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Released GFRα1 Potentiates Downstream Signaling, Neuronal Survival, and Differentiation via a Novel Mechanism of Recruitment of c-Ret to Lipid Rafts

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Summary

Although both c-Ret and GFRα1 are required for responsiveness to GDNF, GFRα1 is widely expressed in the absence of c-Ret, suggesting alternative roles for "ectopic" sites of GFRα1 expression. We show that GFRα1 is released by neuronal cells, Schwann cells, and injured sciatic nerve. c-Ret stimulation in *trans* by soluble or immobilized GFRα1 potentiates downstream signaling, neurite outgrowth, and neuronal survival, and elicits dramatic localized expansions of axons and growth cones. Soluble GFRα1 mediates robust recruitment of c-Ret to lipid rafts via a novel mechanism requiring the c-Ret tyrosine kinase. Activated c-Ret associates with different adaptor proteins inside and outside lipid rafts. These results provide an explanation for the tissue distribution of GFRα1, supporting the physiological importance of c-Ret activation in *trans* as a novel mechanism to potentiate and diversify the biological responses to GDNF.

Introduction

Signaling by many neurotrophic factors involves the activation of receptors with intrinsic tyrosine kinase activity. The c-Ret receptor tyrosine kinase is an essential component of the receptor for a class of structurally and functionally related neurotrophic factors, the GDNF ligand family (Durbec et al., 1996; Trupp et al., 1996; Vega et al., 1996). GDNF (glial cell line–derived neurotrophic factor) was originally discovered as a potent survival factor for ventral midbrain dopaminergic neurons (Lin et al., 1993) but later found to also have pronounced effects on motorneurons, sensory neurons, and other neuronal subpopulations (Airaksinen et al., 1999). Outside the nervous system, GDNF has important roles as a morphogenetic factor in developing kidney and in the differentiation of spermatogonia (Sariola and Saarma, 1999; Meng et al., 2000). c-Ret is unable to bind GDNF on its own but can be activated in a complex with GFRα1 (GDNF family receptor α-1), a glycosyl phosphatidylinositol (GPI)-anchored protein that binds GDNF with high affinity (Jing et al., 1996; Treanor et al., 1996). In cells coexpressing c-Ret and GFRα1, GDNF induces and/or stabilizes the formation of a complex between the two receptors, resulting in dimerization and activation of the c-Ret kinase (*cis* signaling). The two receptors also interact with low affinity in the absence of ligand, resulting in the formation of a less selective receptor complex capable of interacting with other members of the GDNF ligand family as well as several GDNF mutants with defective GFRα1 binding (Sanicola et al., 1997; Trupp et al., 1998; Eketjäll et al., 1999). Three close mammalian homologs of GDNF have been identified, all of which utilize c-Ret as signaling receptor with the aid of different members (GFRα1–4) of the GFRα family of GPI-linked coreceptors (see Airaksinen et al. [1999] for a recent review).

Thus, in the GDNF system, the tasks of ligand binding and transmembrane signaling are divided between the GFRα and c-Ret receptors. Because both receptors are necessary for GDNF signaling, this would predict a high degree of colocalization of c-Ret and GFRα subunits in vivo. However, GFRαs are in fact much more widely expressed than c-Ret in nervous tissue, suggesting alternative roles for these "ectopic" sites of GFRα expression (Trupp et al., 1997; Yu et al., 1998). One possibility is that GFRαs may also signal independently of c-Ret, presumably in collaboration with novel transmembrane proteins. In fact, recent evidence obtained in c-Ret-deficient cell lines and sensory neurons isolated from c-Ret knockout mice indicates the existence of alternative signaling mechanisms mediated by GFRαs acting in a cell-autonomous manner independently of c-Ret (Poteryaev et al., 1999; Trupp et al., 1999). On the other hand, GFRαs also bind ligand and activate c-Ret when provided exogenously in soluble form or immobilized on agarose beads (Treanor et al., 1996; Klein et al., 1997; Yu et al., 1998). Thus, another possibility is that "ectopic" GFRαs may also function in a non-cell-autonomous manner to capture and concentrate diffusible GDNF family ligands from the extracellular space and then present these factors in *trans* to afferent c-Ret-expressing cells (*trans* signaling) (Trupp et al., 1997; Yu et al., 1998).

The relative importance of *cis* and *trans* mechanisms of c-Ret activation is, however, not understood. Other GPI-anchored receptors, such as the ciliary neurotrophic factor (CNTF) receptor α (CNTFRα), are released by expressing cells and act as soluble mediators of the biological activities of their ligands (Davis et al., 1993). However, it is unknown whether GFRαs are normally released by producing cells in vivo, nor whether soluble receptors are able to mediate physiologically relevant responses to GDNF family ligands. Expression studies of GDNF and its receptors indicate that many GDNF-responsive neurons expressing both c-Ret and GFRα1 project to sites rich in GFRα1 expression, suggesting that they may normally be exposed to both *cis* and *trans* signaling in vivo (Trupp et al., 1997; Yu et al., 1998). It is not obvious, however, what benefit a cell that already expresses endogenous GFRα1 may gain from an additional supply of exogenous GFRα molecules. The possibility that *cis* and *trans* mechanisms cooperate in the overall GDNF response of a cell has not been investigated.

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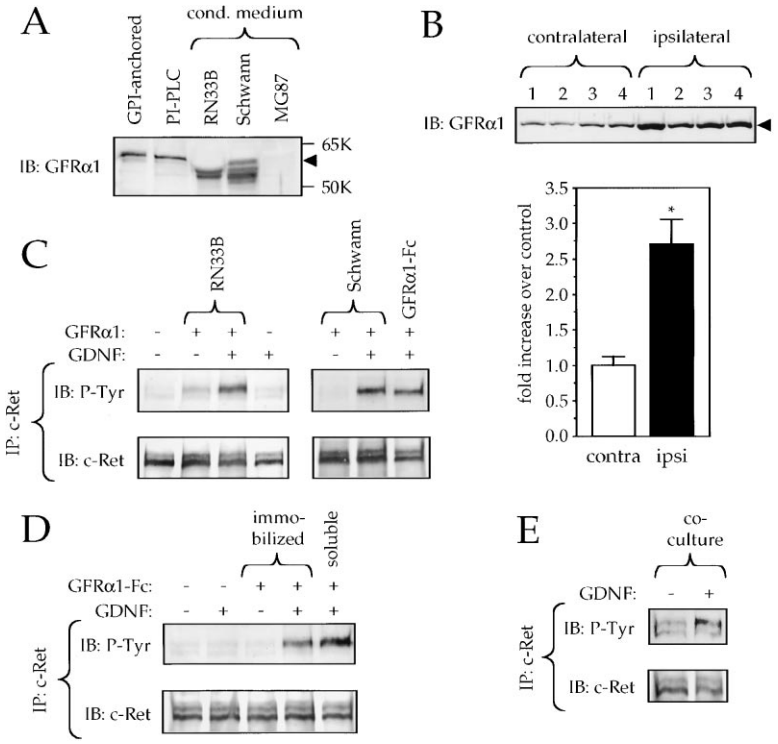

Figure 1. Release of GFRα1 from Schwann Cells and Sciatic Nerve Explants, and Activation of c-Ret in *trans* by Soluble and Immobilized Receptors

(A) GFRα1 in membrane rafts of RN33B cells (GPI anchored), released by PI-PLC treatment (PI-PLC) or collected from conditioned medium of RN33B cells or Schwann cells.

(B) GFRα1 in conditioned medium of explants of control (contralateral) or lesioned (ipsilateral) rat sciatic nerves. Results from four different animals are shown (numbers). Asterisk, $p < 0.017$, Student's t test.

(C) MG87-Ret cells were treated with GDNF and soluble GFRα1 derived from conditioned medium of RN33B or Schwann cells and analyzed for c-Ret phosphorylation (IP, immunoprecipitation; IB, immunoblotting).

(D) MG87-Ret cells grown on dishes coated with GFRα1-Fc (+) or with a control IgG (−) were stimulated with GDNF as indicated and analyzed for c-Ret phosphorylation. Soluble GFRα1-Fc was included as positive control.

(E) Cocultures of RN33B (75%) and MG87-Ret (25%) cells were stimulated with GDNF as indicated and analyzed for c-Ret tyrosine phosphorylation as above.

One difference between activation of c-Ret by GPI-anchored (*cis*) versus soluble (*trans*) GFRαs may lie on the localization of these receptors at the cell membrane. The glycolipid moiety of GPI-anchored receptors is known to have affinity for specialized regions of the plasma membrane known as lipid rafts. These are liquid-ordered phase microdomains produced by lateral packing of sphingolipids and cholesterol, scattered within a fluid, disordered phase of the lipid bilayer (Simons and Ikonen, 1997; Ikonen and Simons, 1998). In addition to GPI-anchored receptors, several transmembrane receptors, including G protein–coupled receptors and some receptor tyrosine kinases, as well as signaling proteins that are anchored to the cytosolic side of the plasma membrane by saturated acyl chains, have also affinity for rafts (Thomas and Brugge, 1997). Raft microdomains may help to compartmentalize sets of signaling molecules at both sides of the plasma membrane, allowing them to interact with each other in a regulated manner, and at the same time preventing them from interacting with proteins excluded from rafts (Simons and Toomre, 2000). It is therefore possible that the *cis* and *trans* mechanisms of c-Ret activation differ in the engagement of raft microdomains during signal transduction. In fact, Tansey et al. (2000) recently reported that c-Ret can be recruited to lipid rafts upon GDNF stimulation of cells expressing GPI-anchored GFRα1. These authors argued that stimulation of cells in *trans* with soluble GFRα1 is unable to promote the recruitment of c-Ret to lipid rafts, although this was actually never tested in their study, and concluded that stimulation in *cis* is a more efficient means of activating c-Ret and its downstream pathways.

In the work presented here, we have investigated whether activation of c-Ret in *trans* represents a physiologically relevant mechanism by examining the release of GFRα1 from primary cells and tissues, as well as alternative mechanisms for the presentation of GDNF in *trans* to the c-Ret receptor. We have compared downstream signaling and biological activities following activation of c-Ret in *cis* and in *trans*, and studied the possible role of membrane rafts in the compartmentalization and activation of c-Ret by the two mechanisms. Our results indicate that c-Ret can be recruited to lipid rafts by different mechanisms, independently of the presence of GPI-anchored GFRα1, and support the physiological importance of the activation of c-Ret in *trans* as a means to potentiate and diversify the biological responses to GDNF family ligands.

Results

Neuronal Precursors, Activated Schwann Cells, and Injured Sciatic Nerve Release Biologically Active GFRα1 to the Extracellular Space

A prerequisite for the action of GFRα1 as a soluble mediator of GDNF responses in vivo is its release from the membrane of GFRα1-expressing cells. GFRα1 was readily detected in conditioned medium of differentiated RN33B cells (Figure 1A), a conditionally immortalized neuronal precursor line that expresses GFRα1 endogenously but not c-Ret (White et al., 1994; Trupp et al., 1999). We also detected soluble GFRα1 in conditioned medium of primary cultures of proliferating Schwann cells extracted from the newborn rat sciatic nerve (Figure 1A). No cell death or disintegration could be observed at the time of harvesting, indicating physiological release of GFRα1. No GFRα1 molecules could be detected in supernatants or lysates of cells that did not express this receptor (Figure 1A).

In the presence of GDNF, conditioned medium from

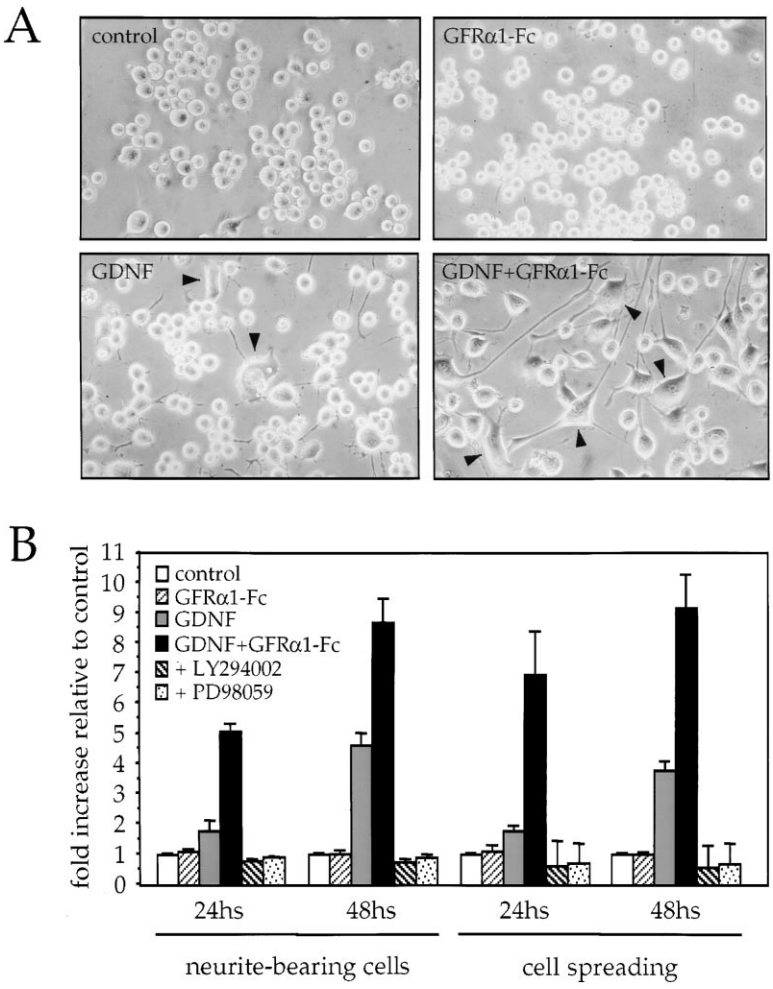

Figure 2. Soluble GFRα1 Potentiates Neuronal Differentiation of the Immortalized Motorneuron Cell Line MN1 in Response to GDNF

(A) Phase contrast images of cultures of MN1 cells grown in control conditions or treated with 1.66 nM GFRα1-Fc, 6 nM GDNF, or 1.66 nM GFRα1-Fc plus 6 nM GDNF. GDNF-induced cell spreading is indicated with arrowheads.

(B) Quantification of neurite outgrowth and cell spreading responses in cultures of MN1 cells after 24 or 48 hr treatment with GDNF, GFRα1-Fc, or their combination. Columns labeled +LY294002 and +PD98059 indicate the response obtained in MN1 cells stimulated with GDNF and GFRα1-Fc in the presence of 50 μM of the indicated inhibitors.

differentiated RN33B cells and Schwann cells induced significant levels of c-Ret activation in *trans* in fibroblast cells stably expressing c-Ret (Figure 1C), indicating the presence of biologically active soluble GFRα1. Comparable effects were observed with a dimeric GFRα1-Fc fusion, indicating similar activities of monomeric and dimeric GFRα1. Medium from RN33B cells had a small effect on its own due to small amounts of GDNF produced by these cells (Miles Trupp and C. F. I., unpublished data).

Release of GFRα1 could also be demonstrated ex vivo in conditioned medium of explants of adult rat sciatic nerve after 48 hr in culture (Figure 1B). Interestingly, release of GFRα1 was greatly stimulated in sciatic nerve explants taken 1 week after a crush lesion (Figure 1B), indicating physiological roles for the released receptors in the injury response.

Activation of c-Ret in *trans* Can Also Be Mediated by GFRα1 Immobilized on the Extracellular Matrix or Anchored to the Membrane of Adjacent Cells

We also examined alternative ways of c-Ret activation in *trans* by exogenous, but nonsoluble, GFRα1 molecules. GDNF induced rapid and robust phosphorylation of c-Ret in MG87-Ret cells grown on a substrate containing GFRα1-Fc molecules immobilized by passive adsorption but not in cells cultured on a control substrate (Figure 1D). This response was GDNF dependent, as cells grown on immobilized GFRα1-Fc did not show c-Ret phosphorylation in the absence of GDNF (Figure 1D). We also examined the possibility that c-Ret may be activated in *trans* by GFRα1 molecules presented on the membrane of adjacent standby cells, which themselves do not express c-Ret. Cocultures of MG87-Ret and RN33B cells were washed extensively prior to stimulation so as to eliminate soluble GFRα1 molecules released to the conditioned medium. GDNF stimulated significant c-Ret phosphorylation in these cocultures (Figure 1E), indicating that GFRα1 can also mediate c-Ret activation in *trans* from the membrane of neighboring cells.

Stimulation in *trans* with Soluble GFRα1 Potentiates Neuronal Differentiation and Survival in Response to GDNF

We investigated the biological significance of stimulation in *trans* by GDNF and soluble GFRα1 in a GDNF-responsive neuronal cell line and in primary cultures of sensory neurons. GDNF had significant effects on cell morphology and neurite outgrowth in MN1 cells treated for 48 hr, although only marginal effects could be seen at 24 hr (Figure 2). Addition of soluble GFRα1-Fc dramat-

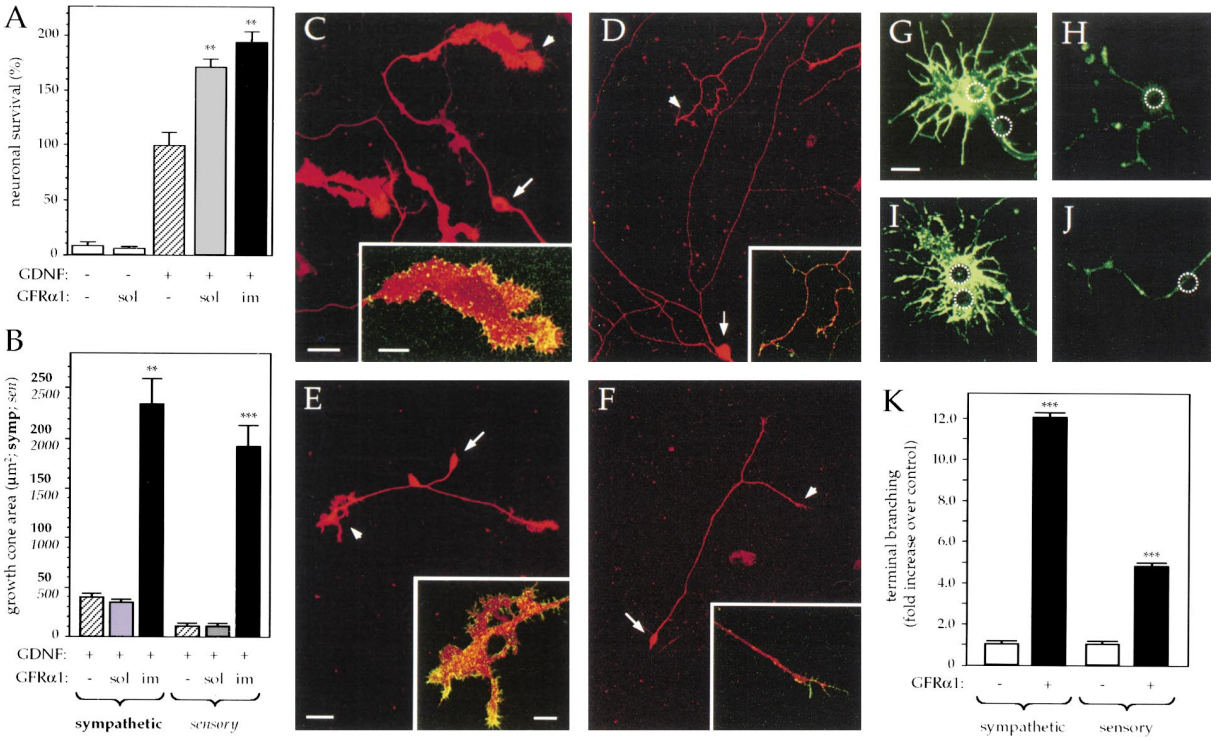

Figure 3. Soluble and Immobilized GFRα1 Potentiate and Diversify the Neurotrophic Activities of GDNF in Sensory and Sympathetic Neurons

(A) Survival responses of embryonic chick nodose ganglion neurons to GDNF in the presence or absence of soluble (sol) or immobilized (im) GFRα1-Fc. Double asterisk, $p < 0.005$ versus GDNF alone.

(B) Quantification of growth cone area of E9 chick nodose (sensory) and sympathetic ganglion neurons in response to GDNF alone, GDNF plus soluble GFRα1-Fc (sol), or GDNF plus immobilized GFRα1-Fc (im). Units in the y axis are in boldface for sympathetic and in italics for sensory growth cones. Results are average ± SEM of at least 50 growth cones from each sample. Double asterisk, $p < 0.003$; triple asterisk, $p < 0.0001$.

(C–F) Morphological differentiation of axons and growth cones of sensory (C and D) and sympathetic (E and F) neurons plated on immobilized GFRα1-Fc (C and E) or control IgG (D and F) and treated with GDNF for 48 hr. Neurons and terminals were stained with anti-GAP43 antibodies. Cell bodies (arrows) and corresponding growth cones (arrowheads) are indicated. The insets show higher magnification micrographs of selected growth cones (arrowheads) stained with anti-GAP43 (red) and phalloidin (green). Scale bar, 40 μm (C and D), 20 μm (C and D, insets), 40 μm (E and F), 10 μm (E and F, insets).

(G–J) Microspeheres (dotted circles) coated with GFRα1-Fc (G and I) or with BSA (H and J) were added to cultures of sensory (G and H) and sympathetic (I and J) neurons grown in the presence of GDNF. Shown are representative examples of terminals stained with phalloidin. Scale bar, 10 μm.

(K) Relative frequency of induction of growth cone branching and intense phalloidin staining (as exemplified by [G] and [I]) in nerve terminals in contact with microspheres in three wells of sensory and sympathetic neurons growing in the presence of GDNF. Double asterisk, $p < 0.0001$.

ically potentiated the response to a saturating dose of GDNF at both 24 and 48 hr (Figure 2), indicating an acceleration of the response. Soluble GFRα1-Fc had no effect on its own at either time point (Figure 2). Stimulation of MN1 cells with GDNF and soluble GFRα1-Fc in the presence of PD98059 and LY294002, specific inhibitors of MEK and PI3K kinases, respectively, failed to provoke changes in cell morphology or neurite outgrowth (Figure 2B), indicating that activation of Erk and Akt kinases is required for the biological response of MN1 cells to GDNF. Developing sensory neurons from the nodose ganglion show a robust survival response to GDNF that saturates between 10 and 20 ng/ml (i.e., 400–800 pM) (Buj-Bello et al., 1995; Trupp et al., 1995). As expected, a saturating dose of GDNF promoted a strong survival response in these cells 48 hr after plating (Figure 3A). In the presence of subnanomolar amounts of soluble GFRα1-Fc, the response to GDNF was nearly doubled (Figure 3A), indicating an increased efficacy of the combined treatment. Increased survival could also be observed in neurons grown on an extracellular matrix containing immobilized GFRα1-Fc (Figure 3A). Together, these data indicated that exogenous GFRα1 in *trans* can potentiate differentiation and survival responses to GDNF even in cells expressing endogenous GPI-anchored receptors.

Immobilized GFRα1 Elicits Localized Differentiation of Growth Cones and Axons

We also examined the effects of exogenous GFRα1 on the morphological differentiation of nodose ganglion sensory neurons as well as paravertebral sympathetic neurons, another neuronal subpopulation highly responsive to GDNF (Buj-Bello et al., 1995; Trupp et al., 1995). Neurons grown on immobilized GFRα1-Fc developed striking lamellipodia-like expansions of axons and growth

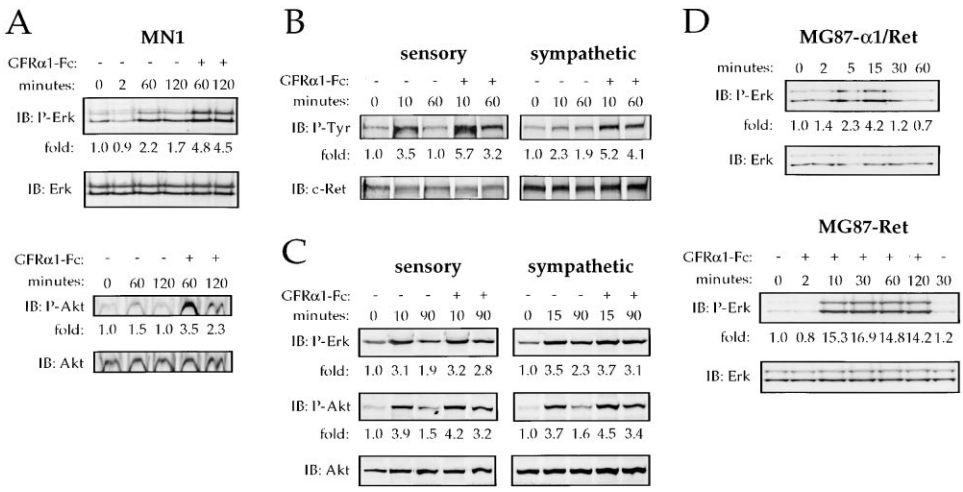

Figure 4. Stimulation with Exogenous GFRα1 in *trans* Potentiates and Prolongs Downstream Signaling in Response to GDNF

(A) Erk (top) and Akt (bottom) phosphorylation in total extracts of MN1 cells treated with GDNF in the presence or absence of soluble GFRα1-Fc.

(B) c-Ret phosphorylation (P-Tyr) in lysates of sensory and sympathetic neurons treated with GDNF in the presence or absence of soluble GFRα1-Fc.

(C) Erk (top) and Akt (bottom) phosphorylation in total extracts of sensory and sympathetic neurons treated with GDNF in the presence or absence of soluble GFRα1-Fc.

(D) Erk phosphorylation in total extracts of MG87-α1/Ret cells treated with GDNF (*cis* signaling, top panels). The bottom panels show Erk phosphorylation in total extracts of MG87-Ret cells treated with GDNF and soluble GFRα1-Fc (*trans* signaling).

cones in response to GDNF (Figures 3C–3F). Giant growth cones several times the size of the corresponding cell body were observed in the majority (>80%) of the neurons cultured on the GFRα1-Fc substrate and were only rarely (<5%) seen in neurons grown with GDNF alone (Figures 3C–3F). Phalloidin staining of polymerized actin revealed numerous fillopodia-like protrusions decorating the growth cones of cells cultured on immobilized GFRα1-Fc (Figures 3C and 3E, insets), indicating active cytoskeletal reorganization. Giant growth cones were not found in neurons cultured on immobilized GFRα1-Fc with brain-derived neurotrophic factor (BDNF) or nerve growth factor (NGF), potent survival and differentiation factors for nodose ganglion and sympathetic neurons, respectively (data not shown). Intriguingly, and despite its ability to potentiate neuronal survival, soluble GFRα1-Fc had no effect on growth cone differentiation; the morphology of cultures grown with GDNF and soluble GFRα1-Fc was indistinguishable from GDNF alone. Quantification of the size of growth cones by image analysis revealed a dramatic increase in the average growth cone area in cells grown on immobilized GFRα1-Fc (Figure 3B).

In vivo, nerve terminals are likely to encounter localized sources, rather than uniform fields, of GFRα1 immobilized to the extracellular matrix or to supporting cells. We modeled this situation by supplying GFRα1-Fc immobilized onto the surface of polystyrene microspheres to neuronal cultures growing in the presence of GDNF. In 2-day-old cultures, microspheres coated with GFRα1, but not control microspheres, produced branching and reorganization of the actin cytoskeleton when associated with the terminal regions of axons (Figures 3G–3K). This effect was only observed in terminals that were in direct contact with GFRα1-coated microspheres, not in axons located at a distance from the beads. In addition, microspheres coated with GFRα1 were visited more frequently by axons than control microspheres coated with BSA (2.2 ± 0.5–fold increase in nodose neurons, n = 3, 2 experiments; 2.7 ± 0.2–fold increase in sympathetic neurons, n = 3, 3 experiments). Thus, localized delivery of GFRα1 can elicit spatially restricted differentiation responses in nerve terminals exposed to a uniform GDNF concentration by activation of local subpopulations of c-Ret receptors in *trans*.

Stimulation with Exogenous GFRα1 Potentiates and Prolongs Downstream Signaling in Response to GDNF

In agreement with the ability of soluble GFRα1 to potentiate the biological response of MN1 cells to GDNF, stimulation with GDNF and soluble GFRα1-Fc resulted in a more sustained phosphorylation of Erk and Akt kinases compared to treatment with GDNF alone (Figure 4A). Significant activation of Erk and Akt was still detected 60 and 120 min after stimulation with GDNF and GFRα1-Fc, at a time when the activity induced by stimulation with GDNF alone was already declining or almost back to basal levels (Figure 4A). Stimulation with soluble GFRα1-Fc of sensory and sympathetic neurons was also accompanied by a marked potentiation and prolongation of c-Ret tyrosine phosphorylation in response to GDNF (Figure 4B). Moreover, stimulation in *trans* also prolonged the activation of Akt and, to a lesser extent, Erk in sensory and sympathetic neurons (Figure 4C).

To compare *cis* versus *trans* stimulation directly in the absence of endogenous GPI-anchored GFRα1, downstream signaling was examined in MG87 fibroblasts stably transfected with different complements of GDNF receptor subunits. In MG87 cells expressing GFRα1 and c-Ret (MG87-α1/Ret), GDNF stimulated rapid phosphorylation of Erk kinases that returned to

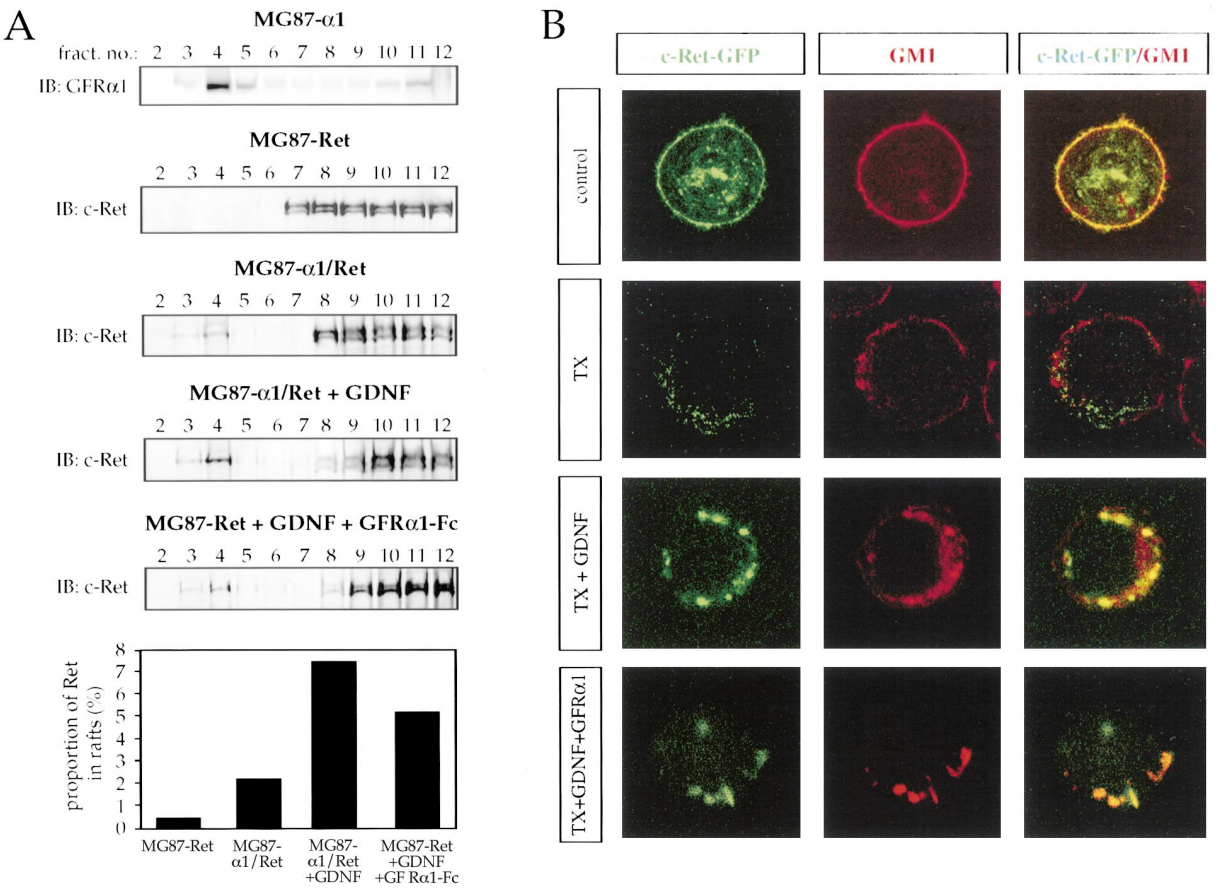

Figure 5. Both GPI-Anchored and Soluble GFRα1 Receptors Mediate Recruitment of c-Ret to Lipid Rafts

(A) Sucrose gradient fractions of Triton X-100 lysates prepared from the indicated cell lines treated with GDNF and soluble GFRα1-Fc as indicated analyzed for GFRα1 and c-Ret. Raft proteins are in fractions 3 to 5. Detergent-soluble proteins are at the bottom of the gradient in fractions 9 to 12. The histogram shows the amount of c-Ret present in rafts relative to the total amount of c-Ret in the gradient.

(B) Visualization of c-Ret in detergent-insoluble membrane compartments in situ by confocal microscopy. The top three rows show Neuro2A-α1 cells (stably expressing GFRα1) transiently transfected with a c-Ret-GFP construct (green) either fixed directly (control) or treated with Triton X-100 prior to fixation (TX) and staining with a rhodamine-labeled cholera toxin fragment B (red). Treatment with GDNF was done for 10 min. The last row shows parental Neuro2A cells transfected and treated as above except that soluble GFRα1-Fc was added together with GDNF, and cells were stimulated for 60 min.

baseline at about 60 min (Figure 4D). In contrast, treatment of MG87 cells expressing only c-Ret (MG87-Ret) with GDNF and soluble GFRα1-Fc produced a more sustained stimulation of Erk phosphorylation that lasted for at least 120 min (Figure 4D). Activation of Akt was also more sustained in MG87-Ret cells stimulated in *trans* compared to MG87-α1/Ret cells stimulated in *cis* (data not shown). Together, these data suggested that *cis* and *trans* signaling activate downstream pathways with different kinetics and that both mechanisms can cooperate to achieve optimal signaling in neurons coexpressing GFRα1 and c-Ret.

Both GPI-Anchored and Soluble GFRα1 Receptors Mediate Recruitment of c-Ret to Lipid Rafts

The striking effects of stimulation in *trans* on the biological and signaling responses to GDNF appear to be at odds with a recent report arguing that activation of c-Ret in *trans* by soluble GFRα1 is inefficient and promotes only weak survival and differentiation responses (Tansey et al., 2000). These authors postulated that the apparent inefficiency of stimulation in *trans* could be due to an inability of soluble GFRα1 to mobilize c-Ret to lipid rafts. We investigated the localization of GFRα1 and c-Ret in cells stably expressing these receptors individually or together, and after stimulation with GDNF in the presence or absence of soluble GFRα1. While GPI-anchored GFRα1 was highly enriched in raft fractions of MG87-α1 cells, c-Ret was predominantly found outside rafts when expressed in the absence of GFRα1 (Figure 5A). Stimulation with GDNF had no effect on the distribution of individually expressed receptors (data not shown). Coexpression of c-Ret with GFRα1 (MG87-α1/Ret cells) resulted in a small relative increase in the amount of c-Ret within rafts (Figure 5A), presumably due to low-affinity, ligand-independent interactions between the two receptors. In these cells, stimulation with GDNF caused a significant increase in the amount of c-Ret in rafts (Figure 5A). Unexpectedly, concomitant stimulation of MG87-Ret

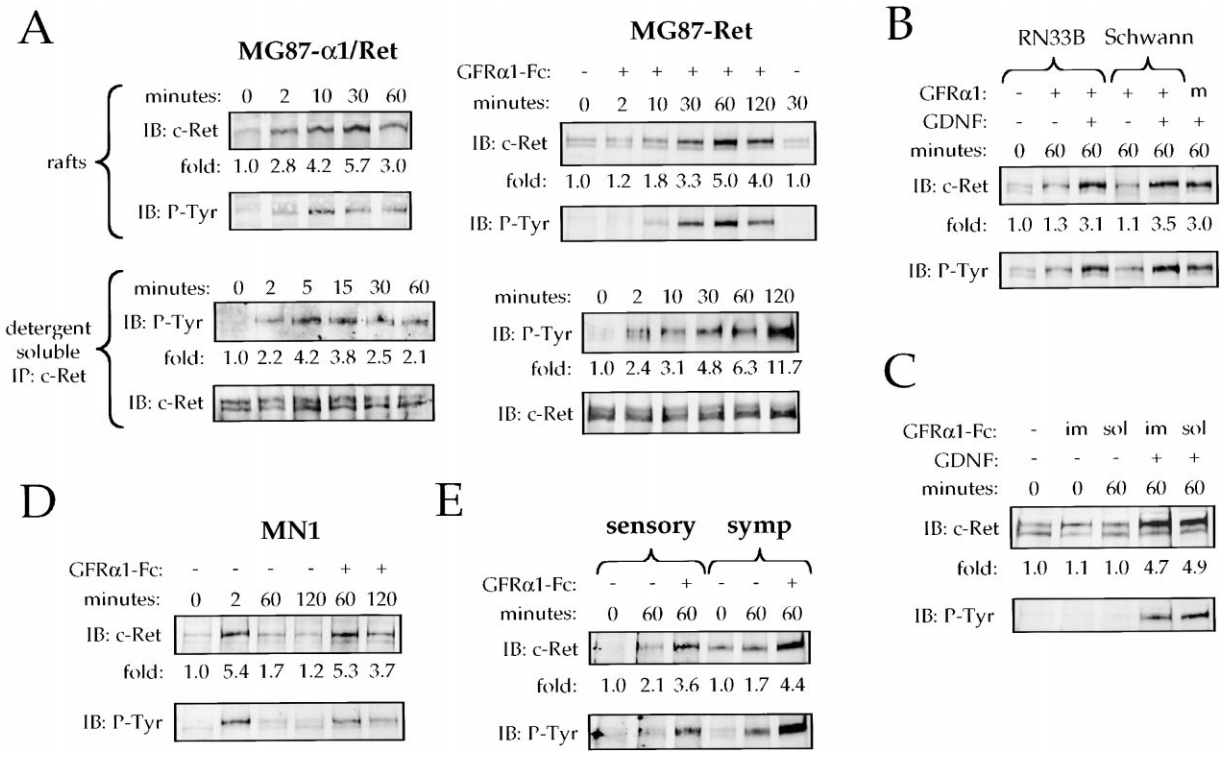

Figure 6. Exogenous GFRα1 Prolongs the Presence and Activation of c-Ret in Lipid Rafts

(A) Rafts and detergent soluble fractions from MG87-α1/Ret and MG87-Ret cells treated as indicated analyzed by Western blotting with c-Ret and phosphotyrosine antibodies.

(B and C) Rafts from MG87-Ret cells. m, monomeric GFRα1; sol, soluble GFRα1-Fc; im, immobilized GFRα1-Fc.

(D) Rafts from MN1 cells.

(E) Detergent-resistant membranes from nodose (sensory) and sympathetic ganglion neurons.

cells with GDNF and soluble GFRα1 also induced robust recruitment of c-Ret to lipid rafts (Figure 5A).

Mobilization of c-Ret to membrane rafts was also visualized in situ using a c-Ret construct carrying a green fluorescence protein (GFP) tag and the raft marker GM1, visualized with a rhodamine-conjugated cholera toxin B fragment. Extraction with Triton X-100 resulted in a punctated pattern of detergent-resistant GM1 staining and low amounts of c-Ret-GFP that was not in association with GM1 (Figure 5B, TX). Stimulation with GDNF of cells expressing GPI-anchored GFRα1 followed by detergent extraction resulted in a patched pattern of c-Ret-GFP that colocalized with GM1 (Figure 5B, TX+GDNF). Importantly, stimulation of cells that did not express GPI-anchored GFRα1 with GDNF and soluble GFRα1-Fc also produced colocalization of c-Ret-GFP and GM1 (Figure 5B). Thus, activation of c-Ret in *trans* results in the mobilization of c-Ret to lipid raft membranes even in cells that lack endogenous GPI-anchored GFRα1.

Stimulation with Exogenous GFRα1 Prolongs the Presence and Activation of c-Ret in Lipid Rafts

The kinetics of recruitment of c-Ret to lipid rafts induced by the *cis* and *trans* stimulation regimes were very different. GDNF treatment of cells expressing GPI-anchored GFRα1 (e.g., MG87-α1/Ret and MN1 cells) resulted in rapid (<5 min) tyrosine phosphorylation and recruitment of c-Ret to lipid rafts, with a peak within the first 30 min, returning to baseline levels between 60 and 120 min (Figure 6A, left). GDNF also stimulated rapid tyrosine phosphorylation of c-Ret outside rafts (Figure 6A, left). In contrast, activation and recruitment of c-Ret to rafts in *trans* in MG87-Ret cells treated with GDNF and soluble GFRα1-Fc was delayed and sustained, with a peak at 60 min, and still high 120 min after stimulation (Figure 6A, right). The recruitment of c-Ret mediated by GDNF and soluble GFRα1-Fc was not affected by pretreatment with PI-PLC (data not shown) and could not be induced by treatment with GDNF alone at any time point analyzed (Figure 6A and data not shown), confirming the requirement of soluble GFRα1 and the absence of endogenous GFRα1 in these cells. Although recruitment of c-Ret to lipid rafts in *trans* was a delayed event, GDNF and soluble GFRα1-Fc induced rapid phosphorylation of c-Ret outside rafts in detergent-soluble fractions of MG87-Ret cells, typically 15–30 min ahead of that observed inside rafts (Figure 6A, right). These data suggested that during *trans* signaling, c-Ret may first be activated outside rafts and then subsequently recruited into this compartment.

Sustained recruitment and activation of c-Ret in lipid rafts could also be mediated by monomeric GFRα1 produced by proteolytic cleavage of GFRα1-Fc (Figure 6B). More importantly, soluble GFRα1 released from RN33B cells and Schwann cells was also capable of promoting sustained recruitment of c-Ret to lipid rafts in MG87-Ret

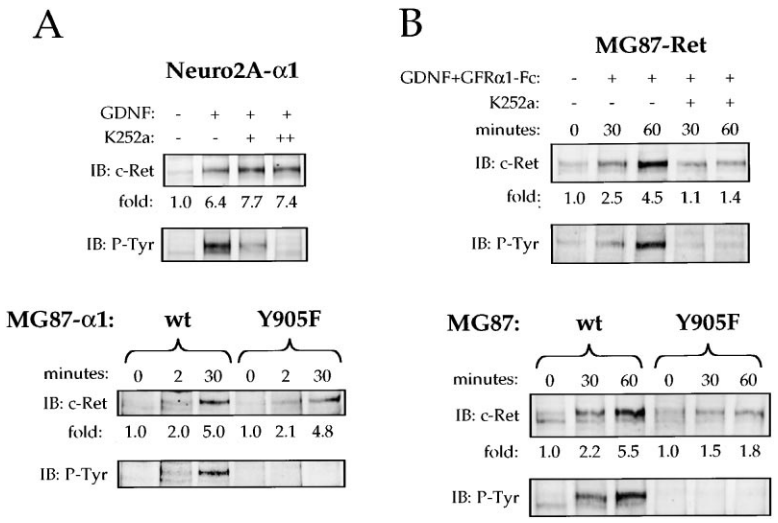

Figure 7. Recruitment of c-Ret to Lipid Rafts in *trans*, but Not in *cis*, Depends upon an Active c-Ret Tyrosine Kinase

(A) Rafts from Neuro2A-α1 cells (top) pretreated with K252a at 200 nM (plus sign) or 1 μM (double plus sign) as indicated were analyzed for c-Ret and phosphotyrosine by immunoblotting. Rafts from MG87-α1 cells (bottom) transiently transfected with wild type or Y905F mutant c-Ret were analyzed for c-Ret and phosphotyrosine by immunoblotting.

(B) Rafts from MG87-Ret cells (top) pretreated with K252a (1 μM) were analyzed for c-Ret and phosphotyrosine by immunoblotting. The bottom part shows rafts from MG87 cells transiently transfected with wild type or Y905F mutant c-Ret analyzed for c-Ret and phosphotyrosine by immunoblotting after stimulation with GDNF and soluble GFRα1.

cells treated with GDNF (Figure 6B). Finally, sustained recruitment of c-Ret to detergent insoluble compartments could also be induced by GDNF treatment of MG87-Ret fibroblasts that were plated on immobilized GFRα1-Fc (Figure 6C), indicating that GFRα1 molecules can still mobilize c-Ret to lipid rafts in *trans* even if they are themselves immobilized to the substrate.

The effects of stimulation in *cis* and *trans* on c-Ret recruitment to lipid rafts were additive. Treatment with GDNF in combination with GFRα1-Fc prolonged the presence of c-Ret in rafts of MN1 cells for at least an additional 60 min (Figure 6D), resulting in an extended wave of c-Ret tyrosine phosphorylation (Figure 6D). Costimulation with soluble GFRα1-Fc also potentiated the recruitment of c-Ret to detergent-insoluble membranes in sensory and sympathetic neurons (Figure 6E). Together, these results indicated that stimulation in *trans* can potentiate and prolong the recruitment and activation of c-Ret in lipid rafts even in cells expressing endogenous GFRα1.

Recruitment of c-Ret to Lipid Rafts in *trans*, but Not in *cis*, Depends upon an Active c-Ret Tyrosine Kinase

How is c-Ret mobilized to lipid rafts in the absence of GPI-anchored GFRα1? Blockade of the c-Ret kinase activity by pretreatment with the alkaloid K252a or by a point mutation within the kinase activation loop (Y905F) did not affect the recruitment of c-Ret to rafts in cells expressing GPI-anchored GFRα1 (Figure 7A). In contrast, recruitment of c-Ret to rafts induced by GDNF and soluble GFRα1-Fc in MG87-Ret cells was greatly attenuated by pretreatment with K252a (Figure 7B). Moreover, the Y905F point mutation also impaired the ability of c-Ret to be efficiently translocated to membrane rafts by GDNF and soluble GFRα1-Fc (Figure 7B). Thus, recruitment of c-Ret to lipid rafts in *trans* is mediated by an intracellular mechanism, regulated by the c-Ret tyrosine kinase.

Activated c-Ret Associates with Distinct Adaptor Proteins Inside and Outside Lipid Rafts

The requirement of an active tyrosine kinase for the mobilization of c-Ret to lipid rafts in *trans* suggested that the recruitment could be mediated by the interaction of the activated receptor with an intracellular target specifically localized to lipid rafts. In MN1 cells stimulated in *cis* with GDNF for 10 min, c-Ret was mainly found associated with SHC in detergent-soluble fractions but not in raft fractions (Figure 8A), suggesting a preferential association between c-Ret and SHC outside rafts. On the other hand, c-Ret was predominantly recovered in raft fractions after precipitation of FRS2 with $p13^{Suc1}$ beads (Figure 8A), suggesting that c-Ret associates with this target primarily within raft compartments. In both cases, the association of c-Ret with these targets was rapid and ligand dependent. FRS2 was found to be a resident protein of lipid rafts in MN1 cells. It became rapidly phosphorylated upon arrival of c-Ret to this compartment, returning back to basal levels by 60 min (Figure 8B).

Stimulation of MG87-Ret cells with GDNF and soluble GFRα1 in *trans* also resulted in the association of c-Ret with SHC outside and with FRS2 inside rafts, but with very different kinetics. Interaction between c-Ret and both these adaptors was detected 60 min after GDNF stimulation (Figure 8C), at a time when activation in *cis* had already extinguished. In contrast to stimulation in *cis*, no c-Ret could be recovered associated with FRS2 from rafts of MG87-Ret cells after 10 min of stimulation in *trans* (data not shown), indicating a delayed but sustained activation of this adaptor during *trans* signaling. Thus, distinct time courses of c-Ret activation after stimulation in *cis* or *trans* translate into different kinetics of interaction of the receptor with downstream adaptor molecules inside and outside lipid rafts.

Mutation of Tyr-1062 in the Cytoplasmic Domain of c-Ret Abolishes Activation of FRS2 and Impairs Recruitment of c-Ret to Lipid Rafts in *trans*

Having identified FRS2 as a target that associates preferentially with c-Ret inside lipid rafts, we examined whether interference with this interaction affected the recruitment of c-Ret to lipid rafts in *trans*. Mutation of Tyr-1062 (Y1062F) in the cytoplasmic domain of c-Ret abolished the activation of FRS2 in response to GDNF stimulation (Figure 8D). Other experiments confirmed that the mutant receptor retained its ability to recruit and activate other targets, such as Grb2 (Besset et al.,

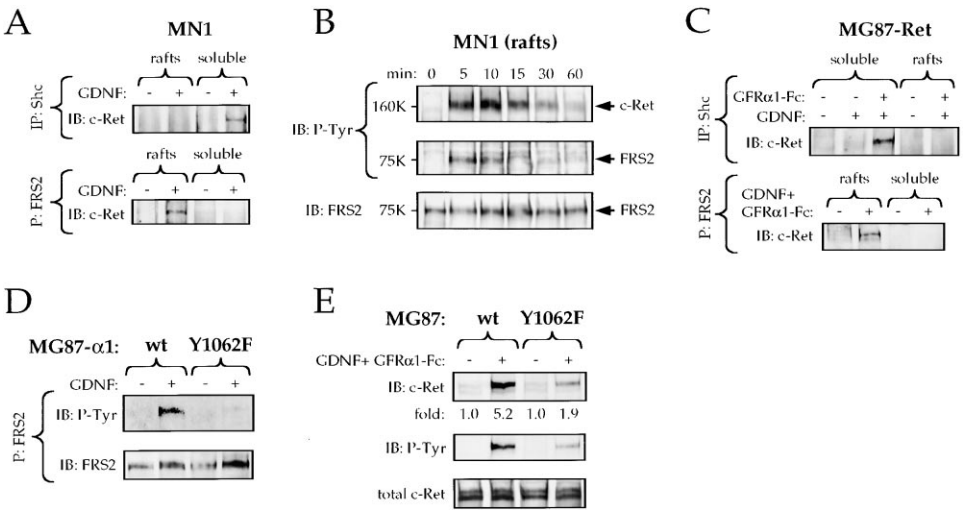

Figure 8. Activated c-Ret Associates with Distinct Adaptor Proteins Inside and Outside Lipid Rafts

(A) Rafts and detergent soluble fractions from MN1 cells stimulated with GDNF for 10 min were precipitated with SHC antibodies (top) or with $p13^{Suc1}$ beads (bottom) and analyzed by Western blotting with c-Ret antibodies.

(B) Rafts from MN1 cells treated with GDNF analyzed by Western blotting with phosphotyrosine or FRS2 antibodies.

(C) Raft and detergent soluble fractions from MG87-Ret cells stimulated with GDNF and soluble GFRα1-Fc for 60 min were precipitated with SHC antibodies (top) or with $p13^{Suc1}$ beads (bottom) and analyzed by Western blotting with c-Ret antibodies.

(D) Rafts from MG87-α1 cells stably transfected with wild type or Y1062F mutant c-Ret treated with GDNF for 15 min were analyzed by Western blotting with phosphotyrosine and FRS2 antibodies.

(E) Rafts from MG87 cells transiently transfected with wild type or Y1062F mutant c-Ret treated with GDNF and GFRα1-Fc were analyzed for c-Ret and phosphotyrosine. Immunoprecipitation of c-Ret from the detergent-soluble fractions is shown below.

2000). Despite retaining catalytic activity, the Y1062F mutant receptor was impaired in its ability to mobilize to lipid rafts when stimulated with GDNF and soluble GFRα1-Fc (Figure 8E), indicating the involvement of this phosphotyrosine residue in the recruitment of c-Ret to rafts in *trans*.

Discussion

Comparison of the expression patterns of c-Ret and GFRα1 has revealed striking discrepancies that were not anticipated by the functional roles originally attributed to these two receptors (Trupp et al., 1997; Yu et al., 1998). Although it is now clear that most cell types expressing c-Ret in the absence of GFRα1 express other member(s) of the GFRα family (Airaksinen et al., 1999), no satisfactory explanation has been found for the widespread expression of GFRαs in the absence of c-Ret. Here, we propose that one function for these "ectopic" sites of GFRα expression is to potentiate and diversify the response to GDNF by activating c-Ret receptors on afferent neurons in *trans*. It has been previously noted that many of the "ectopic" sites of GFRα1 expression in the brain correspond to targets of GDNF-responsive neurons (Yu et al., 1998). In the periphery, sympathetic and sensory axons are exposed to exogenous GFRα1 produced by Schwann cells in peripheral nerves (Naveilhan et al., 1997; Trupp et al., 1997), and growth cones from cutaneous sensory nerve endings are found in close interaction with terminal Schwann cells expressing GFRα1 (Fundin et al., 1999). Thus, both in the central and peripheral nervous systems, "ectopic" sites of GFRα1 expression are associated with terminals or axons from GDNF-responsive neurons, in line with the notion that GFRα1 molecules produced by supporting and target cells engage in *trans* signaling.

Different Modes of Action of Exogenous GFRα1 Molecules in the Activation of c-Ret in *trans*

We present evidence for three different modes of activation of c-Ret molecules in *trans* via exogenous GFRα1 (Figure 9A). First, soluble GFRα1 molecules can bind GDNF in the extracellular space and present it in *trans* to c-Ret-expressing neurons. In this way, GFRα1 can function as part of an heteromeric ligand together with GDNF on c-Ret-expressing axons and cell bodies located at a distance. Second, released GFRα1 can be immobilized to the extracellular matrix by passive adsorption and present GDNF to cell bodies and terminals growing on this substrate. Immobilization of GFRα1 to the extracellular matrix could be a way to spatially confine activation of c-Ret in *trans* and to create and stabilize a precise ligand distribution, such as the gradients of GDNF/GFRα1 that develop in injured peripheral nerves (see below). Third, GPI-anchored GFRα1 molecules on standby cells can bind GDNF and present it in *trans* to c-Ret receptors on adjacent cells. This mechanism may allow highly localized activation of c-Ret receptors on terminals or en passant axons by cell–cell contact, and could provide a trophic signal with great spatial resolution, even in a field of uniformly distributed ligand. The results of our experiments with microspheres coated with GFRα1 are in agreement with this idea and suggest that stimulation of c-Ret in *trans* by cell–cell contact may participate in the guidance, formation, or maintenance of neuronal connections.

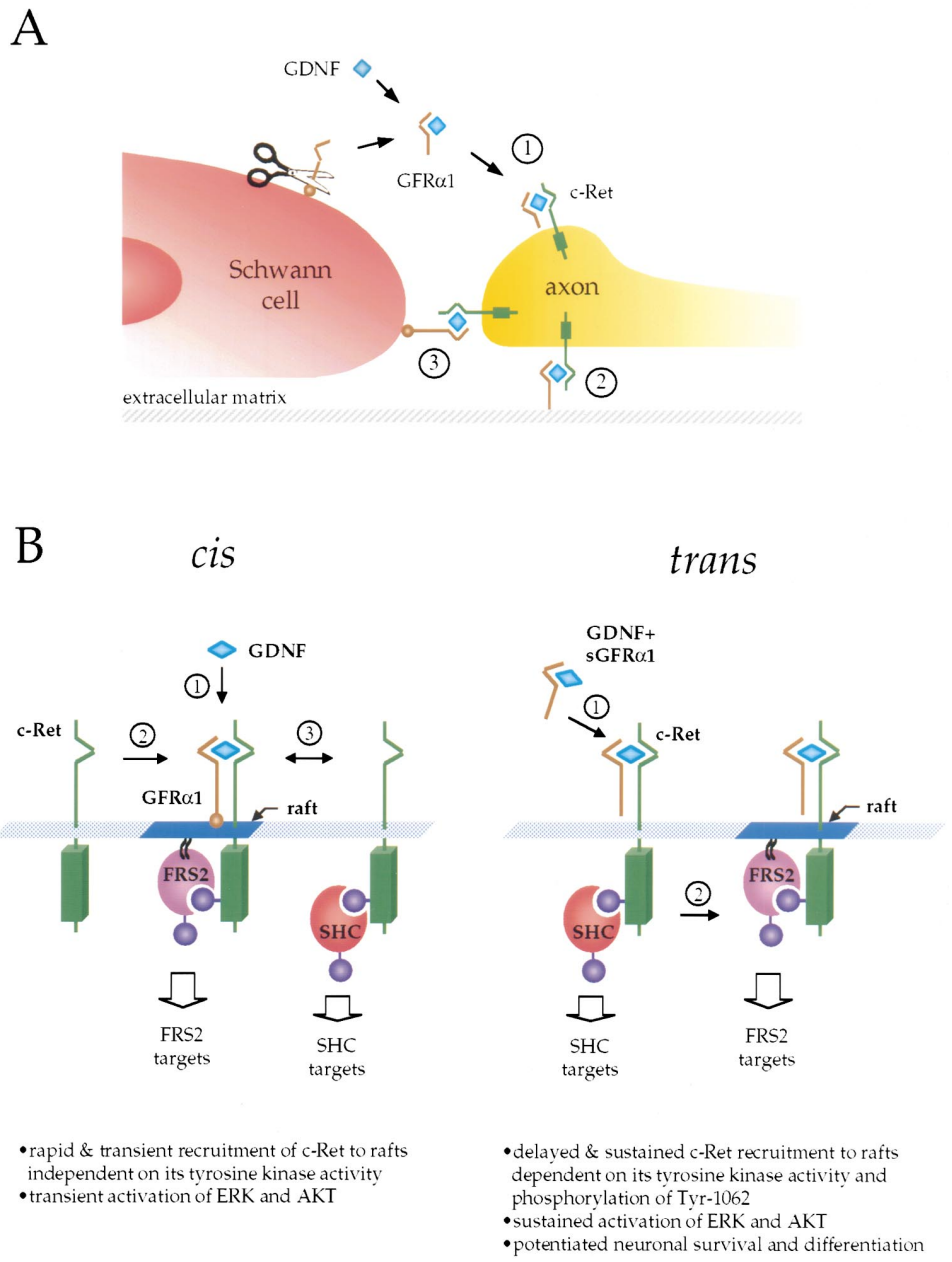

Figure 9. Summary of Results

(A) Different modes of action of exogenous GFRα1 molecules in the activation of c-Ret in *trans*. GPI-anchored GFRα1 can be released by the action of membrane phospholipases or proteases. Soluble GFRα1 molecules can capture GDNF in the extracellular space and activate c-Ret in *trans* on target cells (1). Released GFRα1 can also be immobilized to the extracellular matrix and present GDNF to axons growing on this substrate (2). GPI-anchored GFRα1 molecules on standby cells can bind GDNF and present it in *trans* to c-Ret receptors on adjacent cells (3).

(B) Initial signaling events in rafts for the GDNF receptor complex. During activation in *cis* (left), GDNF binds to GPI-anchored GFRα1 receptors in lipid rafts (1), resulting in the recruitment and activation of c-Ret in this compartment (2). c-Ret associates with and activates FRS2 inside and SHC outside lipid rafts. Activated c-Ret is in equilibrium between raft and nonraft compartments (3). During activation in *trans* (right), a complex of GDNF and soluble GFRα1 (sGFRα1) released from neighboring cells binds to and activates c-Ret outside rafts (1), where the activated receptor associates with and phosphorylates SHC. c-Ret is then recruited to rafts by a mechanism dependent on its tyrosine kinase activity and phosphorylation of Tyr-1062 (2). Inside lipid rafts, c-Ret associates with and activates FRS2. Both c-Ret and GFRα1 are believed to function as homodimers; for simplicity, only monomers are represented here.

Effects of Immobilized GFRα1 on Morphological Differentiation of Sensory and Sympathetic Neurons

Neurons plated on immobilized GFRα1 and stimulated with GDNF developed striking growth cones and axonal protrusions, involving the formation of large lamellipodia and actin-rich fillopodia. These responses were not observed in cells stimulated with the neurotrophins BDNF or NGF, indicating that they are dependent on GDNF signaling, and not due to unspecific effects of GFRα1

on cell adhesion. Intriguingly, soluble GFRα1 was unable to elicit these effects, suggesting that aggregation of exogenous GFRα1 is required. As it has been observed in immune cells (Viola et al., 1999), localized exposure to immobilized GFRα1 in *trans* may promote redistribution and clustering of lipid rafts leading to sustained c-Ret activation and signal amplification. A number of immune cell receptors can be activated by preclustered or immobilized antibodies, and in the nervous system, ligands for Eph-related receptor tyrosine kinases require membrane attachment or clustering for activity (Davis et al., 1994). In these examples, cell–cell contact plays a major role in the activation mechanism, suggesting that the effects seen here of immobilized GFRα1 may represent activities normally mediated by cell–cell interactions. In addition, the fact that microspheres coated with GFRα1 were visited by sensory and sympathetic axons more frequently than control microspheres indicates that immobilized GFRα1 stabilizes axonal contact, suggesting that, in vivo, GDNF may help to stabilize axon trajectories along sites of GFRα1 expression.

Release of GFRα1 during Nerve Injury and Its Possible Role in Nerve Regeneration

Lesion of the sciatic nerve produces a dramatic upregulation of the expression of both GDNF and GFRα1 in Schwann cells in a proximodistal gradient, such that cells in the distal stump of the lesioned nerve express higher levels of these molecules the further away they are from the transection site (Naveilhan et al., 1997; Trupp et al., 1997). It has been postulated that this could represent a mechanism to aid the regeneration of sensory and motor axons (Trupp et al., 1997). Our present results, showing that nerve lesion stimulates the release of Schwann cell–derived GFRα1, bring support to this notion. Based on these results, we propose that GFRα1 molecules released in a proximodistal gradient in the lesioned nerve stabilize gradients of increasing concentration of GDNF preassociated with GFRα1 that may help to stimulate and guide the regrowth of axons during nerve regeneration. Furthermore, we suggest that exogenous administration of GFRα molecules could be used to enhance and diversify therapeutic activities of GDNF family ligands in the treatment of nerve injury and neurodegeneration.

Recruitment of c-Ret to Lipid Rafts by *cis* and *trans* Mechanisms

Mobilization to lipid rafts is a robust feature of c-Ret signaling that can be induced even in the absence of GPI-anchored GFRα1. This observation appears to be in contradiction with a recent report arguing that only GPI-anchored GFRα1 is able to recruit c-Ret to rafts (Tansey et al., 2000). Although these authors concluded that c-Ret could not be mobilized to rafts when stimulated with soluble GFRα1 in *trans*, this was actually never demonstrated in their study. We have examined the cell line used in that work (Neuro2A) and found that stimulation of c-Ret in *trans* does indeed induce robust recruitment of this receptor to lipid rafts with sustained kinetics (Figure 5B; G. P. and C. F. I., unpublished data). In the same study, it was also argued that only stimulation in *cis* affords efficient downstream signaling and biological responses to GDNF. However, Tansey et al. (2000) did not examine signaling events beyond 30 min after stimulation in *trans*, and biological responses were tested after transient overexpression of receptors in neurons that do not normally respond to GDNF. As shown here, downstream signaling after stimulation in *trans* is at least as efficient as that induced in *cis* and produces sustained activation of Erk and Akt kinases. This, however, does not become evident until after 30 min of stimulation. Moreover, our results demonstrate that cells that normally respond to GDNF, such as sensory and sympathetic neurons, show enhanced survival and differentiation responses to GDNF in the presence of soluble or immobilized GFRα1. Thus, we disagree with the model proposed by Tansey et al. (2000) and argue instead that activation of c-Ret in *trans* by exogenous GFRα1, particularly clustered GFRα1 molecules, represents a physiological mechanism to potentiate responses to GDNF family ligands and to elicit novel biological effects, including growth cone differentiation. A direct test for the relative roles of *cis* and *trans* activation in vivo will require tissue-specific ablations of GFRα1.

Upon activation in *cis*, c-Ret is recruited to membrane rafts by a rapid extracellular mechanism, likely driven by its affinity for newly formed GDNF/GFRα1 complexes, independently of its tyrosine kinase activity (Figure 9B). The fact that activated c-Ret can also be seen outside rafts suggests that once activated, c-Ret may be in equilibrium between raft and non-raft compartments. In contrast, recruitment in *trans* is delayed, sustained, and requires an active c-Ret tyrosine kinase, suggesting the involvement of intracellular events. What mechanism(s) could account for the recruitment of c-Ret to lipid rafts in *trans*? Our results suggest that, after activation and autophosphorylation, the c-Ret kinase acquires affinity for an intracellular component of rafts, possibly via the interaction of its phosphorylated tyrosine residues with an SH2 or PTB domain-containing protein (Figure 9B). We found that phosphorylation of Tyr-1062 is required for the efficient recruitment of c-Ret to lipid rafts in *trans* and that this residue is also necessary for activation of the FRS2 adaptor. This protein associates with the plasma membrane via a saturated acyl chain (Kouhara et al., 1997), and we have also shown that it normally resides within lipid rafts. Based on this evidence, we propose that one mechanism by which c-Ret can get recruited to lipid rafts after activation in *trans* is via the direct or indirect association of FRS2 to phosphorylated Tyr-1062 in the activated receptor. Thus, recruitment in *trans*, like recruitment in *cis*, is also mediated by proteins that normally reside in rafts, except that in *trans* these are intracellular proteins, while in *cis* it is the GPI-anchored GFRα1 receptor.

Teleology: Why GPI-Anchored Receptors and Recruitment to Lipid Rafts?

The function of the GPI linkage in GFRα1 cannot be the recruitment of c-Ret to lipid rafts as proposed by Tansey et al. (2000), since c-Ret can also be recruited in *trans* in the absence of GPI-anchored receptors. In contrast, our results highlight the importance of membrane anchorage (*cis*) as well as release (*trans*) for the function of GFRα1. Membrane-bound GFRα molecules allow ligand specificity, preventing indiscriminate signaling by different GDNF family ligands on c-Ret-expressing cells. On

the other hand, released receptors are able to act at a distance and promote unique localized biological effects by activation of c-Ret receptors in *trans*. Thus, the GPI linkage appears to be a suitable compromise, allowing both membrane anchorage and regulated release (Faivre-Sarraih and Rougon, 1997).

c-Ret differs from other receptor tyrosine kinases in its ability to be specifically recruited to membrane rafts. The NGF and EGF receptors, for example, are constitutively associated with rafts, and while NGF treatment has no effect on TrkA localization (Huang et al., 1999), EGF stimulation induces rapid exit of EGFR from raft membranes (Mineo et al., 1999). Compartmentalization of signal transduction at the cell surface allows a single class of receptors to transmit different signals from alternate locations in the cell. Our results provide evidence that at least two distinct signaling targets, FRS2 and SHC, differentially associate with c-Ret depending on whether the activated receptor is in raft or non-raft compartments. Intriguingly, activation of both these proteins by GDNF requires the same phosphotyrosine residue in c-Ret (i.e., Y1062), suggesting that competition between these two adaptor proteins for the same site in c-Ret results in the observed segregation of different pools of activated receptors bound to either adaptor in raft versus non-raft compartments. Altering the location of active receptors and the complement of potential signaling targets present in different membrane compartments could represent strategies used to regulate and diversify intracellular signal transduction.

Experimental Procedures

Cell Lines

RN33B is a conditionally immortalized neuronal precursor cell line isolated from embryonic rat raphe nucleus (White et al., 1994; Trupp et al., 1999). MG87-α1, MG87-α1/Ret, and MG87-Ret lines were derived from MG87 fibroblasts by stable transfection of GDNF receptor subunits. Neuro2A-α1 cells were generated by stable transfection of the neuroblastoma Neuro2A with GFRα1. MN1 is an immortalized motorneuron cell line (Salazar-Grueso et al., 1991; Trupp et al., 1999).

Cell Transfection, Plasmids, and Pharmacological Treatments

Transient transfections were performed using the calcium phosphate method. Enhanced GFP was fused C-terminally to a human c-Ret cDNA and subcloned in pCDNA3. c-Ret mutants Y905F and Y1062F were made by site-directed mutagenesis as described elsewhere (Besset et al., 2000). GDNF produced in insect cells as described (Trupp et al., 1995) was used at 50 ng/ml (2 nM) and GFRα1-Fc (R&D Systems) was used at 150 ng/ml (833 pM), unless otherwise indicated. Monomeric soluble GFRα1 was obtained by Factor Xa digestion of GFRα1-Fc, followed by purification on protein G agarose. For immobilization of GFRα1-Fc to the culture substrate, dishes or cover slips were treated with 10 μg/ml GFRα1-Fc in PBS for 2 hr at 37°C and then washed extensively with PBS. Control coatings were done with human IgG. Pretreatments with K252a (Calbiochem) were done for 30 min at 37°C prior to stimulation. MEK and PI3K kinase inhibitors PD98059 and LY294002 (Calbiochem) were used at 50 μM.

Schwann Cell and Sciatic Nerve Explant Cultures

Schwann cells were extracted from P1 rat sciatic nerves by collagenase treatment and cultured in serum-containing medium supplemented with bFGF and forskolin. At confluency, cultures were switched to serum-free medium and incubated for an additional 48 hr at which time conditioned medium was harvested, clarified by high-speed centrifugation, and concentrated by ultrafiltration in Amicon chambers. The left sciatic nerve was exposed at its midthigh portion in anesthetized adult rats and crushed three times using microforceps. One week later, the right (contralateral) and left (ipsilateral) nerves were removed, weighted, and incubated in serum-free medium in 24-well plates for 48 hr. Conditioned medium was harvested and treated as above.

Preparation of Rafts, Detergent-Soluble Fractions, Detergent-Resistant Membranes, and Total Cell Lysates

Cell monolayers were lysed for 60 min at 4°C in buffer containing 1% Triton X-100 and protease and phosphatase inhibitors. Samples were taken to 40% sucrose in an ultracentrifuge tube, and a discontinuous 5%–25% sucrose gradient was layered on top, followed by centrifugation 16 hr at 175,000 × g. In some cases (Figure 5A), 1 ml fractions (12 in total) were collected from the top of the gradient for direct SDS–PAGE analysis. Rafts (fractions 3–5) were collected, washed in buffer, recentrifuged for 1 hr, and resuspended in buffer with inhibitors. Protein was quantified and normalized among different raft preparations. Detergent soluble fractions (9–12) were pooled for subsequent immunoprecipitation. When material was limiting, only detergent-resistant membranes were prepared instead of rafts. Cells were lysed as above, and detergent-soluble and -insoluble fractions were separated by microcentrifugation at 20,000 × g. The pellet (detergent-resistant membranes) was solubilized in 60 mM β-octyl-D-glucopyranoside (Pierce) prior to immunoprecipitation or lysed directly in SDS–PAGE sample buffer. For total cell lysates, cells were lysed in buffer containing 0.1% SDS, 1% Nonidet P-40, and 0.5% Na-deoxycholate plus inhibitors.

Immunoprecipitation and Western Blotting

Immunoprecipitations and Western blotting analysis of detergent-soluble fractions or rafts solubilized in 60 mM β-octyl-D-glucopyranoside were performed as previously described (Trupp et al., 1999). All blots were scanned in a Storm 840 fluorimager (Molecular Dynamics), and quantifications were done with ImageQuant software (Molecular Dynamics). Numbers below the lanes indicate fold induction relative to control normalized to total levels of target protein. The antibodies were obtained from various sources as follows: anti-phosphotyrosine, anti-Ret, and anti-FRS2 were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Erk, anti-P-Erk (Thr-202/Tyr-204), anti-Akt, and anti-P-Akt (Ser-437) were from New England Biolabs; anti-SHC and $p13^{Suc1}$ beads were from Upstate Biotechnology (Lake Placid, NY); anti-GFRα1 was kindly provided by Michele Sanicola, Biogen.

Neuronal Differentiation, Neurite Outgrowth, and Survival Assays

For MN1 cell differentiation assays, the numbers of cells with neurites longer than two cell diameters, and cells displaying spreading (defined as large, phase-dark cells with a visible nucleus) were quantified relative to the total number of neurons counted in random fields of four different wells in each experiment. GDNF was used at a saturating dose (150 ng/ml, i.e., 6 nM) and soluble GFRα1-Fc at a subsaturating dose (300 ng/ml, i.e., 1.66 nM). The results reported are average of three independent experiments. Neuronal survival assays in dissociated cultures of E9 chick nodose ganglion and paravertebral sympathetic neurons were performed as previously described (Trupp et al., 1995). To ensure that any increase in the response observed after addition of soluble GFRα1 would reflect an enhanced efficacy of the combined treatment and not merely a suboptimal supply of factor, GDNF was used at a saturating dose (150 ng/ml, i.e., 6 nM) and soluble GFRα1-Fc at a subsaturating dose (150–300 ng/ml, i.e., 0.83–1.66 nM). For differentiation assays, following coating with GFRα1-Fc (see above), cover slips were further treated with polyornithine (1 mg/ml) and laminin (100 μg/ml). Polysterene 5 μm microspheres (Polysciences) were coated with 25 μg/ml GFRα1-Fc or BSA in borate buffer (pH 8.0) overnight at room temperature, followed by several washes in PBS. For biochemical analyses, cultures of nodose or sympathetic neurons were maintained in the presence of BDNF or NGF, respectively, for 48 hr prior to acute stimulation with GDNF or GDNF plus soluble GFRα1.

Confocal Microscopy and Immunohistochemistry

The analysis of c-Ret-GFP distribution in Neuro2A cells was done essentially as described by Ledesma et al. (1998) (Ledesma et al., 1998). Rhodamine-conjugated cholera toxin B fragment was from List Biological Laboratories. For immunohistochemistry, primary cultures were fixed in 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100. Anti-GAP43 antibodies were from Sigma, and Alexa-conjugated phalloidin was from Molecular Probes. Confocal microscopy was performed in a Zeiss confocal microscope with 25× or 63× objective lenses using laser excitation wavelengths 488 and 543 nm.

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Note Added in Proof

A recent study showed that GFRα1 is released by gut cells and that soluble GFRα1 potentiates survival of enteric neurons by GDNF: Worley, D.S., Pisano, J.M., Choi, E.D., Walus, L., Hession, C.A., Cate, R.L., Sanicola, M., and Birren, S.J. (2000). Developmental regulation of GDNF response and receptor expression in the enteric nervous system. Development *127*, 4383–4393.