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Ret-dependent and -independent Mechanisms of Glial Cell Line-derived Neurotrophic Factor Signaling in Neuronal Cells*

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Glial cell line-derived neurotrophic factor (GDNF) has been shown to signal through a multicomponent receptor complex consisting of the Ret receptor tyrosine kinase and a member of the GFRα family of glycosylphosphatidylinositol-anchored receptors. In the current model of GDNF signaling, Ret delivers the intracellular signal but cannot bind ligand on its own, while GFRαs bind ligand but are thought not to signal in the absence of Ret. We have compared signaling pathways activated by GDNF in two neuronal cell lines expressing different complements of GDNF receptors. In a motorneuron-derived cell line expressing Ret and GFRαs, GDNF stimulated sustained activation of the Ras/ERK and phosphatidylinositol 3-kinase/Akt pathways, cAMP response element-binding protein phosphorylation, and increased c-*fos* expression. Unexpectedly, GDNF also promoted biochemical and biological responses in a line of conditionally immortalized neuronal precursors that express high levels of GFRαs but not Ret. GDNF treatment did not activate the Ras/ERK pathway in these cells, but stimulated a GFRα1-associated Src-like kinase activity in detergent-insoluble membrane compartments, rapid phosphorylation of cAMP response element-binding protein, up-regulation of c-*fos* mRNA, and cell survival. Together, these results offer new insights into the dynamics of GDNF signaling in neuronal cells, and indicate the existence of novel signaling mechanisms directly or indirectly mediated by GFRα receptors acting in a cell-autonomous manner independently of Ret.

GDNF[1] was originally characterized as a survival factor for dopaminergic neurons (1). Subsequently, biological effects of GDNF on the survival and differentiation of several other neuronal populations (2–7) and in kidney morphogenesis (8) have considerably extended the range of activities of this polypeptide. GDNF utilizes a unique receptor system comprised of a signaling component encoded by the c-*ret* proto-oncogene (9–12) and a glycosylphosphatidylinositol (GPI)-anchored co-receptor, GDNF family receptor α1 (GFRα1), which is required for ligand binding (13, 14). Three close mammalian homologues of GDNF have been identified, all of which utilize Ret as signaling receptor with the aid of different members (GFRα1–4) of the GFRα family of GPI-linked co-receptors (15–17). GFRα receptors have been shown to provide some degree of ligand specificity, although promiscuity between the different receptors is also possible (reviewed in Ref. 18). GDNF, for example, can activate Ret via binding to either GFRα1 or GFRα2 (19–21).

Naturally occurring oncogenic mutations in *ret* have been utilized prior to the identification of endogenous ligands as tools to dissect the intracellular signaling pathways activated by the Ret kinase, mostly in non-neuronal cell types (22). Oncogenically activated Ret engages Grb2 adaptor and Shc docking proteins which results in activation of the Ras/ERK pathway (23–25) and activates other signaling targets, such as phospholipase C-γ (PLC-γ), which are required for transformation by several of the oncogenic forms of *ret* (26). In comparison, much less is known about the intracellular signaling mechanisms triggered by the interaction of wild type Ret with GDNF and GFRα receptors in neuronal cells.

The current model of GDNF signaling proposes a rather stringent division of labor between the Ret and GFRα receptor subunits. According to this view, Ret is the signaling receptor component but cannot bind ligand in the absence of GFRα receptors, while the latter do bind ligand with high affinity but, because they do not cross the lipid bilayer, they are believed not to signal in the absence of Ret. Although at face value this model would predict a high degree of co-localization of Ret and GFRα receptor subunits *in vivo*, GFRαs are in fact much more widely expressed than Ret in nervous tissue, an unexpected discrepancy that remains unsatisfactorily explained. Thus, for example, GFRα1 is highly expressed in the lateral geniculate nucleus, superior colliculus, and hippocampus, all areas largely devoid of Ret expression (27). The expression pattern of other members of the GFRα family also show discrepancies with that of Ret. In adult rat brain, for example, GFRα2 is highly expressed in extensive regions of the cerebral cortex and septum which show no Ret expression (21, 28). In the peripheral nervous system, Schwann cells are a rich source of GDNF and GFRα1, particularly after nerve lesion (27, 29), while they express no detectable levels of Ret or Ret-like GDNF-binding proteins (27). Based on these observations, and on the ability of GFRα receptors to bind ligand and activate Ret when provided exogenously in soluble form or immobilized on agarose beads (14, 28), we and others have proposed that GFRα receptors might also function in a non-cell autonomous way to capture and concentrate diffusible GDNF family ligands from the ex-

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[1] The abbreviations used are: GDNF, glial cell line-derive neurotrophic factor; GPI, glycosylphosphatidylinositol; PLC, phospholipase C; PAGE, polyacrylamide gel electrophoresis; CREB, cAMP response element-binding protein; GST, glutathione *S*-transferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PI-PLC, phosphatidylinositol-phospholipase C; PI3K, phosphatidylinositol 3-kinase; ATF-1, activating transcription factor 1.

tracellular space and then present these factors in *trans* to afferent Ret-expressing cells (27, 28). The possibility that GFRα receptors may in addition have cell-autonomous signaling functions independently of Ret has not been explored.

We have identified neuronal cell lines with endogenous expression of GDNF receptors which are therefore likely to also express natural downstream effectors of GDNF. In the work presented here, we compare the activation of intracellular signaling pathways by GDNF in two such lines expressing different complements of endogenous GDNF receptors. MN1 is a prototypic GDNF-responsive cell line (10) and expresses Ret as well as GFRα receptors. RN33B, on the other hand, expresses GFRα receptors but not Ret and is in this respect a representative of a large number of GFRα-expressing neurons in the nervous system. We find that GDNF elicits distinct signal transduction pathways in both these cell types and present evidence that supports a cell-autonomous signaling role for GFRα receptors independently of Ret.

EXPERIMENTAL PROCEDURES

Cell Lines—The generation and characterization of the raphe nucleus cell line RN33B (30, 31) and the motorneuron hybrid cell line 2F1.10.14 (referred to here as MN1) (32) have been previously described. L6 is a rat myoblast cell line and was obtained from the American Type Culture Collection (ATCC).

Protein Purification and Iodination—All binding and biochemical studies were carried out with recombinant GDNF produced in *Escherichia coli* (Peprotech, Inc.) or in Sf21 insect cells using a baculovirus expression system as described (6). GDNF was labeled with $Na^{125}I$ by either the chloramine-T or Bolton-Hunter methods to a specific activity of approximately $0.3–1.5 \times 10^8$ cpm/μg.

Biochemical Analysis of GDNF Receptor Complexes—For affinity labeling, 20–50 ng/ml ^{125}I-GDNF was first allowed to bind to cell monolayers for 2 h at 4 °C in binding buffer (Dulbecco's phosphate-buffered saline supplemented with 1 mM $CaCl_2$ and 1 mg/ml bovine serum albumin). Ligand-receptor complexes were chemically cross-linked for 30 min at room temperature using 1-ethyl-3-(-3-dimethyl-aminopropyl)-carbodiimide hydrochloride supplemented with *N*-hydroxysulfosuccinimide. Following quenching of the cross-linking reactions with glycine, cells were washed twice with 10 mM Tris/HCl-buffered saline, pH 7.5, pelleted and subsequently lysed in a small volume of Nonidet P-40 lysis buffer (10 mM Tris-HCl, pH 7.5, 137 mM NaCl, 2 mM EDTA, 10% glycerol, 1% Nonidet P-40) supplemented with a commercial mixture of protease inhibitors (Roche Molecular Biochemicals). Cleared lysates were boiled for 5 min in SDS/β-mercaptoethanol buffer, fractionated by SDS-PAGE on 4–20% gradient or 7.5% polyacrylamide electrophoresis gels, and visualized by PhosphorImaging in a Storm 840 (Molecular Dynamics). For phosphatidylinositol-phospholipase C (PI-PLC) treatment, cell monolayers were washed in serum-free medium and then incubated with 1 unit/ml PI-PLC (Roche Molecular Biochemicals) in Dulbecco's modified Eagle's medium for 90 min at 37 °C, followed by affinity labeling as above. For immunoprecipitation of affinity labeled receptor complexes, following binding and cross-linking with iodinated ligands cell lysates were cleared and incubated overnight at 4 °C with agarose beads conjugated to anti-phosphotyrosine monoclonal antibodies (UBI, New York). Immunocomplexes were collected, washed in ice-cold lysis buffer, and boiled for 5 min before SDS-PAGE and autoradiography as above.

Western Blotting, Detergent Fractionation, Immunoprecipitation, and Kinase Assays—MN1 cell monolayers in 10-cm plates were changed to serum-free media 16 h prior to incubation at 37 °C with 100 ng/ml GDNF for the indicated time periods and immediately lysed with 1 ml of ice-cold Nonidet P-40 lysis buffer (as above) supplemented with a mixture of protease inhibitors and a mixture of phosphatase inhibitors (1 mM sodium orthovanadate, 20 mM sodium fluoride, 10 mM β-glycerolphosphate). After 15 min lysis on ice, cell lysates were cleared by centrifugation. Immunoprecipitations were done by 4 °C overnight incubation of cell lysates with the corresponding antibodies plus 100 μl of Protein G-Sepharose bead slurry (GammaBind, Amersham Pharmacia Biotech, Uppsala, Sweden). Beads were washed five times with lysis buffer and boiled in SDS/β-mercaptoethanol buffer. Immunoprecipitates were fractionated by SDS-PAGE (10% polyacrylamide) and blotted to polyvinylidene difluoride membranes. Blots were probed with the indicated antibodies, followed by alkaline phosphatase-conjugated anti-IgG and developed with the ECF Western Detection System (Amersham Pharmacia Biotech, United Kingdom). All blots were scanned in a Storm 840 fluorimager (Molecular Dynamics) and quantified using ImageQuant software. Prior to reprobing, blots were first stripped by a 60-min incubation at room temperature in 0.2 M glycine-HCl, pH 2.4. Antibodies were obtained from various sources as follows: anti-phosphotyrosine, anti-Shc, anti-Grb2, anti-PLC-γ, anti-$p85^{PI3K}$ were from Upstate Biotechnology Inc. (Lake Placid, NY); anti-ERK, anti-Ret (long isoform), polyclonal anti-GFRα1 (used for immunoprecipitation), and anti-Src were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-Ras and monoclonal anti-GFRα1 (used for Western blotting) were from Transduction Laboratories (Lexington, KY); anti-CREB, anti-P-CREB (Ser-133), anti-Akt and anti-P-Akt (Ser-437) were from New England Biolabs. The activated Ras interaction assay was performed as described previously (33) using the pGEX-RBD plasmid encoding a GST-Raf1 (amino acids 1–149) fusion protein generously provided by Stephen Taylor, Cornell University, Ithaca, NY.

For total cell lysates (used for analyses of CREB phosphorylation), cells were lysed in SDS lysis buffer (0.5% SDS, 10 mM Tris-HCl, pH 7.5, 137 mM NaCl, 2 mM EDTA, 10% glycerol) supplemented with a mixture of protease inhibitors (as above) and a mixture of phosphatase inhibitors (as above). DNA in cell lysates was sheared by repeatedly passing the lysates through a G-26 needle.

Detergent fractionation of RN33B cell lysates was done by first lysing the cells for 15 min on ice with ice-cold Triton X lysis buffer (1% Triton X-100, 10 mM Tris-HCl, pH 7.5, 137 mM NaCl, 2 mM EDTA, 10% glycerol) supplemented with a mixture of protease inhibitors (as above) and a mixture of phosphatase inhibitors (as above). After mild centrifugation at 2000 rpm for 5 min, the cleared supernatant was saved (Triton X-soluble lysate) and the pellet was resuspended in ice-cold Triton X lysis buffer with supplements as above plus 60 mM β-octyl-D-glucopyranoside (Pierce), which solubilizes membrane rafts. After 15 min lysis on ice, lysates were cleared at high speed and supernatants were saved (Triton X-insoluble lysate). For kinase assays, following immunoprecipitation and washing, beads were further washed twice in kinase buffer (20 mM HEPES, pH 7.0, 3 mM $MgCl_2$, 2 mM $MnCl_2$, 150 mM NaCl, 1 mM sodium orthovanadate, 20 mM sodium fluoride, 10 mM β-glycerolphosphate) supplemented with protease inhibitors, and incubated at room temperature for 20 min in 50 μl of kinase buffer containing 20 μCi of [γ-^{32}P]ATP. After two washes with ice-cold kinase buffer, beads were resuspended in SDS/β-mercaptoethanol sample buffer, boiled, fractionated by SDS-PAGE, and blotted onto polyvinylidene difluoride membranes. The membranes were exposed to phosphor screens, which were subsequently scanned in a Storm 840 PhosphorImager (Molecular Dynamics) and quantified using ImageQuant software. After exposure, the membranes were probed with different antibodies as above. Pretreatments with 100 μM PD98059 (Calbiochem) or 10 μM PP1 (BioMol Research Laboratories) were done for 30 min at 37 °C prior to GDNF stimulation.

Analysis of mRNA Expression—For analysis of c-*fos* mRNA expression in cell lines, monolayers in 10-cm plates were changed to serum-free media 6–16 h prior to addition of 100 ng/ml GDNF. At the indicated time intervals, media was removed, cells solubilized in guanidine isothiocyanate containing β-mercaptoethanol, and RNA extracted as described previously (6). For Northern blotting, 20 μg of total RNA was fractionated on 1% agarose gels containing 0.7% formaldehyde and transferred to Hybond-C membranes (Amersham Pharmacia Biotech). Blots were hybridized with an [α-^{32}P]dCTP-labeled rat c-*fos* gene fragment, washed at high stringency and visualized by autoradiography on x-ray films. For RNase protection, riboprobes for rat *GFRα1*, *GFRα2*, c-*Ret,* and a c-*fos* were made as described previously (21, 27) using reagents from Promega; hybridization and digestion was according to protocols from Ambion Inc. As control, a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) riboprobe was added in the same reaction. After electrophoresis, dried gels were exposed to phosphor screens scanned in a Storm 840 PhosphorImager (Molecular Dynamics) and quantified using ImageQuant software. Reverse transcriptase-polymerase chain reaction analysis of c-*Ret* mRNA expression was made with a kit from Perkin-Elmer using adult rat brain RNA as positive control.

Survival Assays—Three independent passages (P35, P39, and P46) of RN33B cells were concurrently seeded at 10^5 cells/cm^2 in uncoated Costar 24-well tissue culture plates. After reaching 80% confluency under proliferating conditions, the cells were shifted to 39 °C in 1:1 Dulbecco's modified Eagle's medium:F-12 medium containing 0.1% bovine serum albumin and the B27 supplements (Life Technologies, Inc.). Cells were grown for 7 days with the indicated concentrations of GDNF. For PI-PLC treatments, the enzyme was added at 0.2 units/ml. The media was changed every 2 days and PI-PLC (were indicated) was added daily. Cell survival was scored by counting the number of differ-

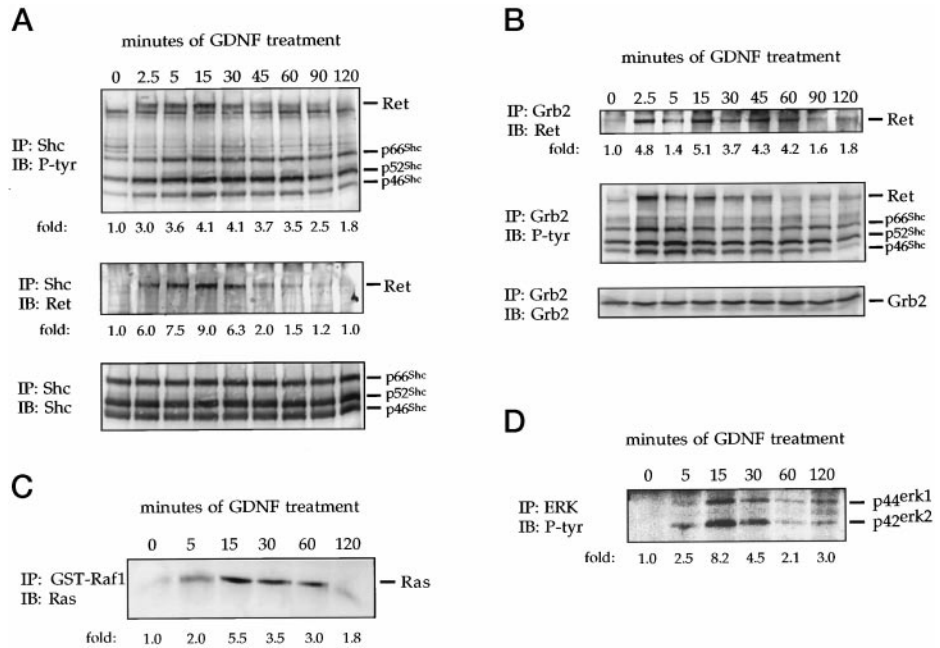

FIG. 1. **Activation of the Ras/ERK pathway by GDNF in MN1 cells.** *A*, all three isoforms of ShcA get rapidly phosphorylated upon stimulation of serum-starved MN1 cell monolayers with GDNF (*upper panel*). The *numbers below* the *lanes* indicate the fold induction of $p52^{Shc}$ phosphorylation relative to control. Also seen in this gel is the time course of tyrosine phosphorylation of Ret molecules that were pulled down together with Shc during the immunoprecipitation. Reprobing of the same filter with anti-Ret antibodies shows that Ret associates transiently with Shc after GDNF stimulation (*second panel*). The *numbers below* the *lanes* indicate the fold induction of Ret/Shc association relative to control. Note that most of the Shc remains phosphorylated after dissociating from Ret. The *bottom panel* shows the last reprobing of the same blot with Shc antibodies and demonstrates equal amounts of Shc protein in all the lanes. The results shown are representative of three independent experiments. *IP*, immunoprecipitation; *IB*, immunoblotting. *B,* Grb2 associates very rapidly with Ret after ligand stimulation of MN1 cells (*upper panel*). The *numbers below* the *lanes* indicate the fold induction of Ret/Grb2 association relative to control. Note the biphasic time course of the association between Grb2 and Ret. The *middle panel* shows tyrosine-phosphorylated Shc and Ret molecules pulled down together with Grb2 during the immunoprecipitation. The *lower panel* shows the final reprobing of the same blot with Grb2 antibodies and demonstrates equal amounts of Grb2 protein in all the lanes. The results shown are representative of two independent experiments. *C,* pull-down assay of activated GTP-loaded Ras with a GST-Raf fusion construct. GST-Raf will only bind to activated GTP-Ras, which after SDS-PAGE can be detected by immunoblotting with anti-Ras antibodies. The *numbers below* the *lanes* indicate the fold induction of GTP-Ras relative to control. The results shown are representative of two independent experiments. *D,* Western blot of MN1 lysates immunoprecipitated with anti-ERK antibodies and probed with anti-phosphotyrosine (*P-tyr*) antibodies. Both $p44^{erk1}$ and $p42^{erk2}$ get rapidly phosphorylated in MN1 cells stimulated with GDNF. The *numbers below* the *lanes* indicate the fold induction of $p44^{erk1}$ phosphorylation relative to control. The results shown are representative of two independent experiments.

entiated RN33B cells under phase optics in 5 random fields/well from three independent wells in each experiment. Data were analyzed by one way analysis of variance (ANOVA) and significant differences between individual treatment groups were analyzed by Tukey post-hoc T tests.

RESULTS

Ret-dependent Signaling in MN1 Cells, a Motor Neuron-derived Cell Line—MN1 is a line derived from mouse embryonic motorneurons immortalized by cell fusion with mouse neuroblastoma cells and selected for expression of choline acetyltransferase activity (32). MN1 cells express Ret (10) as well as several GFRα receptors, including GFRα1 (see below). GDNF binds to both types of receptors in MN1 cells and promotes cell survival upon serum withdrawal (10). GDNF stimulation of serum-starved MN1 monolayers results in rapid phosphorylation of tyrosine residues in the intracellular domain of the Ret protein (10). We sought to characterize effects downstream of this event, and first examined the activation of signaling components in the Ras/ERK pathway. Recruitment of Shc docking proteins by many receptor tyrosine kinases is a common route to Ras activation. In MN1 cells, we see a rapid increase in phosphorylation of the three ShcA isoforms p46, p52, and p66 starting 2.5 min after GDNF treatment with a peak at 15 min (Fig. 1*A*). Increased phosphorylation of Shc proteins is maintained for up to 2 h after GDNF treatment (Fig. 1*A*). In pull-down experiments, we see a rapid association between Shc and Ret beginning 2.5 min after ligand stimulation (Fig. 1*A*). This interaction is transient, peaks at 15 min, and decays 45 min after GDNF treatment, correlating with the dephosphorylation of Ret molecules co-immunoprecipitated with Shc (Fig. 1*A*). At this time, however, Shc still retains high levels of tyrosine phosphorylation (Fig. 1*A*).

A major link between receptor tyrosine kinases, Shc and the Ras pathway is the adaptor protein Grb2. It has recently been shown that Grb2 can be directly recruited to oncogenically activated Ret by binding to phosphorylated Y1096, in the C terminus of the long Ret isoform (34). We find that stimulation of MN1 cells with GDNF promotes a very rapid association between Grb2 and Ret (Fig. 1*B*). Interestingly, this interaction shows a biphasic time course, with a peak at 2.5 min and a re-association at 15 min (Fig. 1*B*). Grb2 can also be recruited indirectly to activated Ret via its interaction with Shc. We find high levels of Grb2 constitutively associated with Shc in MN1 cells, an interaction that seems to be only marginally affected by treatment with GDNF (data not shown). We note that the interaction between Grb2 and Ret seen at 15 min coincides with the maximal association between Shc and Ret, while the peak seen at 2.5 min could represent the direct association of Grb2 to the receptor.

Next, we investigated activation of Ras in MN1 cells treated with GDNF using a Raf1-GST fusion protein which selectively binds to the activated, GTP-loaded form of the Ras protein. We detect a dramatic increase in the GTP bound form of Ras beginning 5 min after treatment, peaking at 15 min and lasting for at least 1 h (Fig. 1*C*). As might be expected from Ras activation, we also detect increased and sustained levels of tyrosine-phosphorylated ERK1 and ERK2 15 min after stimu-

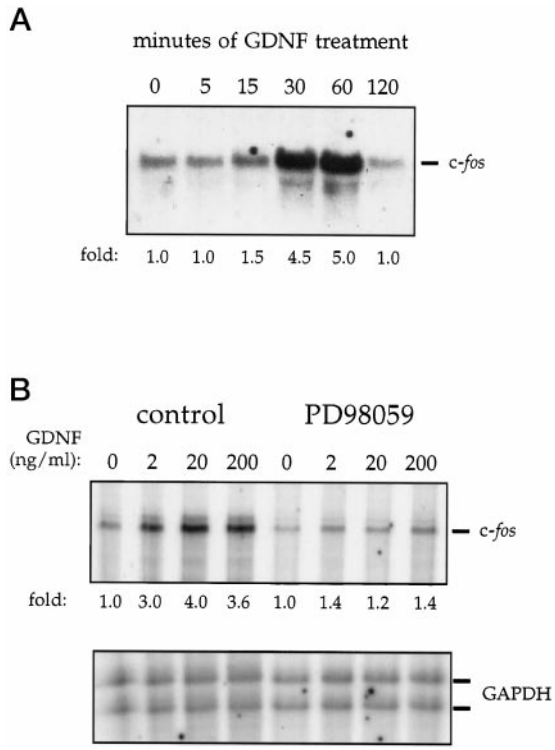

FIG. 2. **Up-regulation of c-*fos* mRNA in MN1 cells treated with GDNF is blocked by the MEK1 inhibitor PD98059.** *A,* Northern blot showing up-regulation of c-*fos* mRNA expression after GDNF treatment in MN1 cells. Twenty micrograms of total RNA was used per lane. The *numbers below* the *lanes* indicate the fold induction of c-*fos* mRNA expression relative to control. The results shown are representative of three independent experiments. *B,* RNase protection assay of MN1 cells treated with the indicated concentrations of GDNF (in ng/ml) for 30 min after no pretreatment (*control*) or pretreatment with PD98059 (*upper panel*). The *lower panel* shows the signals obtained by simultaneous hybridization with a glyceraldehyde 3′-OH phosphate dehydrogenase (*GAPDH*) riboprobe used as control for RNA loading. The *numbers below* the *lanes* in the *upper panel* indicate the fold induction of c-*fos* mRNA expression relative to control after normalization to the signal obtained with the *GAPDH* riboprobe.

lation with GDNF (Fig. 1*D*). Phosphorylation of ERKs is known to result in their translocation to the nucleus where they phosphorylate transcription factors that induce expression of specific immediate-early genes. In MN1 cells treated with GDNF, we see an up-regulation of c-*fos* mRNA 30 min after treatment which is sustained for up to 1 h (Fig. 2*A*). The increase in c-*fos* mRNA by GDNF treatment is dose-dependent, saturates between 20 and 200 ng/ml GDNF, and can be completely prevented by pretreatment with PD98059, a specific inhibitor of MEK1, the upstream regulator of ERK1 and ERK2 (Fig. 2*B*). Together, these results indicate that GDNF stimulation of cells which endogenously express Ret results in the activation of the Ras/ERK signaling cascade which leads to activation of ERKs and up-regulation of immediate early genes.

In addition to the Ras/ERK cascade, we also find that GDNF treatment of MN1 cells triggers rapid phosphorylation of the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K) (Fig. 3*A*). One of the downstream targets of PI3K, the serine-threonine kinase Akt (Fig. 3*B*), also gets rapidly phosphorylated on Ser-437 upon GDNF treatment, an event known to correlate with activation of Akt. Phosphorylation of Akt is sustained and lasts between 1 and 2 h after GDNF stimulation (Fig. 3*B*). Activation of the PI3K/Akt pathway by GDNF could be responsible for the survival activities of this neurotrophic factor on MN1 cells (10).

Finally, we also see increase in tyrosine phosphorylation of PLC-γ (Fig. 3*C*), as well as transient phosphorylation of CREB (cAMP responsive element-binding factor) in Ser-133 and the CREB-related protein ATF-1 (activating transcription factor-1) (Fig. 3*D*). CREB phosphorylation, which results in activation of this transcription factor, is first seen 5 min after GDNF stimulation, peaks at 10 min, and returns to basal levels by 30 min (Fig. 3*D*).

Ret-independent Signaling in RN33B Cells, a Conditionally Immortalized Neuronal Precursor—The neuronally restricted RN33B precursor cell line was developed by infecting embryonic rat raphe nucleus neuroblasts with a retrovirus encoding the temperature-sensitive mutant of the SV40 large T antigen. Following a switch to the non-permissive temperature (39 °C) in defined serum-free medium, RN33B cells withdraw from the cell cycle and differentiate into neurons (31). Although immortalized, RN33B cells are not transformed, will not form tumors, and will differentiate and incorporate into normal brain tissue upon transplantation (35, 36). Undifferentiated RN33B cells express high levels of GFRα1 and lower levels of GFRα2 mRNAs as determined by RNase protection assay (Fig. 4*A*). No Ret mRNA expression was detected in these cells using riboprobes derived from two different regions of the Ret transcript (one of which included the tyrosine kinase domain) (Fig. 4*A*). Reverse transcriptase-polymerase chain reaction experiments also failed to detect any *ret* mRNA in either undifferentiated or differentiated RN33B cells (data not shown). As expected, MN1 cells express high levels of *ret* mRNA (Fig. 4*A*), as well as moderate levels of GFRα1, GFRα2 (Fig. 4*A*), and GFRα3 (not shown) mRNAs.

Cross-linking of RN33B cells with ^{125}I-GDNF results predominantly in the labeling of a complex of 75,000–90,000 corresponding to GFRα receptors (Fig. 4*B*). Low amounts of 150,000–160,000 complexes can also be seen in some gels, which likely correspond to a GFRα dimer cross-linked to ^{125}I-GDNF. The high molecular weight smear of >200,000 resembles complexes formed after cross-linking of other GPI-linked receptors in several cell types (37), and probably corresponds to high molecular weight aggregates of GFRα receptors. All the complexes labeled by ^{125}I-GDNF in RN33B cells are sensitive to pretreatment with PI-PLC (Fig. 4*B*), indicating the requirement of GPI-linked receptors for the binding of GDNF to these cells. No ^{125}I-GDNF-labeled complex corresponding to the Ret receptor can be seen in undifferentiated RN33B cells (Fig. 4*B*) or in differentiated cells (not shown). Moreover, although we can immunoprecipitate a Ret-GDNF complex from affinity-labeled MN1 cells using anti-phosphotyrosine antibodies (10), we cannot pull down any of the cross-linked complexes from RN33B cells with these antibodies (data not shown), suggesting that GDNF does not associate with receptor tyrosine kinases in these cells.

Cell survival of RN33B cells during differentiation can be increased by the addition of trophic factors such as brain derived neurotrophic factor to the differentiation medium.[2] Despite the lack of Ret expression in RN33B cells, addition of GDNF to cells grown at 39 °C results in a dose-dependent 2-fold increase in the number of surviving differentiated cells (Fig. 5*A*). The survival effect of GDNF is abolished when the cells are pretreated with PI-PLC (Fig. 5*B*), indicating a requirement for GPI-anchored receptor molecules in the cell membrane.

As a first step to investigate possible signal transduction mechanisms activated by GDNF in RN33B cells, we looked at the Ras/ERK and PI3K/Akt pathways. Unlike MN1 cells, GDNF treatment of RN33B cells does not result in Shc phos-

[2] S. R. Whittemore, unpublished observations.

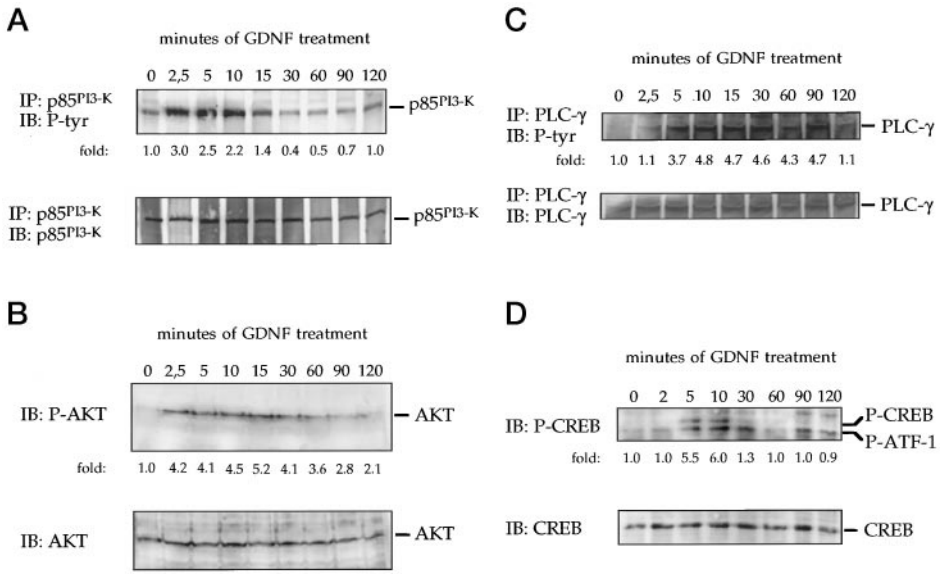

FIG. 3. **Stimulation of MN1 cells with GDNF leads to activation of the PI3K/Akt pathway, transient phosphorylation of PLCγ, and phosphorylation of CREB.** *A,* rapid tyrosine phosphorylation of the regulatory subunit of PI3K p85^{PI3K} (*upper panel*). The *numbers below* the *lanes* indicate the fold induction of p85^{PI3K} phosphorylation relative to control. The *lower panel* shows a reprobing of the same filter with anti-p85^{PI3K} antibodies and demonstrates comparable amounts of p85^{PI3K} protein in all the lanes. The results shown are representative of two independent experiments. *B,* rapid and sustained phosphorylation of Akt in Ser-437 demonstrated by immunoblot of total cell lysates with a specific anti-phospho-Akt antibody (*upper panel*). The *numbers below* the *lanes* indicate the fold induction of Akt phosphorylation relative to control. The *lower panel* shows a reprobing of the same filter with anti-Akt antibodies and demonstrates comparable amounts of Akt protein in all the lanes. The results shown are representative of three independent experiments. *C,* tyrosine phosphorylation of PLCγ in MN1 cells stimulated with GDNF. The *numbers below* the *lanes* indicate the fold induction of PLCγ phosphorylation relative to control. The *lower panel* shows a reprobing of the same filter with anti-PLCγ antibodies and demonstrates comparable amounts of PLCγ protein in all the lanes. *D,* phosphorylation of CREB in Ser-133 demonstrated by immunoblot of total cell lysates with a specific anti-phospho-CREB antibody (*upper panel*). Also seen in this gel is the phosphorylation of the CREB-related protein ATF-1. The *numbers below* the *lanes* indicate the fold induction of CREB phosphorylation relative to control. The *lower panel* shows a reprobing of the same filter with anti-CREB antibodies and demonstrates comparable amounts of CREB protein in all the lanes. The results shown are representative of two independent experiments.

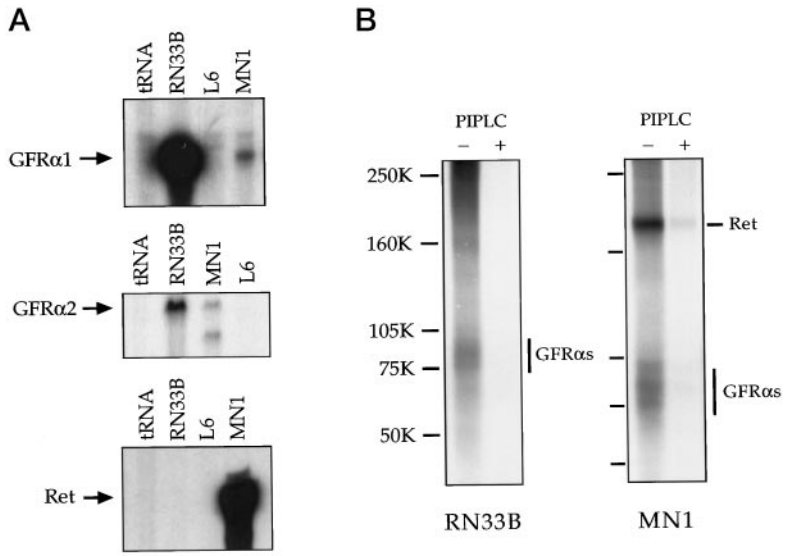

FIG. 4. **Expression of GFRα receptors but not Ret in RN33B cells.** *A,* RNase protection assays showing expression of GFRα1 (*upper panel*), GFRα2 (*middle panel*), and Ret (*lower panel*) in different cell lines. Ten micrograms of total RNA was used in all cases, except in the RN33B sample hybridized with the GFRα1 riboprobe in which 5 μg of poly(A)$^+$ RNA was used. MN1 cells express high levels of *ret* mRNA and moderate levels of *GFRα1* and *GFRα2* mRNA. RN33B cells express high levels of *GFRα1* mRNA, lower levels of *GFRα2* mRNA. *Ret* mRNA was undetectable in RN33B cells using a riboprobe derived from the extracellular region of the rat *ret* gene. Additional assays were carried out with a probe spanning the region encoding the Ret tyrosine kinase domain with identical results (not shown). No mRNA for any of the known GDNF receptors was detected in the rat myoblast line L6. Yeast tRNA (tRNA) was assayed in parallel to control for nonspecific fragments. *B,* affinity labeling of RN33B cells with iodinated GDNF (*left panel*). Binding of ^{125}I-GDNF to RN33B cells followed by chemical cross-linking and SDS-PAGE reveals one major complex of 75,000–90,000 corresponding to GFRαs cross-linked to a monomer of ^{125}I-GDNF. A smear of ≥200,000 can also be seen which resembles complexes formed after cross-linking of several other GPI-linked receptors. Treatment of RN33B cells with PI-PLC abolishes all GDNF binding to RN33B cells. A similar experiment performed on MN1 cells (*right panel*) is shown for comparison.

phorylation, activation of Ras, or phosphorylation of p85^{PI3K} or Akt (data not shown). However, GDNF stimulation elicits a very rapid increase in c-*fos* mRNA in RN33B cells with a peak 15 min after treatment (Fig. 6*A*), much faster than the time course of the responses normally elicited by activation of receptor tyrosine kinases. Consistent with the lack of Ras activation,

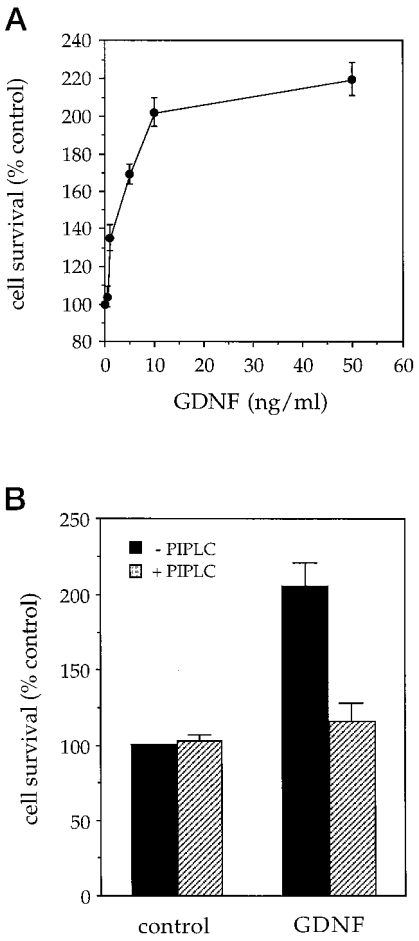

FIG. 5. **Protective effects of GDNF in differentiating RN33B cells.** *A,* dose-response curve for the survival effects of GDNF on differentiated RN33B cells. Data are normalized to the percent control for each of three independent cell preparations and are expressed as the mean ± S.E. *B,* effects of PI-PLC on GDNF-induced RN33B cell survival. Cells were treated ± 50 ng/ml GDNF and ± 0.2 units/ml PI-PLC. Data are normalized to the percent control for each of three independent cell preparations and are expressed as the mean ± S.E. ANOVA showed that the data were statistically significant (d.f. = 3, 8; F = 26.18; $p < 0.001$). Tukey's post-hoc *t* test showed that GDNF treatment in the absence of PI-PLC was statistically different from all other treatment groups ($p < 0.001$) and that there was no statistical difference between control, PI-PLC, and GDNF/PI-PLC groups.

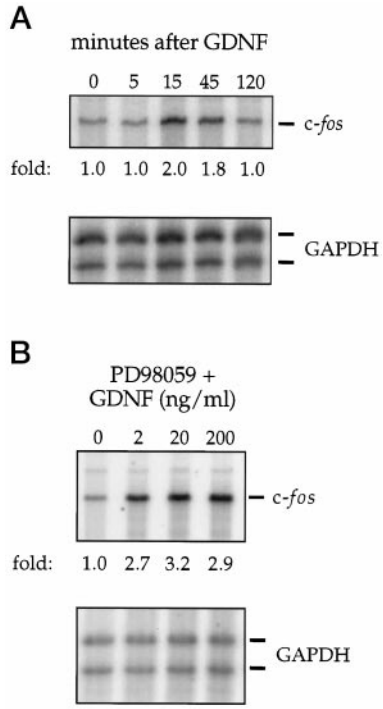

FIG. 6. **Rapid and transient up-regulation of c-*fos* mRNA in RN33B cells treated with GDNF.** *A,* RNase protection assay showing the time course of increase in c-*fos* mRNA levels induced by GDNF in RN33B cells. The *numbers below* the *lanes* indicate the fold induction of c-*fos* mRNA levels relative to control normalized to the levels of GAPDH mRNA. The *lower panel* shows GAPDH expression in the same samples. The results shown are representative of three independent experiments. *B,* dose-dependent up-regulation of c-*fos* mRNA in RN33B cells treated with GDNF in the presence of the MEK1 inhibitor PD98059. The *numbers below* the *lanes* indicate the fold induction of c-*fos* mRNA levels relative to control normalized to the levels of GAPDH mRNA. The *lower panel* shows GAPDH expression in the same samples.

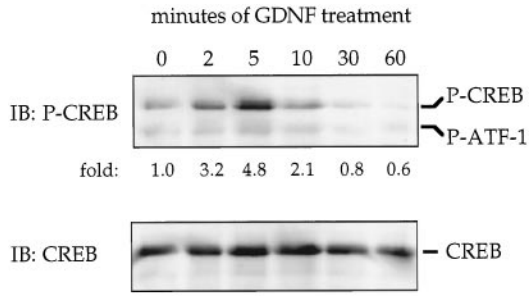

FIG. 7. **Rapid increase in CREB phosphorylation after GDNF treatment of RN33B cells.** GDNF treatment of RN33B cells induces a rapid increase in phosphorylation of CREB in Ser-133 (*upper panel*). ATF-1 phosphorylation in RN33B cells is not affected by GDNF treatment. The *numbers below* the *lanes* indicate the fold induction of CREB phosphorylation relative to control normalized to the levels of CREB. The *lower panel* shows the reprobing of the same filter with anti-CREB antibodies. The results shown are representative of three independent experiments.

and in contrast to MN1 cells, pretreatment of RN33B cells with the MEK1 inhibitor PD98059 does not block up-regulation of c-*fos* mRNA in GDNF-treated cells (Fig. 6*B*). The distinct kinetics and insensitivity to PD98059 of the c-*fos* mRNA response in RN33B cells indicate a signal transduction mechanism for GDNF that is different from that seen in MN1 cells.

As the involvement of the ERK pathway seemed unlikely, we turned our attention to CREB, also known to be a major regulator of c-*fos* transcription (38). We find that CREB phosphorylation in Ser-133 increases with very rapid kinetics following GDNF stimulation of RN33B cells (Fig. 7). Increased CREB phosphorylation can be seen 2 min after GDNF stimulation, peaks at 5 min, and decays quickly thereafter, typically below baseline levels (Fig. 7). Unlike MN1 cells, however, GDNF treatment of RN33B cells does not result in phosphorylation of the CREB-related protein ATF-1 (compare Figs. 3*D* and 7).

A prevalent signaling event induced by clustering or ligation of a number of GPI-linked receptors is the stimulation of a detergent-insoluble, receptor-associated Src family kinase activity (39, 40). We investigated the presence of kinase activity in GFRα1 immunoprecipitates from RN33B cells stimulated with GDNF. Immunoprecipitation of Triton X-soluble and -insoluble RN33B cell lysates with GFRα1 antibodies followed by *in* vitro kinase assay reveals one major endogenous substrate of 110,000 in both lysates as well as two additional phosphorylated proteins of 55,000 and 60,000 in the detergent-insoluble fraction (Fig. 8, *A* and *C, arrowheads*). Stimulation of RN33B cells with GDNF results in a transient increase in kinase activity toward all three substrates in the Triton X-insoluble fraction (Fig. 8, *A* and *B*). No increased phosphorylation can be detected in the immunoprecipitated Triton X-soluble fraction (Fig. 8*C*), or in the total detergent-insoluble fraction prior to

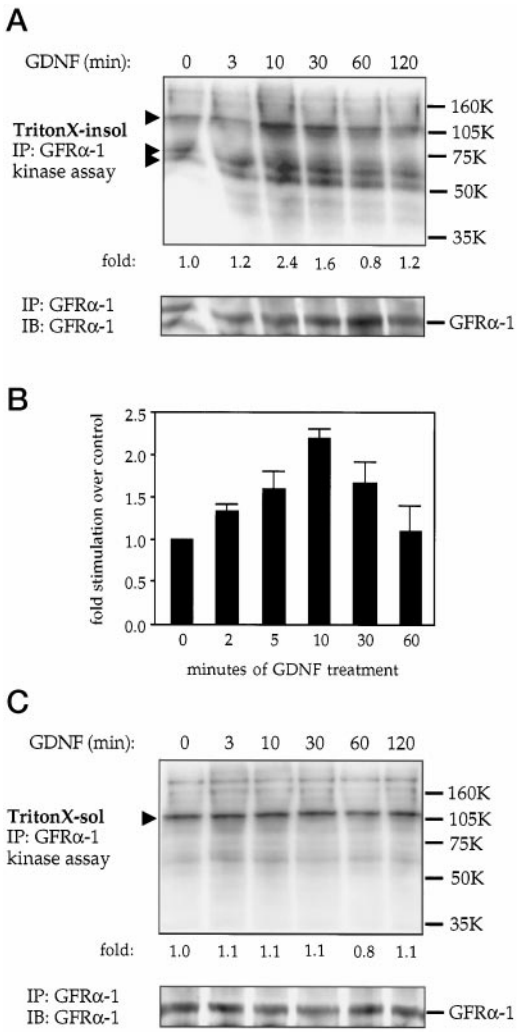

FIG. 8. **GDNF induces the stimulation of a kinase activity associated with GFRα1 in detergent-insoluble cell compartments.** *A,* an *in vitro* kinase assay was performed on GFRα1 immunoprecipitates (*IP*) from Triton X-insoluble lysates of RN33B cells treated with GDNF (*upper panel*). Endogenous phosphorylated substrates of 55,000, 60,000, and 110,000 are indicated with *arrowheads*. Increased phosphorylation of all three proteins can be seen following GDNF treatment. The *numbers below* the *lanes* indicate the fold induction in phosphorylation of the 110,000 protein relative to control normalized to the levels of GFRα1. The *lower panel* shows a reprobing of the same filter with anti-GFRα1 antibodies and demonstrates comparable amounts of GFRα1 protein in all lanes. *B,* time course of GDNF stimulation of autokinase activity in GFRα1 immunoprecipitates of Triton X-insoluble cell lysates. Data are normalized to control for each of three independent experiments and are expressed as the mean ± S.E. ANOVA indicates significant increase ($p < 0.005$) in autokinase activity at 10 min compared to control. *C, in vitro* kinase assay performed on GFRα1 immunoprecipitates from Triton X-soluble lysates of RN33B cells treated with GDNF. An endogenous phosphorylated substrate of 110,000 is indicated by the *arrowhead*. Note that, in detergent-soluble lysates, GDNF treatment does not result in increased phosphorylation of this protein. The *lower panel* shows a reprobing of the same filter with anti-GFRα1 antibodies and demonstrates comparable amounts of GFRα1 protein in all lanes.

immunoprecipitation (not shown). These data suggest that in RN33B cells GDNF signaling begins within domains of ligand-activated GFRα1 receptors in detergent-insoluble compartments, presumably membrane rafts.

To investigate the nature of the kinase activity that co-immunoprecipitates with GFRα1 in RN33B cells, we utilized PP1, a specific inhibitor of Src family kinases (41). Addition of PP1 during the *in vitro* kinase assay of GFRα1 immunoprecipitates from RN33B cells attenuates by 80% the phosphorylation of endogenous substrates (Fig. 9*A*), indicating that a substantial fraction of the *in vitro* kinase activity associated with GFRα1 comes from a Src-like kinase. We then examined the role of Src family kinases in the stimulation of GFRα1-associated kinase activity and CREB phosphorylation induced by GDNF in RN33B cells. Pretreatment with PP1 of RN33B cell monolayers prevents the rapid increase in kinase activity induced by GDNF in GFRα1 immunoprecipitates isolated from detergent-insoluble cell lysates (Fig. 9*B*). Basal phosphorylation in unstimulated cells is unchanged after PP1 treatment, indicating that the drug blocks a trans- or autophosphorylation event involved in kinase activation.

Consistent with the involvement of a Src-like kinase, a Src family member of 60,000–65,000 can be detected in GFRα1 immunoprecipitates after immunoblotting with a pan-Src antibody (Fig. 9*B, middle panels*). GDNF stimulation causes a moderate increase in the amounts of Src-like proteins in GFRα1 immunoprecipitates from both control and PP1-treated cells, suggesting that ligation of GFRα1 stimulates the recruitment of a Src-like kinase to membrane compartments containing this receptor. This notion is supported by the presence of GFRα1 in immunoprecipitates of Src-like proteins prepared from Triton X-insoluble lysates of cells stimulated with GDNF (Fig. 9*C, middle panel*). Moreover, a 2-fold increase in tyrosine phosphorylation of Src-like proteins can be seen in detergent-insoluble compartments after GDNF treatment of RN33B cells (Fig. 9*C, upper panel*). PP1 treatment also blocks the early increase in CREB phosphorylation induced by GDNF in RN33B cells (Fig. 9*D*), showing that this event is downstream of the activation of a Src-like kinase.

Finally, as a first step to verify whether this pathway may also be operational in GFRα-only neurons, we investigated the stimulation of GFRα1-associated kinase activity in differentiated RN33B cells. As can be seen in Fig. 10*A*, a dramatic change in the morphology of RN33B cells occurs upon 7-day differentiation in defined medium. Differentiated RN33B cells have phase-bright, round cell bodies and elaborate a dense network of neurites. These cells express no active oncogene, and by all criteria examined so far they are irreversibly differentiated, postmitotic neuronal cells. As in mitotically active cells, increased kinase activity toward substrates of 55,000, 60,000, and 110,000 can be seen in GFRα1 immunoprecipitates prepared from Triton X-insoluble lysates of 7-day differentiated cells stimulated with GDNF (Fig. 10*B*). Interestingly, there appears to be a more robust and sustained increase in kinase activity in these cells as compared with undifferentiated cells. This response can be prevented by pretreatment of differentiated RN33B cell monolayers with the Src family kinase inhibitor PP1 (Fig. 10*C*).

DISCUSSION

Although much effort has been devoted to understanding of the intracellular pathways activated by oncogenic forms of Ret, considerably less is known about the signaling mechanisms activated by its endogenous ligands and co-receptors in neuronal cells. We have investigated and compared the kinetics of activation of several signal transduction pathways in two cell lines of neuronal origin expressing different complements of endogenous GDNF receptors. We present evidence of at least two distinct signal transduction pathways for GDNF in neuronal cells (Fig. 11). Activation of Ret via complex formation with GDNF and GFRα1 in MN1 cells leads to a series of signaling events similar to those observed downstream of other neurotrophic receptor tyrosine kinases, including rapid recruitment and phosphorylation of adaptor and docking proteins, sustained activation of the Ras/ERK and PI3K/Akt pathways, phosphorylation of phospholipase C-γ and the transcription

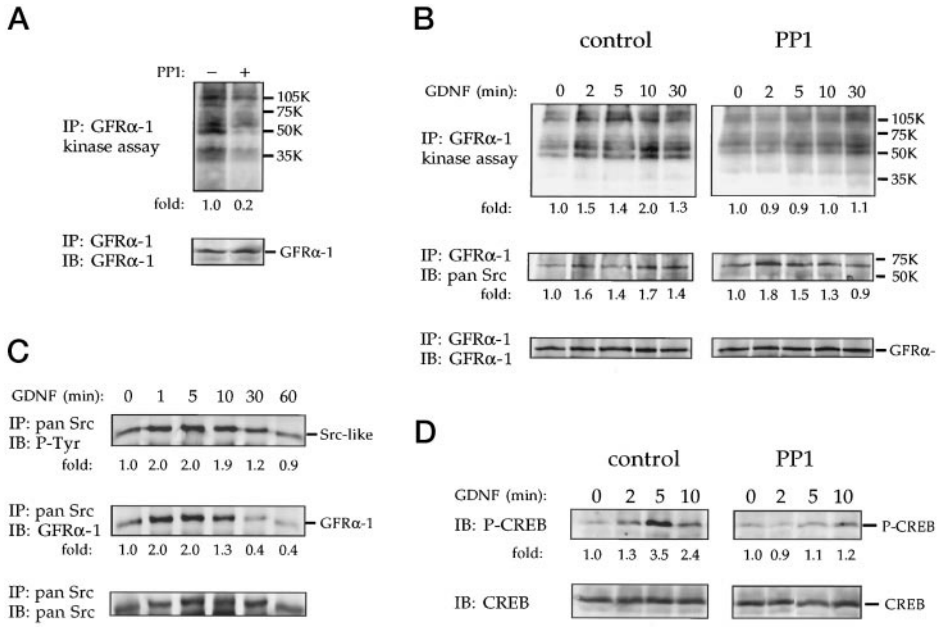

FIG. 9. **Role of Src family kinases in GDNF signaling in RN33B cells.** *A, in vitro* kinase assays were perfomed on GFRα-1 immunoprecipitates (*IP*) from RN33B cells in the presence or absence of the Src family kinase inhibitor PP1. Addition of the inhibitor results in 80% reduction in phosphorylation of endogenous substrates. The *lower panel* shows a reprobing of the filter with anti-GFRα1 antibodies. *B,* RN33B cells were pretreated with PP1 and then stimulated with GDNF for the indicated times. *In vitro* kinase assays were performed on GFRα1 immunoprecipitates from Triton X-insoluble lysates (*upper panels*). The *numbers below* the *lanes* indicate the fold induction in phosphorylation of the 60,000 protein relative to control normalized to the levels of GFRα1. After exposure, filter membranes were probed with a pan-Src antibody (*middle panels*) demonstrating the presence of a Src family kinase in GFRα1 immunoprecipitates. The *numbers below* the *lanes* indicate the fold induction of co-immunoprecipitation of Src-like kinase relative to control normalized to the levels of GFRα1. The *lower panel* shows a reprobing of the filter with anti-GFRα1 antibodies. The results shown are representative of three independent experiments. *C,* tyrosine phosphorylation of Src-like proteins stimulated by GDNF treatment in detergent-insoluble compartments of RN33B cells. Cells were treated with GDNF for the indicated times. Src-like proteins were immunoprecipitated from Triton X-insoluble cell lysates using anti-pan-Src antibodies and then analyzed for phosphotyrosine (*upper panel*) and GFRα1 co-immunoprecipitation (*middle panel*) by Western blotting. *Numbers below* the *lanes* indicate fold induction relative to control normalized to the level of Src-like proteins in each lane. The *bottom panel* shows a reprobing of the same filter with anti-pan-Src antibodies. *D,* treatment of RN33B cells with PP1 prevents the rapid increase in CREB Ser-133 phosphorylation induced by GDNF (*upper panels*). The *numbers below* the *lanes* indicate the fold induction of CREB phosphorylation relative to control normalized to the levels of CREB. The *lower panel* shows the reprobing of the same filter with anti-CREB antibodies and demonstrates comparable amounts of CREB protein in all lanes. The results shown are representative of two independent experiments.

factor CREB, and increased c-*fos* mRNA expression. On the other hand, in cells expressing high levels of GFRα receptors in the absence of Ret, such as RN33B, GDNF binding to GFRα1 stimulates the activation and/or recruitment of a receptor-associated Src-like kinase activity that triggers a signaling pathway leading to rapid phosphorylation of CREB and increased c-*fos* transcription independent of the Ras/ERK pathway.

Several lines of evidence support the existence of GDNF signaling mechanisms that are independent of Ret in RN33B cells: (i) no Ret expression can be detected in these cells by either a sensitive RNase protection assay or reverse transcriptase-polymerase chain reaction; (ii) no Ret-like receptor can be detected by affinity labeling of RN33B cell monolayers with ^{125}I-GDNF; (iii) no affinity labeled GDNF receptor complexes in RN33B cells can be immunoprecipitated with anti-phosphotyrosine-specific antibodies. While it is difficult to base an argument on the failure to detect a molecule, several other features of the intracellular signaling events seen in RN33B cells suggest that these are not the result of the activation of a Ret-like receptor tyrosine kinase. In particular, the fact that GDNF treatment does not result in activation of Ras, while still being able to increase c-*fos* mRNA levels in the presence of a MEK1 inhibitor, indicates that the mechanisms of GDNF signaling in RN33B cells are qualitatively different from those typically activated by receptor tyrosine kinases.

Based on our results, we would instead argue that GDNF binding to GFRα1, and perhaps also GFRα2, on RN33B cells activates signaling pathways not unlike those seen after ligation of other GPI-linked receptors, such as Thy-1, the lipopolysaccharide receptor (CD14), FcγRIIIb (CD16), urokinase-type plasminogen activator receptor (CD87), F3 and contactin (F11) (for recent reviews on GPI-anchored protein signaling, see Refs. 39 and 40). Despite some variability in the more downstream signaling events, one of the first components engaged by all GPI-linked receptors studied so far appears to be a member of the Src family of protein tyrosine kinases (39). However, it is not clear how GPI-anchored receptors may couple with Src-like kinases, as these two molecules are found on opposite sides of the lipid bilayer. One model proposes that a transmembrane co-receptor links GPI-anchored receptors and Src family kinases. A contactin-associated transmembrane protein, p190^{Caspr}, has recently been identified which contains a proline-rich sequence in its cytoplasmic domain that could mediate coupling with Fyn through the Fyn SH3 domain (42). In the case of the urokinase-type plasminogen activator receptor, two integrins have been shown to associate with this complex along with Src family kinases (43). A second model suggests that Src kinases colocalize with GPI-linked proteins via their N-terminal fatty acylation modifications, myristylation and palmitylation. Thus, rather than providing direct interaction, the lipid modification may colocalize Src family kinases and GPI-linked proteins in distinct glycoprotein-rich membrane domains. Detergent-resistant membranes containing both GPI-anchored proteins and Src family kinases can be isolated from several cells including neurons (44, 45). Membrane rafts that are formed or stabilized when GPI-anchored proteins are clustered may play an important role in signaling through GPI-

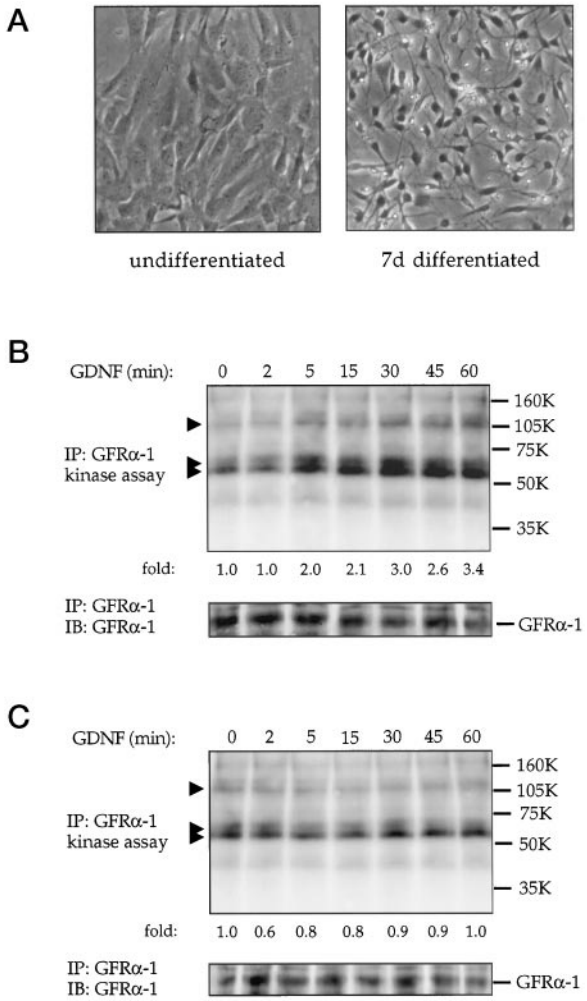

FIG. 10. **Stimulation of Src-like kinase activity in postmitotic, irreversibly differentiated RN33B cells.** *A,* morphology of mitotically active RN33B cells growing at the permissive temperature (33 °C) in the presence of serum (*left panel*) and 7-day differentiated cells growing at 39 °C in defined medium (*right panel*). Note that differentiated cells have round, phase-bright, small cell bodies and elaborate a dense network of neurites. Both panels represent phase-contrast photomicrographs taken at the same magnification. *B, in vitro* kinase assay performed on GFRα1 immunoprecipitates (*IP*) from Triton X-insoluble lysates of 7-day differentiated RN33B cells treated with GDNF (*upper panel*). Endogenous phosphorylated substrates of 55,000, 60,000, and 110,000 are indicated with *arrowheads*. The *numbers below* the *lanes* indicate the fold induction in phosphorylation of the 55,000 and 60,000 proteins relative to control normalized to the levels of GFRα1. The *lower panel* shows a reprobing of the same filter with anti-GFRα1 antibodies and demonstrates comparable amounts of GFRα1 protein in all lanes. The results shown are representative of two independent experiments. *C, in vitro* kinase assay after pretreatment with the Src family kinase inhibitor PP1. The *lower panel* shows a reprobing of the same filter with anti-GFRα1 antibodies.

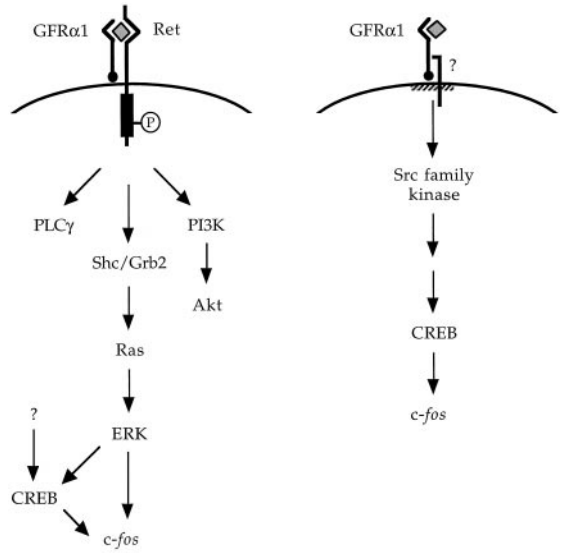

FIG. 11. **Ret-dependent and -independent mechanisms of GDNF signaling in neuronal cells.** The *left side* summarizes Ret-dependent signaling pathways in MN1 cells. The *right side* summarizes Ret-independent pathways in RN33B cells. *Arrows* do not implicate direct interactions but the deduced or presumed order of different signaling components. On the *right*, the *hatched box* represents detergent-insoluble membrane rafts. A hypothetical transmembrane adaptor protein for GFRα signaling is indicated.

linked receptors (37, 46). Thus, it is possible that proteins that are downstream of GPI-linked receptors in signaling might be concentrated and activated simply by partitioning into these rafts, without binding the GPI-anchored protein directly; rafts in the outer bilayer leaflet may somehow be coupled to rafts in the inner leaflet, possibly through monolayer coupling (40).

Our results do not exclude the existence of a transmembrane co-receptor linking GFRα molecules with Src family kinases. In fact, some of the phosphorylation substrates that co-immunoprecipitate with GFRα1 from detergent-insoluble membrane compartments could constitute good candidates for such function. The fact that both the increase in kinase activity as well as CREB phosphorylation can be prevented by treatment with the Src family inhibitor PP1 indicates that such kinases may be important components of the signaling pathway activated by GDNF in cells expressing GFRα receptors in the absence of Ret. Intriguingly, although MN1 cells also express GFRα receptors, all the c-*fos* mRNA response in these cells can be blocked by the MEK1 inhibitor PD98059, suggesting that in cells expressing GFRαs together with Ret, the pathways activated by the Ret tyrosine kinase are dominant over Ret-independent signaling mechanisms. It is worth noting, however, that MN1 cells express much lower levels of GFRα receptors than RN33B cells, suggesting that perhaps these receptors must be expressed above a certain level in order to signal independently of Ret. It is also possible that, upon ligand binding, Ret sequesters GFRα receptors away from the membrane compartments from which they normally signal.

The pronounced discrepancies in the expression patterns of Ret and GFRα receptors have led to the proposal that these GPI-anchored proteins might also work as ligand-presenting molecules in a non-cell autonomous fashion. An alternative role for GFRα receptors expressed in the absence of Ret, which does not exclude non-cell autonomous functions, is suggested by the data presented here showing that these GPI-anchored molecules can directly or indirectly mediate a cell-autonomous response to ligand independently of Ret. Our observation of increased kinase activity in postmitotically differentiated RN33B cells treated with GDNF suggests that a similar pathway may also be operational in primary neurons. Biological responses that could be mediated by direct signaling from GFRα receptors include acute plasticity-related responses, changes in gene expression, and cell survival. Genes regulated by transcription factors of the CREB family could be primary targets of the GFRα signaling pathway. Interestingly, members of the Src family of protein tyrosine kinases have recently been shown to directly modulate the activity of *N*-methyl-D-asparatate glutamate receptors in the hippocampus, a rich site of GFRα expression, and in this way regulate synaptic efficacy (47, 48). Local activation of Src family kinases by GDNF ligands could thus constitute a possible mechanism by which these factors influence synaptic transmission in the brain. Unfortunately, com-

parisons of the phenotypes of mutant mice generated by homologous recombination lacking expression of Ret, GDNF, or GFRα1 are not likely to be very useful in revealing possible roles of GFRα signaling *in vivo* as the early postnatal death of all these animals precludes the study of a mature nervous system (49–54).

In conclusion, the evidence presented here indicates that, in addition to its expected effects on Ret-expressing cells, GDNF is also able to signal in cells lacking Ret receptors via a mechanism that involves binding to GFRα1 receptors, stimulation of a cytoplasmic GFRα1-associated Src-like kinase activity, and activation of CREB. These findings considerably extend the range of target cells that could potentially be affected by GDNF family ligands.

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