Gliatal Cell Line-derived Neurotrophic Factor Signals through the RET Receptor and Activates Mitogen-activated Protein Kinase*

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Gliatal cell line-derived neurotrophic factor (GDNF), a member of the transforming growth factor-β family of growth factors, was first identified by its ability to promote the survival of midbrain dopaminergic neurons in culture. We demonstrate that GDNF treatment of several neuroblastoma cell lines leads to dose-dependent tyrosine phosphorylation of the RET receptor and that other transforming growth factor-β family members are not able to activate the RET receptor. GDNF treatment of neuroblastoma cells also results in increased transcription of an Elk luciferase reporter gene, suggesting that GDNF activates the mitogen-activated protein kinase signal transduction pathway.

Isolation and characterization of the c-ret proto-oncogene revealed that it resembled a receptor tyrosine kinase (1, 2). Genetic studies have subsequently demonstrated that germ line mutations in the retn oncogene are the direct cause of multiple endocrine neoplasia (MEN)1 2A, MEN 2B, and familial medullary thyroid carcinoma. MEN 2A and familial medullary thyroid carcinoma are characterized by a congenital absence of enteric ganglia along a variable length of the intestine (8, 9). GDNF was first isolated by virtue of its ability to induce dopamine uptake and cell survival in cultures of embryonic ventral midbrain dopaminergic neurons (10, 11). Its amino acid sequence indicated that it was a member of the TGF-β supergene family. Its ability to rescue dopaminergic neurons supported the idea that GDNF might ameliorate degeneration of dopaminergic neurons in patients with Parkinson’s disease. Indeed, studies employing rhesus monkeys that have the symptomatology of Parkinson’s disease induced by neurotoxin treatment have demonstrated that GDNF treatment resulted in significant improvements in several of the cardinal symptoms of parkinsonism (12).

Using chicken sympathetic neurons Trupp et al. (13) demonstrated that GDNF could bind to an unidentified receptor with an apparent dissociation constant of 9 × 10⁻⁹ M. We have demonstrated that the neuroblastoma cell line, LA-N-5, which expresses the RET receptor on its surface, binds GDNF with a similar dissociation constant (14). Recently, Trupp et al. (15) and Durbee et al. (16) have similarly reported that RET can serve as a functional receptor for GDNF. In the present study, we demonstrate that GDNF leads to dose-dependent RET phosphorylation in several different neuroblastoma cell lines. Furthermore, we show that GDNF is unique among the TGF-β family members in its ability to activate the RET receptor. Finally, we identify several reporter genes, which respond by increased transcriptional activation in response to GDNF treatment of neuroblastoma cells. Activation of the Elk reporter gene in response to GDNF suggests that RET phosphorylation results in activation of the mitogen-activated protein kinase (MAPK) signal transduction cascade.

MATERIALS AND METHODS

Cell Culture and Transient Transfection—Neuroblastoma cells were grown in an 8% CO₂ environment using Dulbecco’s modified Eagle’s medium containing 15% fetal bovine serum. Subconfluent LA-N-5 cells in 35-mm dishes were transfected using a total of 2.5 μg of DNA and 8 μl of LipofectAMINE reagent as described by the manufacturer (Life Technologies, Inc.). For drug treatment, GDNF (50 ng/ml) or PD98059 (10 μM) was added 24–36 h prior to harvesting the cells.

Chloramphenicol Acetyltransferase, Luciferase, and β-Galactosidase Assays—48 h post-transfection, cells were harvested by scraping, pelleted at 250 × g for 10 min, and resuspended in 200–400 μl of extraction buffer (250 mM Tris, pH 7.8, 5 mM EDTA) for the CAT assay or (25 mM glycyglicine, pH 7.5, 15 mM MgSO₄, 4 mM EGTA, 1 mM dithiothreitol and 0.1% Triton X-100) for the luciferase assay (17). Cells were lysed by three cycles of freeze-thawing for the CAT assay and by addition of the extraction buffer for the luciferase assay. In both cases, the extracts were cleared of cell debris by centrifugation at 12,000 × g for 5 min. The extract was divided, and the aliquot to be used in the CAT assays was heated at 65°C for 15 min to remove endogenous deacetylating activities and cleared as described previously (15).

The CAT assay was performed as described by Nielsen et al. (19), and the luciferase assay was performed as described by Brasier et al. (17). The β-galactosidase assay was performed using the Luminescent β-galactosidase detection kit (Clontech) per the manufacturer’s instructions. The CAT and luciferase activity were adjusted for variability between experiments by division with the corresponding β-galactosidase activity.

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1 The abbreviations used are: MEN, multiple endocrine neoplasia; GDNF, glial cell line-derived neurotrophic factor; TGF, transforming growth factor; MAPK, mitogen-activated protein kinase; CAT, chloramphenicol acetyltransferase; PAGE, polyacrylamide gel electrophoresis; PMA, 12-O-tetradecanoyl phorbol-13-acetate; SAPK, stress-activated protein kinase.
S-transferase. Immunoprecipitation was carried out as described by Liu et al. (20). Samples were run on a 7% SDS-PAGE gel, transferred to Immobilon (Millipore), and immunoblotted with phosphotyrosine antibody (1:10,000) (UBI) in Tris-buffered saline, 0.2% Tween 20, and 4% ovalbumin or a commercial RET antibody (1:200) (Santa Cruz Biotechnicals) in Tris-buffered saline, 0.1% Tween 20, and 5% milk. For both immunoblots, secondary antibody conjugated to horseradish peroxidase (Life Technologies, Inc.) was used at a 1:10,000 dilution, and the signal was visualized by ECL using recommended protocols (Amersham Corp.).

Inhibitor Studies—LA-N-5 cells in 35-mm dishes were treated with GDNF (50 ng/ml) or 12-O-tetradecanoyl phorbol-13-acetate (PMA) (1 μM) for the indicated times in the absence or the presence of 10 μM PD98059. Inhibitor was added 10 min prior to growth factor addition. After removal of the medium, cells were lysed in 100 μl of Laemmli sample buffer followed by sonication for 15 s. The samples were separated on a 10% SDS-PAGE gel and subjected to Western analysis using antibodies directed against phosphorylated MAPK (1:1000) (New England Biolabs).

RESULTS AND DISCUSSION

GDNF is a member of the TGF-β super-gene family of growth factors (21). All members of this family studied to date signal through heteromeric serine/threonine kinase receptors and not through receptor tyrosine kinases. As mentioned previously, our laboratory has demonstrated that GDNF binds to RET expressing neuroblastoma cells with an apparent dissociation constant of ~9 nM (14). This dissociation constant is larger than similar constants for known receptor tyrosine kinases such as trk and its ligand, nerve growth factor (Kd = 10^-11 M) (22). The dissociation constants observed for the GDNF-RET interaction might be explained by the necessity of accessory protein(s), which are needed for high affinity ligand binding. Several examples of accessory proteins that play roles in ligand binding have been reported including a recent example involving the
receptor tyrosine kinase, MuSK (23, 24). In the case of the MuSK receptor, an unidentified accessory protein forms a complex with the receptor leading to high affinity binding of the ligand, agrin (23, 24). In order to determine if GDNF could lead to activation of the receptor tyrosine kinase RET in various cellular paradigms and to locate a cell line that exhibits higher affinity binding, we identified several cell lines that express RET mRNA (data not shown). When SK-N-SH, Neuro2A, and LA-N-1 cells are treated with GDNF, an increase in RET tyrosine phosphorylation is seen (Fig. 1A). Furthermore, this increase is dose-dependent as is seen by the addition of increasing amounts of GDNF to Neuro2A cells (Fig. 1B). However, none of the cell lines tested displayed an increased affinity for GDNF because all required at least 10–50 ng/ml (0.3–1.5 nM) GDNF for RET activation. Because this appears to be the first example of a TGF-β family member that leads to receptor tyrosine kinase activation, we felt that it was necessary to demonstrate that this activation was specific for GDNF and that other TGF-β family members could not activate RET. Neuroblastoma cells (LA-N-5) were treated with 50 ng/ml of GDNF, TGF-β1, activin, bone morphogenic protein 7, and bone morphogenic protein 2 for 1 min followed by isolation of the RET receptor by immunoprecipitation (Fig. 1C). Western blot analysis demonstrated that only GDNF was capable of activating the RET receptor as measured by an increase in the tyrosine phosphorylation of the receptor. To date, GDNF is unique among the TGF-β family members in its ability to activate a receptor tyrosine kinase.

We then sought to determine whether the GDNF-RET interaction results in productive cellular signaling. The ability of GDNF to activate various promoters was checked by transient transfection analyses using LA-N-5 cells (Fig. 2A). GDNF treatment results in a 7-fold transcriptional activation of a GAL-Elk/GAL-Luc reporter pair, whereas it has less than a 2-fold effect on both the tyrosine hydroxylase and fos promoters. GDNF did not increase transcription of the Neureptide Y promotor or a GAL-Jun/GAL-Luc reporter pair in these cells. The Gal-Elk and Gal-cjun reporter systems are based on the ability of activated MAPK and stress-activated protein kinase (SAPK) to phosphorylate the transcription factors Elk and cjun, respectively (25, 26). This phosphorylation event in turn leads to activation of these transcription factors. In general, MAPK is activated by treatment of cells with growth factors or serum (26, 27). On the other hand, SAPK can be activated by exposing the cells to ultraviolet light (28). Indeed, exposure of these cells to ultraviolet light resulted in the activation of SAPK as measured by the ability of the extract to phosphorylate cJun (data not shown). The ability of GDNF to activate the Elk but not the cJun reporter suggests that GDNF-RET interactions lead to activation of the MAPK pathway but not the SAPK pathway.

In order to determine if GDNF-RET activation results in phosphorylation of MAPK, we employed an antibody specific for the phosphorylated form of the kinase. When LA-N-5 cells are treated with GDNF for 5 min, there is an increase in MAPK phosphorylation (Fig. 2B). This increase can be completely blocked by addition of PD98059, an inhibitor of MAPK activation (29). PMA is also capable of activating MAPK in these cells and serves as a positive control for robust stimulation. To corroborate this result, SK-N-SH cells were subjected to the aforementioned analysis. Both GDNF and PMA activated MAPK robustly in these cells. The above data are in agreement with van Weering et al. (30), who demonstrated that an epidermal growth factor-Ret chimera receptor could activate MAPK. To further confirm the role of MAPK in RET signaling, LA-N-5 cells were transfected with the Elk reporter and a dominant negative form of MEK (31) (Fig. 2C). When these cells were treated with GDNF, both basal level and GDNF-stimulated promoter activity were significantly reduced. As expected, PD98059 also blocked basal and GDNF-stimulated Elk activation (Fig. 2C) These experiments do not rule out other pathways for GDNF-RET signaling but do establish that the MAPK pathway is one way that RET signals are transmitted to the nucleus of a cell.

Our recent work (14) and that of others (15, 16) have established that GDNF can activate the RET receptor. This is surprising in that RET is a receptor tyrosine kinase, whereas GDNF is in the TGF-β family, a group of growth factors previously believed to signal exclusively through receptor serine/threonine kinases (32). The ability of GDNF to signal through a receptor tyrosine kinase establishes an important and interesting bridge between the families of receptor tyrosine kinases and the TGF-β family members. Our results suggest that there will be additional members of the TGF-β family or other GDNF-like molecules that will also signal through receptor tyrosine kinase activation.

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Addendum—During the review of this paper Jing et al. (33) and Treanor et al. (34) reported that GDNF uses a multi-subunit receptor system that includes RET, GDNF, and an accessory glycosylphosphatidylinositol-linked cell surface receptor, which both groups designated GDNFR-α.

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