead indicates 140-kDa mature Trk. (*Upper B*) Western blot analysis was performed on the Trk immunoprecipitates as outlined in *Upper A* with an anti-Tyr(*P*) antibody (4G10 from Upstate Biotechnology) and an ECL detection system. GM1 also enhances NGF-induced Trk autophosphorylation of tyrosine residues. The arrowhead indicates the presence of 140-kDa mature Trk. (*Upper C*) Immunoprecipitates with an anti-Trk antibody from NGF-treated (lanes 1 and 2) and control (lane 3) cells were used for an *in vitro* kinase assay in the presence of 2 μM GM1 (lane 1). *In vitro*, GM1 activates NGF-induced Trk autophosphorylation. The larger and smaller arrowheads indicate the presence of 140-kDa fully glycosylated mature Trk and 110-kDa partially glycosylated immature Trk, respectively. (*Lower*) Phosphoamino acid analysis of Trk obtained by immunocomplex kinase assay of Trk immunoprecipitates from cells untreated (lane 1) and treated with NGF (50 ng/ml) and 50 μM GM1 (lane 4), NGF (50 ng/ml) alone (lane 3), and 50 μM GM1 alone (lane 2) was performed as described in test. The radioactivity was only detected at the position of Tyr(*P*). Dotted circles indicate the positions of Tyr(*P*), phosphothreonine [Thr(*P*)], and phosphoserine [Ser(*P*)] markers, respectively. The radioactivity in each Tyr(*P*) spot was as follows in lanes 1–4: 1, 0 cpm; 2, 25 ± 3 cpm; 3, 332 ± 38 cpm; 4, 1124 ± 88 cpm (triplicate assays; mean ± SD).

was also evident in whole-cell cultures. Cells exposed to 50 μM GM1 in culture medium demonstrated a >3-fold increase in tyrosine autophosphorylation compared with cells cultured in the absence of GM1 (Fig. 2 *Upper A* and *B*). This was analyzed by either Cerenkov counting or densitometric analysis of the band corresponding to Trk. In phosphoamino acid analysis, addition of the combination of 50 μM GM1 and NGF to culture medium (Fig. 2 *Lower*, lane 4) resulted in a >3-fold increased incorporation of radioactivity into Trk than resulted from addition of NGF alone (Fig. 2 *Lower*, lane 3). GM1 alone (Fig. 2 *Lower*, lane 2) did not alter the incorporation of radioactivity into Trk.

Lipid Extraction from Trk Immunoprecipitates. To determine whether GM1 specifically associates with Trk, cell-free lysates prepared from PC12 cells were divided into three equal portions. Trk, $p75^{NGFR}$, and EGFR were immunoprecipitated from these crude lysates with specific antibodies as described above. Total lipids were extracted from these immunoprecipitates with 3 ml of chloroform/methanol, 2:1 (vol/vol), and further fractionated by using TLC analysis as described above. The immunoprecipitates obtained from control and NGF-treated cells contained 3.1 pmol and 12.4 pmol of GM1, respectively. However, $p75^{NGFR}$ and EGFR immunoprecipitates contained <2% of the GM1 present in Trk immunoprecipitates from NGF-treated PC12 cells (compare in Fig. 3*A* lanes 1 and 3 with lanes 5 and 6, and densitometric analysis). To assess the specificity of GM1 binding to Trk, an anti-Trk antibody was pretreated with synthetic Trk antigen and then used for the immunoprecipitation with continuous presence of Trk antigen. GM1 was not detected in the samples extracted from the immunoprecipitates obtained by such treatment (Fig. 3*A*, lanes 2 and 4). These data indicate an association of GM1 with Trk but not with other proteins in the immunoprecipitates. GM1 was not found in immunocomplexes prepared with preimmune serum (data not shown). To determine the amount of each receptor protein, Western blot analysis of the immunoprecipitates used in lipid extraction was performed. The data show that both $p75^{NGFR}$ and EGFR are more abundant than Trk (Fig. 3*C*). To further examine the specificity of the interaction between GM1 and Trk, PC12 cells were labeled with [^{3}H]palmitic acid in either the presence (50 ng/ml) or absence of NGF. Cell lysates were immunoprecipitated with an anti-Trk antibody. Total lipids were extracted from this immunocomplex and analyzed by TLC followed by autoradiographic analysis. The autoradiogram showed the presence of GM1 but not of polysialogangliosides, although the exact nature of the uppermost band is currently not known (Fig. 3*B*).

Western Blot Analysis. Employing either an anti-GM1 antibody or HRP-conjugated CTB, we examined the association of GM1 with Trk by Western blot analysis of the immunoprecipitates produced with anti-Trk, anti-$p75^{NGFR}$, and anti-EGFR antibodies. In the Trk immunoprecipitates, the anti-GM1 antibody revealed the presence of a 140-kDa protein (Fig. 4*A*, lanes 1 and 2). No band was observed in the immunoprecipitates produced with $p75^{NGFR}$ and EGFR (Fig. 4*B*, lanes 3 and 4, Fig. 4*A*, lanes 3 and 4). Additionally, CTB, which specifically binds to GM1, also revealed the presence of a 140-kDa band (Fig. 4*C*). To confirm the positive band as Trk, an anti-GM1 antibody was stripped from the polyvinylidene difluoride membrane, and the membrane was then reprobed with an anti-Trk antibody. The results showed the positive band to be Trk (data not shown).

To confirm the observation that the association of an anti-GM1 antibody and Trk is due to the presence of endogenous GM1 bound to Trk, Trk from PC12 cells incubated in GM1-containing culture medium (25 μM) was immunoprecipitated. The resultant immunoprecipitates from both treated and untreated cells were then subjected to SDS/PAGE and blotted with an anti-GM1 antibody. As determined by densitometry, bands from treated cells (Fig. 4*B*, lane 2) were >3-fold stronger than those obtained from untreated cells (Fig. 4*B*, lane 1).

We confirmed these data by an alternate approach. PC12 cells were cultured in the presence (Fig. 4*C*, lanes 3 and 4) or absence (Fig. 4*C*, lanes 1 and 2) of 10 μM GM1 and were stimulated additionally with NGF at 50 ng/ml (Fig. 4*C*, lanes 2 and 3). Cell lysates were immunoprecipitated with an anti-Trk antibody and examined by Western blot analysis with HRP-conjugated CTB as a specific probe for GM1. The results show that GM1 loading results in an increased detection of GM1 in the immunoprecipitates and that NGF increases the association of GM1 with Trk. This is consistent with the findings we obtained with an anti-GM1 antibody.

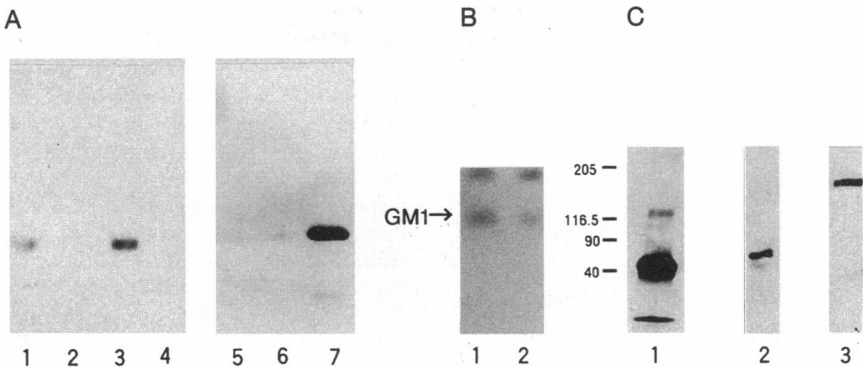

FIG. 3. Detection of GM1 in Trk immunoprecipitates. (*A*) Total lipids were extracted from the anti-Trk antibody-generated immunoprecipitates as described in text. NGF-treated (lanes 3 and 4) PC12 cells contain >3 times the amount of GM1 compared with controls (lanes 1 and 2). The addition of Trk antigen to the antiserum against Trk eliminates the detection of GM1 (lanes 2 and 4). Immunoprecipitates with an anti-EGFR antibody (lane 5) (ab-4, Oncogene Science) or anti-$p75^{NGFR}$ antibody (lane 6) (monoclonal 192 IgG, Boehringer) fail to demonstrate the presence of GM1. Lane 7 represents 40 pmol of authentic bovine brain GM1. (*B*) Lipid extraction was performed on anti-Trk antibody-generated immunoprecipitates from lysates of PC12 cells incubated with [^{3}H]palmitic acid for 1 day followed by treatment with (lane 1) and without (lane 2) NGF at 50 ng/ml for the final 5 min. A positive band corresponding to monosialoganglioside GM1 was observed. No bands corresponding to polysialogangliosides were seen. (*C*) To determine the amount of protein present for each receptor, Western blot analysis was performed on the immunoprecipitates used for the lipid extraction analysis. We used antibodies specific for each receptor: anti-Trk (lane 1), polyclonal anti-$p75^{NGFR}$ antibody (lane 2; gift from Dr. Chao, Cornell University), and anti-EGFR (lane 3) (ab-1, Oncogene Science). The data show that both $p75^{NGFR}$ and EGFR are more abundant than Trk. The large smears seen in lane 1 represent immunoglobulins and the gel front.

DISCUSSION

We demonstrate in the present study that GM1 binds to Trk; GM1 added to culture medium is taken up by cells and forms a tight association with Trk in a manner that is resistant to the effects of SDS. This association of GM1 with Trk appears to enhance the effects of NGF, as demonstrated by the enhancement of NGF induction of neurite outgrowth and neurofilament expression. These effects are most likely due to enhancement of the Trk-associated tyrosine kinase activity elicited by NGF. It has been shown that GM1 functions as a neurotrophic agent (1–3) and that it potentiates the action of NGF in responsive cells, both *in vitro* and *in vivo* (4–6). Although attempts have been made to elucidate the mechanism by which GM1 elicits neurotrophic effects, its precise function has not been apparent. Our previous work with CTB as a specific probe for cell surface GM1 showed that CTB enhances the action of NGF on PC12 cells. However, this effect was not observed in cells treated with cytochalasin B (14). Because CTB has not been shown to interact directly with cytoskeletal proteins and because GM1 does not span the plasma membrane, it is reasonable to assume that cell surface GM1 in PC12 cells associates with a transmembrane-spanning structure such as Trk receptor. The findings presented in the current study are consistent with this hypothesis.

Although the exact site(s) for the binding of GM1 to Trk protein is not known at present, the addition of tunicamycin, a potent inhibitor of N-glycosylation, into culture medium, resulted in a loss of association of GM1 with Trk protein and in the disappearance of the tyrosine kinase activity associated with both the 140-kDa fully glycosylated Trk and the 110-kDa partially glycosylated Trk (data not shown). These data suggest that glycosylation of Trk is required for the specific association of GM1 with Trk. Interestingly, the nonglycosylated 80-kDa precursor form of Trk protein has a weak autophosphorylating activity, while glycosylated $p70^{trk}$, the protein product of *trk oncogene*, has tyrosine kinase activity comparable to that of mature Trk protein (17).

Although a detailed mechanism for the binding of a ganglioside to a glycoprotein remains to be elucidated, studies documenting the binding of a phospholipid to cytoskeletal proteins have been reported (23). Further research is needed to delineate the precise mechanism by which GM1 activates the NGF-dependent Trk-associated tyrosine kinase and how GM1 binds to Trk in a manner that is SDS-resistant. Still, the present observations suggest intriguing innovative therapeutic strategies for trials currently in progress with neurotrophins in neurodegenerative disorders (24).

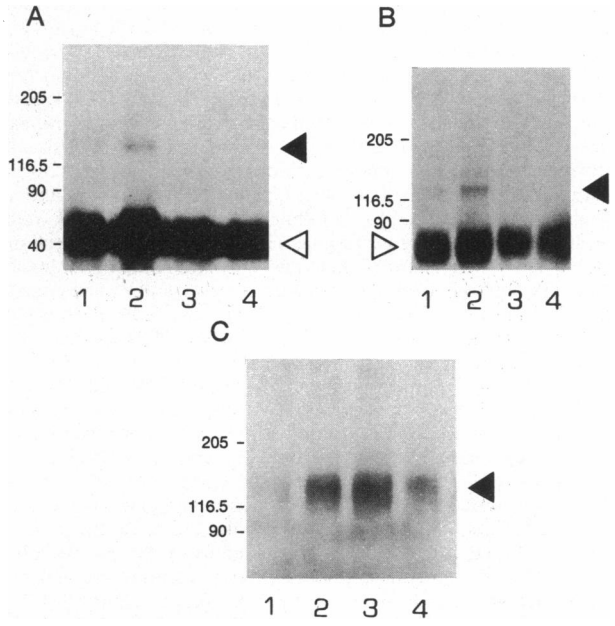

FIG. 4. Immunoblot analysis of Trk with an anti-GM1 antibody (*A* and *B*) and HRP-conjugated CTB (*C*). (*A*) Trk (lanes 1 and 2) and EGFR (lanes 3 and 4) immunoprecipitates from control (lanes 1 and 3), NGF (50 ng/ml)-treated (lane 2), and EGF (100 ng/ml)-treated (lane 4) cells were subjected to SDS/7.5% polyacrylamide gel electrophoresis and analyzed by immunoblotting with an anti-GM1 antiserum (Iatron Laboratories, Tokyo). The 140-kDa band is only detected in Trk immunoprecipitates. (*B*) Immunoblot analysis of the immunoprecipitates from PC12 cells cultured in the presence (lanes 2 and 4) or absence (lanes 1 and 3) of 25 μM GM1 with either anti-Trk antibody (lanes 1 and 2) or anti-$p75^{NGFR}$ antibody (192 IgG) (lanes 3 and 4) was performed and probed with an anti-GM1 antibody. A 140-kDa positive band is observed only in the Trk immunoprecipitates. (*C*) Immunoblot analysis of anti-Trk antibody-generated immunoprecipitates made from PC12 cells cultured in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 10 μM GM1 with HRP-conjugated CTB as a probe in the ECL (Amersham) detection system. Contents of lanes 2 and 3 were stimulated with NGF (50 ng/ml) for 5 min at 37°C. The closed and open arrowheads indicate the presence of the 140-kDa protein and immunoglobulins, respectively.

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