Glial Cell Line-derived Neurotrophic Factor Induces Ret-mediated Lamellipodia Formation*

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Ret is a receptor tyrosine kinase involved in the hereditary human cancer syndrome multiple endocrine neoplasia type 2 and familiar medullary thyroid carcinoma, as well as in the hereditary human colonic aganglionicosis or Hirschsprung’s disease (1–4). During embryonic development, Ret is involved in kidney development and in development of certain parts of the peripheral nervous system, since mice lacking functional Ret die soon after birth showing renal dysgenesis and lack of sympathetic ganglia and enteric neurons in the digestive tract (5).

Recently, GDNF has been identified as a ligand for Ret (6–9). GDNF is a survival factor for central nervous system motor neurons (10–12) and midbrain dopaminergic neurons (13), but also for survival of different classes of neurons from the peripheral nervous system (14). Mice with a targeted deletion of the GDNF gene show a phenotype identical to that of ret knock-out mice. GDNF has been shown to induce Ret tyrosine phosphorylation, mesoderm induction in Ret-expressing Xenopus embryo cells, neurite outgrowth from autonomic neuroblasts in explants from the nephrogenic region of embryonic day 11.0–11.5 mouse embryo, and increased survival and proliferation of Ret-expressing cells (6, 8). However, the signal transduction cascades mediating these GDNF-induced effects have not been investigated in detail.

GDNF does not bind directly to Ret, but through the interaction with GDNFR-α, a glycosyl phosphatidylinositol-linked cell surface receptor lacking a transmembrane or intracellular domain (7, 9). Indeed, Jing et al. (9) and Treanor et al. (7) show that Ret expression is not enough for GDNF binding, but instead GDNFR-α expression mediates GDNF binding, independent of Ret expression. Additionally, treatment of GDNF-responsive cells with phosphatidylinositol-specific phospholipase C, which results in release of GDNF-α from the membrane, results in loss of responsiveness of Ret to GDNF. Addition of soluble forms of GDNF-α restores this responsiveness (7, 9). Apparently, several cell types express GDNFR-α endogenously in the absence of Ret expression, since Trupp et al. (8) and Durbec et al. (6) showed that introduction of Ret alone is sufficient to confer GDNF responsiveness to different cell types.

Here we show that transfection of Ret into the human neuroepithelioma cell line SK-N-MC makes these cells responsive to GDNF. We show that GDNF induces Ret tyrosine phosphorylation and activation of the Ras-ERK2 pathway, which is not observed in the parental cell line. In addition, Ret induces PI3K activity, followed by PI3K-dependent formation of large lamellipodia. As lamellipodia formation is implicated in neuritogenesis, Ret signaling via PI3K may mediate neurite outgrowth as observed in GDNF-stimulated neuroblasts (6).

**EXPERIMENTAL PROCEDURES**

**Cell Lines**—The human neuroepithelioma cell line SK-N-MC and its stably transfected subclones were cultured in DF12 medium supplemented with antibiotics and 10% fetal calf serum. The full-length Ret-expressing cell line SKP2 was generated by stable transfection of Ret-negative SK-N-MC cells with an Rc/CMV-Ret expression plasmid (kindly provided by M. Takahashi (Nagoya University School of Medicine, Nagoya, Japan); Ref. 15), encoding the p9 form of Ret.

**Analysis of the Ras-ERK2 Pathway**—Experimental procedures for the analysis of the Ras-ERK2 pathway have been described previously (16).

**Immunofluorescence**—Cells were grown on glass coverslips and serum-starved overnight. After a 10 min incubation of the cells with GDNF (Pepro Tech) or EGF (Sigma), either with or without a 10-min pretreatment with the PI3K inhibitors wortmannin (Sigma) or LY294002 (Sigma), cells were fixed for 20 min in PBS containing 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS, and nonspecific binding was blocked by incubation with PBS containing 0.5% bovine serum albumin for 45 min. Coverslips were then incubated for 2 h at room temperature with FITC-coupled phalloidin (Sigma), which specifically binds to polymerized actin. After three washes in PBS, the coverslips were embedded in ImmunoMount (Shandon), and analyzed using a Labophot fluorescence microscope (Nikon).

**In Vitro PI3K Assay**—To determine PI3K activity in the cells, serum-starved cells were either left untreated or stimulated for 5 min with 40 ng/ml EGF or 100 ng/ml GDNF. Cells were lysed in a buffer containing 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 1 mM MgCl₂, 2 mM EDTA, 0.1 mM aprotinin, 1 mM leupeptin, 1 mM orthovanadate, and 1 mM phenylmethylsulfonyl fluoride. Tyrosine-

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† The abbreviations used are: GDNF, glial cell line-derived neurotrophic factor; EGF, epidermal growth factor; HERRet, human EGF receptor ligand binding domain-Ret tyrosine kinase domain chimeric receptor; PI3K, phosphatidylinositol 3-kinase; ERK2, extracellular signal-regulated kinase 2; GDNFR, GDNF receptor; PBS, phosphate-buffered saline; FTTC, fluorescein isothiocyanate.
phosphorylated proteins were immunoprecipitated for 2 h at 4 °C using an anti-phosphotyrosine antibody (FB2) coupled to protein A-Sepharose beads. After extensive washing, beads were taken up into kinase assay buffer (30 mM Hepes (pH 7.4), 30 μM adenosine, 0.1 mg/ml phosphatidylinositol), followed by incubation for 20 min at room temperature. Subsequently, ATP and MgCl₂ were added to a final concentration of 40 μM and 30 mM, respectively, followed by an additional incubation for 25 min at room temperature. The kinase reaction was stopped by addition of 2 volumes of 1 M HCl, lipids were isolated using methanol and chloroform, and separated by thin layer chromatography. Chromatograms were exposed to a PhosphorImager screen. Fold inductions of PI3K activity were calculated relative to the unstimulated situation.

RESULTS

GDNF Induces Concentration-dependent Tyrosine Phosphorylation of p170 Ret—Recently, several groups have implicated GDNF as a ligand for the receptor tyrosine kinase Ret (6–9). To study early signal transduction events after GDNF-stimulated Ret tyrosine kinase activity, we used the Ret-negative neuroepithelioma cell line SK-N-MC stably transfected with a full-length Ret expression plasmid (SKP2 cell line). Stimulation of SKP2 cells with different concentrations of GDNF resulted in a concentration-dependent tyrosine phosphorylation of the 170-kDa plasma membrane-bound isoform of Ret (Fig. 1). Maximal Ret tyrosine phosphorylation levels were induced with GDNF concentrations between 40 and 160 ng/ml, which is identical to concentrations reported by others for stimulation of Ret (and GDNF receptor-α)-expressing cells (6–9). The 150-kDa isoform of Ret, which is not present at the plasma membrane (15, 17), is not phosphorylated in response to GDNF stimulation (Fig. 1). From these results we conclude that GDNF can induce auto-phosphorylation of the receptor tyrosine kinase Ret in the SKP2 cell line.

GDNF Induces Activation of the Ras-ERK2 Pathway—GDNF has been suggested to induce activation of mitogen-activated protein kinases since it induces mesoderm formation in Ret-expressing embryonic Xenopus cells as well as increase proliferation of Ret-expressing cells (6–8). To investigate whether GDNF indeed directly activates the Ras-ERK2 signaling pathway, we analyzed GDNF signaling toward ERK2 in SKP2 cells. We first analyzed the effect of GDNF on Shc phosphorylation. As shown by Shc or phosphotyrosine immunoprecipitations followed by immunoblotting with anti-phosphotyrosine or anti-Shc antibodies, respectively, GDNF induces specific tyrosine phosphorylation of the 66-, 52-, and 46-kDa forms of Shc in SKP2 cells (Fig. 2A). We next analyzed the activation of Ras, by measuring the ratio of Ras bound to GDP and GTP before and after GDNF stimulation of SKP2 cells. A consistent, yet small increase in Ras bound to GTP was observed in three independent experiments (Fig. 2B). This increase was not observed in the parental SK-N-MC cells treated with GDNF. The activation of Ras after EGFl stimulation of the HERRet chimeric receptor, was more pronounced, i.e. a 2–3-fold increase (Fig. 2B).

Finally, we analyzed whether GDNF stimulation of SKP2 cells leads to ERK2 activation. As shown in Fig. 2C, GDNF induced a concentration-dependent ERK2 activation, as determined by a shift in mobility of the activated form of ERK2. The ERK2 mobility shift was maximal between 40 and 160 ng/ml GDNF. This correlates well with tyrosine phosphorylation of the 170-kDa isoform of Ret at these concentrations (Fig. 1). ERK2 activation after GDNF stimulation of SKP2 cells was completely inhibited by transient transfection of a dominant negative mutant of Ras (Ras-N17), showing that the small increase in Ras GTP following GDNF stimulation is sufficient for ERK2 activation (Fig. 2D).

GDNF-induced activation of the Ras-ERK2 pathway is specifically mediated by the Ret tyrosine kinase, since GDNF stimulation of the parental Ret-negative SK-N-MC cells did not induce protein-tyrosine phosphorylation, Shc phosphorylation, Ras activation, or ERK2 activation (Fig. 2).

Ret Tyrosine Kinase Activation Induces PI3K Activity and PI3K-dependent Lamellipodia Formation—GDNF has been shown to induce neurite outgrowth from nephrogenic explants of heterozygous, but not homozygous, ret knock-out mice, indicating that GDNF-activated Ret is involved in neurite outgrowth. Since the actin cytoskeleton is involved in neurite formation, we investigated the effect of Ret activation on the actin cytoskeleton of stably transfected SK-N-MC cells. This was tested both in SKP2 cells as well as in SKF5 cells, which are SK-N-MC cells stably transfected with the HERRet chimeric receptor (16). When SKF5 cells were grown in the presence of 0% or 10% serum, several actin-rich cell surface protrusions were present on the cells, as could be seen after staining of the cells with FITC-labeled phalloidin, which specifically recognizes polymerized actin (Fig. 3A). Time lapse video microscopy of living SKF5 cells revealed that these protrusions are continuously formed and retracted by the cells, and thus resemble the filopodia observed at the growth cone of an axon (18, 19). Addition of EGF to activate the Ret tyrosine kinase resulted in the formation of large lamellipodia (Fig. 3B). These lamellipodia were observed within 5 min in more than 90% of the cells at the border of a cell cluster. Lamellipodia were observed for at least 30 min. Time lapse video microscopy revealed that after stimulation lamellipodia are formed by filling in the space between filopodia, which gradually decrease in length and disappear. Dose-response analysis showed that lamellipodia are already formed at 2.5 ng/ml EGF, which corresponds to the minimal concentration necessary for Ret auto-phosphorylation and ERK2 activation (data not shown).

In a concentration-dependent manner, GDNF also induced lamellipodia formation in SKP2 cells. Using 160 ng/ml, approximately 70% of the cells at the border of a cell cluster showed lamellipodia formation (Fig. 3D). At lower concentrations fewer cells responded, with a minimum of 10 ng/ml for the induction of lamellipodia. This also correlates with the minimum concentration of GDNF required for induction of tyrosine phosphorylation on Ret (Fig. 1).

Lamellipodia formation or membrane ruffling in non-neuronal cell lines is dependent on, and mediated by, PI3K activity (20–22). Additionally, neurite outgrowth in neuronal PC12 cells is dependent on PI3K activity (23). To establish the role of PI3K in Ret-induced lamellipodia formation, we first determined the effect of Ret activation on PI3K activity. As shown in Fig. 4, stimulation of SKF5 cells with EGF resulted in a clear activation of PI3K activity. In addition, GDNF stimulation of full-length Ret in SKP2 cells induced PI3K activation (Fig. 4). In the parental SK-N-MC cell line, EGF or GDNF treatment
did not induce PI3K activation. These results show that Ret tyrosine kinase activation results in PI3K activity.

Using two different PI3K inhibitors, wortmannin and LY294002, we investigated the role of PI3K in Ret-induced lamellipodia formation. Pretreatment of SKF5 or SKP2 cells with wortmannin (Fig. 5) or LY294002 (data not shown) followed by stimulation of Ret tyrosine kinase activity with EGF or GDNF, respectively, resulted in a complete inhibition of lamellipodia formation. Titration experiments showed that pretreatment with 25 nM wortmannin is sufficient to completely inhibit PI3K- and Ret-induced lamellipodia formation (data not shown). These results show that PI3K activity is essential for Ret-induced lamellipodia formation.

Signal transduction toward lamellipodia formation in the SK-N-MC-derived SKF5 and SKP2 cell lines is mediated specifically by Ret, since stimulation of the parental SK-N-MC cell line with either EGF or GDNF did not induce changes in the actin cytoskeleton (Fig. 3).

**DISCUSSION**

Recently, GDNF has been identified as a ligand for the receptor tyrosine kinase Ret. With respect to effects of GDNF on cells, only late effects, such as increased proliferation or survival and induction of differentiation, have been documented (6–9, 14, 24, 25). In this paper we describe early events in GDNF-induced signal transduction. GDNF-induced signaling was investigated in stable transfectants of the SK-N-MC neuroepithelioma cell line expressing full-length Ret. We show that GDNF stimulation of SKP2 cells results in a concentration-dependent induction of Ret tyrosine phosphorylation, followed by Shc phosphorylation and Ras and ERK2 activation. Although GDNF treatment of SKP2 cells only results in a small increase in Ras activity, it is clearly essential for the activation of ERK2, since expression of dominant negative Ras-N17 inhibits ERK2 activation completely. A similar situation was shown previously for platelet-derived growth factor, which also induces only a small increase in GTP-bound Ras that is, however, sufficient for ERK2 activation (26). In addition, we show that Ret tyrosine kinase activity, activated by GDNF stimulation of full-length Ret or by EGF stimulation of HERRet, induces PI3K activity. PI3K activation by receptor tyrosine kinases has now been shown for several other receptors, including the platelet-derived growth factor receptor, insulin receptor, insulin-like growth factor receptor, and hepatocyte growth factor receptor (27–30). Ret-induced activation of PI3K mediates the formation of lamellipodia in both SKP2 and SKF5 cells. It is now well established that for lamellipodia formation or membrane ruffling PI3K activity is essential. This has been shown by the use of mutant receptors that fail to bind the p85 regulatory subunit of PI3K, the use of dominant negative mutants of p85, and by the use of two specific PI3K inhibitors, wortmannin and LY294002 (20–22, 29, 31). Indeed, treatment of SKP2 or SKF5 cells with wortmannin or LY294002 completely inhibited Ret-induced lamellipodia formation, confirming...
ing the role for PI3K in this response. The responses described in this paper are specifically mediated by Ret since GDNF or EGF stimulation of the Ret-negative parental SK-N-MC cell line did not result in any of the described signaling events.

The signals induced by GDNF in full-length Ret-expressing SKP2 cells are, in general, weaker than those induced by EGF stimulation of HERRet-expressing SKF5 cells. This may be due to the number of receptors expressed on the SKP2 cell surface, which is at least 10-fold lower than HERRet expression in SKF5 cells. The concentration of GDNF necessary to induce Ret-mediated responses in SKP2 cells, 10 ng/ml, is in the same order of magnitude as the concentration used by others for stimulation of cell lines or primary neurons, including those cell lines that express high levels of GDNFR-α (6–9, 14, 32, 33).

**FIG. 3. Ret tyrosine kinase activity induces lamellipodia formation.** Serum-starved SK-N-MC, SKF5, and SKP2 cells were stimulated with 40 ng/ml EGF (SK-N-MC, SKF5) or 100 ng/ml GDNF (SK-N-MC, SKP2), followed by staining of polymerized actin with FITC-coupled phalloidin. A, SKF5, unstimulated; B, SKF5, EGF; C, SKP2, unstimulated; D, SKP2, GDNF; E, SK-N-MC, unstimulated; F, SK-N-MC, EGF; G, SK-N-MC, GDNF. Bar represents 10 μm.
We therefore conclude that, if GDNFR-α is essential for GDNF-induced Ret activation, SKP2 cells express sufficient GDNF-α molecules. However, it should be noted that Mount et al. (24) reported increased proliferation of primary Purkinje cells using GDNF concentrations as low as 1 pg/ml.

Ret signal transduction has also been investigated in a fibroblast cell line stably transfected with a HERRet expression vector (34). In these cells, Ret tyrosine kinase activity induces Ras activation, but not ERK2 activation, which is in clear contrast to results obtained in both GDNF-stimulated SKP2 cells, as shown in this paper, and EGF-stimulated SKF5 cells (16). In addition, Santoro et al. concluded that in fibroblasts Ret activation does not induce PI3K activity. Also this signaling pathway is activated after Ret activation in SK-N-MC-derived cell lines. Clearly, Ret signaling in fibroblasts is different from Ret signaling in neuronal cells, the cell type in which Ret is normally expressed.

In neuronal cells, lamellipodia formation is a critical event in neuritogenesis. Lamellipodia formation is observed at growth cones of neurites, and inhibition of lamellipodia formation with actin polymerization inhibitors or PI3K inhibitors blocks neurite outgrowth (18, 19, 23, 35, 36). Lamellipodia formation has also been implicated in cellular migration (37). We have shown here that activation of Ret tyrosine kinase activity, either by GDNF stimulation of full-length Ret or by EGF stimulation of HERRet, results in the formation of large lamellipodia. These lamellipodia may represent early events in neurite formation. Indeed, expression of constitutively active Ret in PC12 cells induces neurite outgrowth (38). In addition, autonomic neur-oblasts in explants from the nephrogenic region of embryonic day 11.0–11.5 mouse embryos show Ret-dependent axonal outgrowth toward beads soaked in GDNF (6). Alternatively, Ret-induced lamellipodia may be involved in migration of neuronal precursors. However, it has been shown that Ret expression is up-regulated only after migration of neuroblasts toward the rat fetal gut. In addition, detailed analysis of ret knockout mice showed that Ret-negative neuroblasts do migrate into the anlage for enteric and sympathetic ganglia, despite the lack of expression of functional Ret (39, 40). It is only after reaching the anlage that the Ret-negative neuroblasts die (40). Death of these cells may be the result of the inability of the neuroblasts to form connections with target cells because of the failing neuritogenesis in the absence of functional Ret. Neuroblasts that cannot form functional contacts with target cells die because of deprivation of neurotrophic factors (for review see Ref. 41).

![Figure 4. Ret tyrosine kinase activity induces activation of PI3K.](http://www.jbc.org/content/253/12/266/F1.large.jpg)

**Figure 4.** Ret tyrosine kinase activity induces activation of PI3K. Serum-starved SKF5 or SKP2 cells were stimulated for 5 min with 40 ng/ml EGF or 100 ng/ml GDNF, respectively, to induce Ret tyrosine kinase activity. PI3K activity was determined by incubation of phosphotyrosine immunoprecipitates with the PI3K substrate phosphatidylinositol and labeled ATP. Shown is the fold induction of labeled phosphatidylinositol 3-phosphate in stimulated cell lysates, relative to unstimulated cell lysates.

![Figure 5. PI3K-inhibitor wortmannin inhibits Ret-induced lamellipodia formation.](http://www.jbc.org/content/253/12/266/F2.large.jpg)

**Figure 5.** PI3K-inhibitor wortmannin inhibits Ret-induced lamellipodia formation. Serum-starved SKF5 or SKP2 cells were pre-treated for 10 min with 50 nM wortmannin, followed by a 5-min stimulation with 40 ng/ml EGF or 100 ng/ml GDNF and staining of polymerized actin with FITC-coupled phallidin. A, SKF5, EGF; B, SKP2, GDNF. Bar represents 10 μm.

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