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Signaling Complexes and Protein-Protein Interactions Involved in the Activation of the Ras and Phosphatidylinositol 3-Kinase Pathways by the c-Ret Receptor Tyrosine Kinase*

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Proximal signaling events and protein-protein interactions initiated after activation of the c-Ret receptor tyrosine kinase by its ligand, glial cell line-derived neurotrophic factor (GDNF), were investigated in cells carrying native and mutated forms of this receptor. Mutation of Tyr-1062 (Y1062F) in the cytoplasmic tail of c-Ret abolished receptor binding and phosphorylation of the adaptor Shc and eliminated activation of Ras by GDNF. Phosphorylation of Erk kinases was also greatly attenuated but not eliminated by this mutation. This residual wave of Erk phosphorylation was independent of the kinase activity of c-Ret. Mutation of Tyr-1096 (Y1096F), a binding site for the adaptor Grb2, had no effect on Erk activation by GDNF. Activation of phosphatidylinositol-3 kinase (PI3K) and its downstream effector Akt was also reduced in the Y1062F mutant but not completely abolished unless Tyr-1096 was also mutated. Ligand stimulation of neuronal cells induced the assembly of a large protein complex containing c-Ret, Grb2, and tyrosine-phosphorylated forms of Shc, $p85^{PI3K}$, the adaptor Gab2, and the protein-tyrosine phosphatase SHP-2. In agreement with Ras-independent activation of PI3K by GDNF in neuronal cells, survival of sympathetic neurons induced by GDNF was dependent on PI3K but was not affected by microinjection of blocking anti-Ras antibodies, which did compromise neuronal survival by nerve growth factor, suggesting that Ras is not required for GDNF-induced survival of sympathetic neurons. These results indicate that upon ligand stimulation, at least two distinct protein complexes assemble on phosphorylated Tyr-1062 of c-Ret via Shc, one leading to activation of the Ras/Erk pathway through recruitment of Grb2/Sos and another to the PI3K/Akt pathway through recruitment of Grb2/Gab2 followed by $p85^{PI3K}$ and SHP-2. This latter complex can also assemble directly onto phosphorylated Tyr-1096, offering an alternative route to PI3K activation by GDNF.

The receptor tyrosine kinase c-Ret is one of the first components in the signaling cascade activated by members of the GDNF[1] family, a group of structurally and functionally related polypeptides involved in the control of neuron survival and differentiation, kidney morphogenesis, and spermatogonial cell fate (1–3). Binding of GDNF to c-Ret is mediated by a glycosyl phosphatidylinositol-anchored co-receptor subunit termed GFRα1 (4, 5). Three close mammalian homologues of GDNF have been identified, all of which utilize c-Ret as signaling receptor with the aid of different members (GFRα1 to -4) of the GFRα family of glycosyl phosphatidylinositol-linked co-receptors (reviewed in Refs. 6 and 7). In the absence of c-Ret, GDNF family ligands may still signal in some cell types expressing GFRα receptors via activation of members of the Src family of cytoplasmic tyrosine kinases in lipid raft microdomains, presumably in collaboration with yet unknown transmembrane proteins (8, 9). GFRα receptors can mediate activation of c-Ret by GDNF when expressed on the surface of the same cell (*cis* signaling) or when presented in soluble form or immobilized onto agarose beads (*trans* signaling) (5, 10, 54). Several point mutations and chromosomal rearrangements can also activate the c-Ret kinase. In humans, these mutations turn on the oncogenic potential of the c-*ret* gene, leading to the development of several types of cancers, including multiple endocrine neoplasias type 2A and 2B (MEN2A and MEN2B), familial medullary thyroid carcinomas, and papillary thyroid carcinomas (reviewed in Refs. 12 and 13). Although the c-*ret* gene has been known for more than a decade, most of our knowledge about its signal transduction capabilities derives from studies of its oncogenic forms, several of which appear to activate unique signaling pathways.

Activation of c-Ret initiates many of the same signal transduction pathways activated by other receptor tyrosine kinases including the Ras/Raf pathway, which leads to activation of the mitogen-activated protein kinases Erk1 and Erk2, and the PI3K pathway, which leads to activation of the serine-threonine kinase Akt and cell survival (Ref. 8 and references therein). However, it is still unclear how these two pathways are initiated by the c-Ret receptor. A number of adaptor proteins have been implicated in signaling by various oncogenic and ligand-activated forms of c-Ret, including Shc, Grb2, SNT/FRS2, Gab1, Nck, Crk, and $p62^{Dok}$ (8, 14–23). The Shc adaptor protein has been shown to interact with phosphorylated Tyr-1062 in several oncogenic forms of c-Ret (18, 24). The adaptor Grb2 links Shc to the Ras pathway, and other work has shown that it can also be recruited directly to c-Ret by binding to phosphorylated Tyr-1096 in the tail of the long isoform of this

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[1] The abbreviations used are: GDNF, glial cell line-derived neurotrophic factor; MEN, multiple endocrine neoplasia; Erk, extracellular signal-regulated kinase; PI3K, phosphatidylinositol-3 kinase; SCG, superior cervical ganglion; NGF, nerve growth factor; GST, glutathione *S*-transferase; RBD, Ras binding domain; GFR, GDNF family receptor.

receptor (14). Intriguingly, the short c-Ret isoform, which differs from the long in the 50 C-terminal residues and lacks Tyr-1096, appears to also be able to recruit Grb2 independently of Shc, although the precise mechanism in this case is unknown (14). Whether activation of Ras is coupled to Tyr-1062, Tyr-1096, or to other phosphorylated residues in c-Ret is still unclear. Activation of PI3K, on the other hand, has recently been shown to depend exclusively on phosphorylation of Tyr-1062 in the short isoform of the MEN2A Ret oncoprotein (25). How PI3K activation is triggered by ligand-activated c-Ret and whether this is dependent upon Ras activity is unknown.

The PI3K/Akt pathway is an important regulator of neuronal survival, both in central and peripheral subpopulations (26–28). Survival of sympathetic neurons of the superior cervical ganglion (SCG) induced by nerve growth factor (NGF) is critically dependent upon an intact PI3K pathway (29, 30). GDNF is also able to activate PI3K and to promote survival of SCG neurons (31), although a causal link between these two events has not been established. In the case of NGF, both Ras-dependent and Ras-independent mechanisms of PI3K activation are at work, each accounting for roughly 50% of the survival responses of SCG neurons to NGF (32, 33). The role of Ras in the survival responses elicited by GDNF has not been addressed.

In the work presented here, we have investigated how the Ras and PI3K pathways couple to the c-Ret receptor when activated by GDNF in cells co-expressing the GFRα1 ligand binding subunit. These studies led us to the characterization of distinct macromolecular complexes that assemble in a ligand-dependent manner onto the activated c-Ret receptor, leading to activation of the Ras/Erk and PI3K/Akt pathways.

EXPERIMENTAL PROCEDURES

Cell Lines and Growth Factors—Fibroblast cell lines were derived from mouse MG87 cells, an NIH3T3 subclone. The M23 cell line was derived by stable transfection of full-length rat GFRα1 into MG87 cells. c-Ret-expressing cell lines were derived by stable transfection of wild type and mutated forms of full-length human c-Ret (long isoform) into M23 cells. MN1 is an immortalized motorneuron cell line generated by cell fusion of mouse embryonic motor neurons with a mouse neuroblastoma (34) and expresses endogenous c-Ret and GFRα1. Recombinant rat GDNF was produced in SF21 insect cells and purified as described previously (35). NGF was purchased from Promega.

Site-directed Mutagenesis—Point mutations in human c-Ret (subcloned in pCDNA3, Invitrogen) were introduced by oligonucleotide-mediated site-directed mutagenesis by the Kunkel method (36).

Western Blotting, Immunoprecipitation—Cell monolayers in 10-cm plates were changed to serum-free media 16 h before incubation at 37 °C with 50 ng/ml GDNF for the indicated time periods and immediately lysed with 1 ml of ice-cold 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 mixture of protease inhibitors (Roche Molecular Biochemicals) and a mixture of phosphatase inhibitors (1 mM sodium orthovanadate, 20 mM NaF, 10 mM β-glycerolphosphate). After a 15-min lysis on ice, cell lysates were cleared by centrifugation. Immunoprecipitations were done by 4 °C overnight incubation of cell lysates with antibodies plus 100 μl of protein G-Sepharose bead slurry (Pharmacia Biotech, Uppsala, Sweden). Beads were washed five times with lysis buffer and boiled in SDS/β-mercaptoethanol buffer. Immunoprecipitates were fractionated by SDS-polyacrylamide gel electrophoresis 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). All blots were scanned in a Storm 840 fluorimager (Molecular Dynamics). For reprobing, blots were stripped for 60 min at room temperature in 0.1 M acetic acid, 0.15 M NaCl. Antibodies were obtained from various sources as follows: anti-Shc, anti-Gab1, anti-Gab2, and anti-p85^{PI3K} were from Upstate Biotechnology Inc. (Lake Placid, NY); anti-Grb2, anti-SHP-2, anti-phosphotyrosine, and anti-Ret (long isoform) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); anti-Ras was from Transduction Laboratories (Lexington, KY); anti-Erk, anti-P-Erk, anti-Akt, and anti-P-Akt were from New England Biolabs (Hutchin, UK); anti-glutathione *S*-transferase (GST) was from Amersham Pharmacia Biotech). The protein kinase C inhibitor Gö6983 (Calbiochem) was used at 120 nM.

Kinase Assays—For PI3K assays, total lysates, prepared from cells treated or not with GDNF (50 ng/ml) for 45 min, were immunoprecipitated with anti-p85^{PI3K} antibodies. After immunoprecipitation and washing, beads were further washed twice in TNE (10 mM Tris HCl, 150 mM NaCl, 5 mM EDTA) containing 0.1 mM sodium orthovanadate and protease inhibitors. Pellets were then incubated for 10 min at 37 °C in 50 μl of TNE supplemented with 20 μg of phosphatidylinositol, 20 mM $MgCl_2$, 0.88 mM ATP, and 30 μCi of [γ-^{32}P]ATP. The reaction was stopped by addition of HCl 6 N, and the radiolabeled lipids were extracted with $CHCl_3$:CH_3OH (1:1). Fifty μl of the organic phase were spotted on silicon TLC plates, and lipids were then separated by thin layer chromatography and visualized by autoradiography on phosphorscreens.

For Akt kinase assays, after immunoprecipitation and washing, beads were further washed twice in kinase buffer (20 mM HEPES, pH 7.0, 25 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM sodium orthovanadate, 25 mM β-glycerolphosphate) supplemented with protease inhibitors and incubated at room temperature for 20 min in 50 μl of kinase buffer containing 50 μCi of [γ-^{32}P]ATP, 1 mM ATP, and histone B2 (HB2) as substrate. After a 20-min incubation at 30 °C, the reaction was stopped by the addition of an equal volume of SDS/β-mercaptoethanol sample buffer, boiled, fractionated by SDS/polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membranes. The membranes were exposed to phosphorscreens, 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.

GST Fusion Proteins and Far Western Blotting—Plasmids for bacterial expression of GST fusions with amino acids 1 to 149 from the Ras binding domain (RBD) of Raf1 (GST-RBD), full-length p85^{PI3K} (GST-p85^{PI3K}FL), and full-length Grb2 (GST-Grb2FL) were obtained from Stephen Taylor (Cornell University, Ithaca, NY), Jonathan Backer (Albert Einstein College of Medicine, Bronx, NY), and James Bliska (SUNY, Stony Brook, NY), respectively. GST fusions were produced in *Escherichia coli* and purified by chromatography on glutathione-conjugated-agarose beads (Amersham Pharmacia Biotech). For pull-down assays, the indicated purified GST fusion proteins were incubated with cell lysates overnight at 4 °C together with glutathione-Sepharose beads, followed by washing, SDS/polyacrylamide gel electrophoresis, and Western blotting as above. The activated Ras interaction assay was performed as described previously (37) using the pGEX-RBD plasmid encoding a GST-RBD fusion protein. For Far Western blotting, polyvinylidene difluoride blots were blocked overnight at 4 °C, followed by an incubation of 2 h at room temperature with purified GST fusion proteins (1 μg/ml), washed, and then developed with anti-GST antibodies as above.

SCG Neuron Culture, Survival Assays, and Microinjection—Neuronal survival assays were performed using dissociated cultures of postnatal day 1 (P1) rat SCG. Ganglia were dissociated by 2 incubations of 30 min at 37 °C in phosphate-buffered saline, the first one containing 0.025% trypsin, and the second containing 5 mg/ml collagenase (Sigma) followed by mechanical trituration in neuron basal medium. Neuron basal medium consist of 50% Dulbecco's modified Eagle's medium, 50% Ham's F-12 (Life Technologies, Inc.), and 1 mg/ml bovine serum albumin. Cells were incubated overnight in the presence of 5 ng/ml NGF. This medium was removed on the next day and replaced by fresh medium containing anti-NGF antibodies (Roche Molecular Biochemicals) and GDNF at 100 ng/ml. The PI3K inhibitor LY294002 (Calbiochem) was added together with GDNF (or with NGF at 10 ng/ml when indicated) at 10, 50, or 100 μM in neuron basal medium containing 0.6% methylcellulose to prevent cells from mechanically detaching. Survival was assessed 24 h later by counting phase-bright, neurite-bearing neurons in random fields.

Microinjection was carried out using an inverted fluorescence microscope (Axiovert 100, Zeiss) with an Eppendorf transjector and micromanipulator. Cells were injected into the cytoplasm with 0.5 mg/ml anti-Ras-blocking antibodies (Oncogene Research) or 0.5 mg/ml purified guinea pig IgG (Sigma) as a control and with 5 μg/ml neutral red 70-kDa Texas Red Dextran (Molecular Probes) in 0.5× phosphate-buffered saline. Texas Red Dextran-positive cells were counted 24 h later.

RESULTS

Activation of the Ras/Erk Pathway by Ligand-stimulated c-Ret Is Mediated by Tyr-1062 with Little or No Contribution by

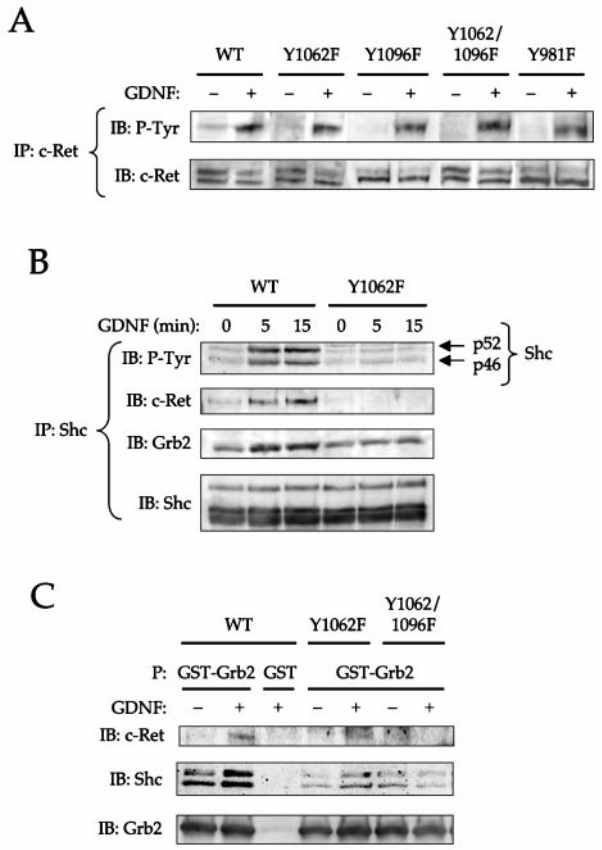

FIG. 1. **Mutation of Tyr-1062 and -1096 affect binding of Shc and Grb2, respectively, to c-Ret.** *A*, characterization of fibroblast clones expressing wild type (*WT*) and mutated c-Ret receptors as indicated. Lysate from cells treated with GDNF (50 ng/ml) were immunoprecipitated (*IP*) with c-Ret antibodies and blotted (*IB*) with anti-phosphotyrosine antibody (*P-Tyr*, *upper panel*). The blot was subsequently reprobed with Ret antibodies to confirm comparable loading among the *lanes* (*lower panel*). *B*, mutation of Tyr-1062 abolishes binding of Shc to c-Ret, Shc tyrosine phosphorylation, and Shc binding to Grb2 after GDNF treatment. Lysates of cells treated with GDNF were immunoprecipitated with Shc antibodies and then blotted with anti-phosphotyrosine, anti-c-Ret, anti-Grb2, and anti-Shc as indicated. *C*, interaction of Grb2 with c-Ret is disrupted by the Y1062F/Y1096F double mutation. Lysates of cells treated with GDNF were precipitated (*P*) with 10 μg of recombinant GST-Grb2 fusion protein (GST was used as control) and then blotted with anti-c-Ret, anti-Shc, or anti-Grb2 antibodies as indicated.

Tyr-1096—We generated fibroblast cell lines stably expressing the GFRα1 receptor together with either wild type or mutant versions of the long isoform of human c-Ret. Tyr-1062 and Tyr-1096 have previously been shown to be phosphorylated in oncogenic forms of c-Ret, where they serve as docking sites for the Shc and Grb2 adaptor proteins, respectively. Tyr-981 lies within a consensus binding site for $p85^{PI3K}$ and could therefore be involved in PI3K activation by ligand-activated c-Ret. Fibroblast lines expressing the Y981F, Y1062F, Y1096F, or the double Y1062F/Y1096F c-Ret mutants were generated and tested for ligand-dependent tyrosine phosphorylation. The four c-Ret mutants could be activated by stimulation with GDNF and showed comparable levels of total tyrosine phosphorylation (Fig. 1*A*).

Mutation of Tyr-1062 abolished binding of Shc to the activated receptor, Shc tyrosine phosphorylation, and Shc binding to Grb2 after GDNF treatment (Fig. 1*B*). Grb2, on the other hand, was still able to interact with the Y1062F mutant in a ligand-dependent manner (Fig. 1*C*). This interaction was eliminated in the double mutant Y1062F/Y1096F (Fig. 1*C*), confirming the capacity of Grb2 to associate with the activated receptor via phosphorylated Tyr-1096 independently of Shc. We then investigated the ability of the c-Ret mutants to activate Ras after GDNF stimulation using a pull-down assay with a fusion construct between GST and the RBD of Raf1 (GST-RBD), which interacts specifically with the GTP-loaded activated form of Ras. In cells expressing the wild type receptor, Ras was activated within 5 min after ligand stimulation, declining back to basal levels by 60 min (Fig. 2*A*). No stimulation of Ras activity could be observed in the Y1062F mutant after ligand treatment (Fig. 2*A*), indicating that activation of Ras by GDNF depends upon phosphorylation of this residue in c-Ret.

The activation of Erk kinases, one of the downstream targets of Ras, was investigated using anti-phospho-Erk antibodies. Rapid Erk phosphorylation was induced by GDNF in cells carrying wild type c-Ret, which lasted up to 45 min after stimulation (Fig. 2*B*). In the Y1062F mutant, Erk phosphorylation was greatly attenuated, although a residual, delayed, and transient activation remained 15 min after ligand stimulation (Fig. 2*B*). In contrast, neither the time course or intensity of Erk phosphorylation were affected after mutation of Tyr-1096 (Fig. 2*B*), confirming that this residue does not participate in activation of the Ras/Erk pathway by c-Ret. Moreover, the residual Erk phosphorylation observed in the Y1062F mutant was not eliminated in the Y1062F/Y1096F double mutant, indicating that it was not mediated via Tyr-1096. Inhibition of protein kinase C could neither block the residual Erk activation in the Y1062F mutant (Fig. 2*C*), ruling out the participation of the phospholipase Cγ pathway in this response. A similar transient peak in Erk phosphorylation was also observed in a fibroblast line carrying the kinase-inactive c-Ret mutant Y905F (Fig. 2*C*), which replaces a crucial tyrosine in the activation loop of the enzyme. This result suggested that the residual Erk activation remaining in the Y1062F mutant was a c-Ret-independent response. In fact, GDNF-dependent phosphorylation of Erk has been detected in cells expressing GFRα1 receptors in the absence of c-Ret (9).[2] This novel pathway is mediated by members of the Src kinase family and appears not to involve Ras activation (8), in agreement with the present findings. Thus, activation of the Ras/Erk pathway by ligand stimulation of c-Ret is mediated by phosphorylation of Tyr-1062 followed by docking of Shc and Grb2, with little or no participation of the Grb2 binding site at Tyr-1096. These results also indicated that binding of Grb2 to phosphorylated Tyr-1096 may lead to the recruitment of other c-Ret targets distinct from the Ras activator Sos.

Activation of the PI3K/Akt Pathway Is Mediated by Both Tyr-1062 and Tyr-1096—We then examined the activity of PI3K in wild type and Y1062F mutant c-Ret after GDNF treatment. Ligand stimulation caused a significant increase in PI3K activity in cells carrying wild type c-Ret (Fig. 3*A*). Mutation of Tyr-1062 greatly diminished this response but did not abolished it completely; a residual 50% increase over base line could be reproducibly detected after GDNF treatment (Fig. 3*A*). Stimulation of the kinase activity of Akt, a target of the PI3K pathway, in response to GDNF was also attenuated, but not totally eliminated, in cells carrying the Y1062F c-Ret mutant, which showed a residual 50% stimulation over base line (Fig. 3*B*). In agreement with this, a reduced and delayed stimulation of Akt phosphorylation by GDNF could still be detected in these cells (Fig. 3*C*). Together, these data indicated the existence of alternative pathways for PI3K/Akt activation independently of phosphorylation of Tyr-1062 and suggested that Tyr-1096, which bound Grb2 but did not contribute to the Ras pathway, could be involved. Indeed, ligand-dependent phosphorylation of

[2] V. Besset, R. P. Scott, and C. F. Ibáñez, unpublished observations.

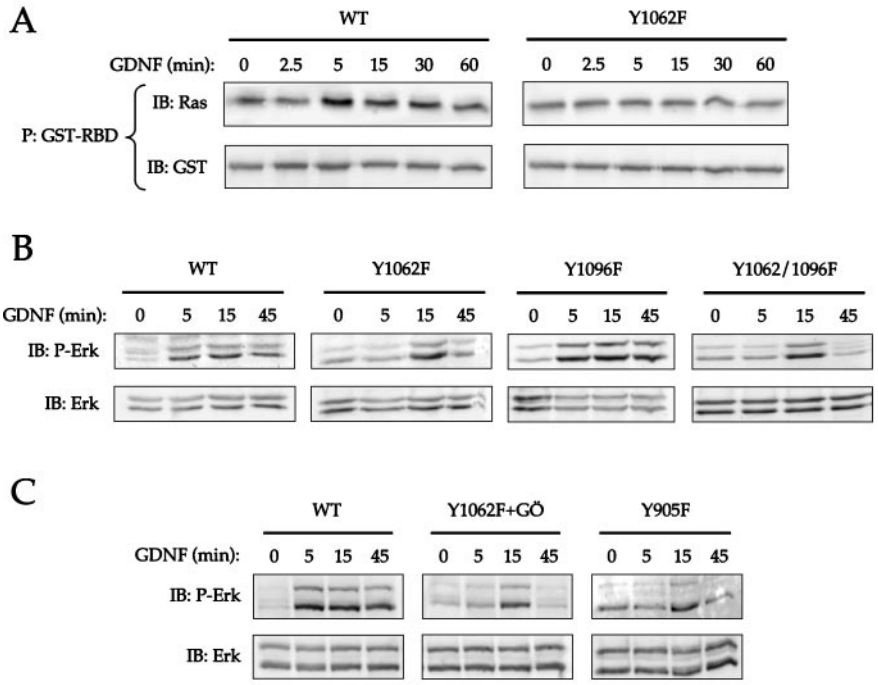

FIG. 2. **Mutation of Tyr-1062 abolishes Ras activation in fibroblast cells but only partially affects Erk phosphorylation.** *A*, pull-down (*P*) assay of activated GTP-loaded Ras with a GST-RBD fusion construct. GST-RBD will only bind to activated GTP-Ras, which after SDS-polyacrylamide gel electrophoresis can be detected by immunoblotting (*IB*) with anti-Ras antibodies (*upper panel*). *WT*, wild type. The blots were then reprobed with anti-GST antibodies (*lower panel*). *B* and *C*, fibroblasts expressing wild type or mutated c-Ret were treated with GDNF (50 ng/ml) for different time points as indicated. Cell lysates were blotted with anti-P-Erk antibodies (*top panel*) and reprobed with anti-total Erk antibodies (*lower panel*). In *panel C*, the fibroblasts carrying the Y1062F c-Ret mutant were pre-treated with the protein kinase C inhibitor Gö6983 at 120 nM for 30 min before treatment with GDNF.

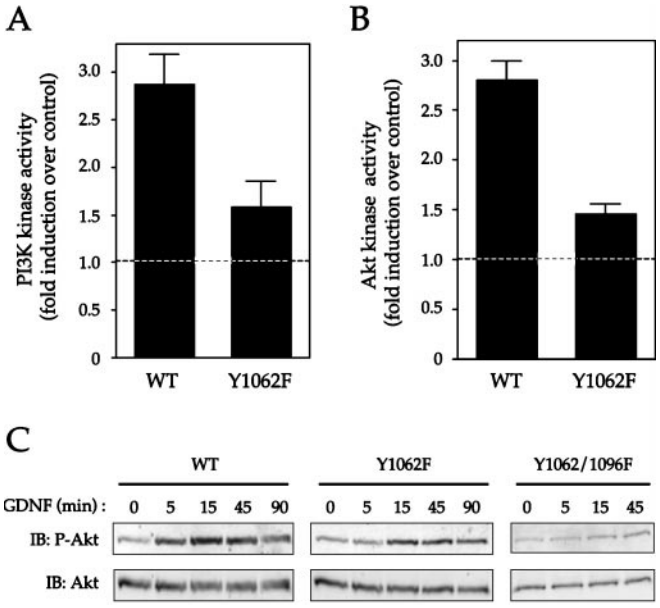

FIG. 3. **Both Tyr-1062 and-1096 contribute to activation of the PI3K/Akt pathway by GDNF.** *A*, PI3-kinase activation induced by GDNF (50 ng/ml, 45 min) in fibroblast cell lines carrying wild type (*WT*) and mutated (Y1062F) c-Ret. Results are the mean ± S.E. of three different experiments and are presented relative to control cells that were not treated with GDNF. *B*, Akt kinase activation induced by GDNF (50 ng/ml, 15 min) in fibroblast cell lines carrying wild type (*WT*) and mutated (Y1062F) c-Ret. Results are the mean ± S.E. of three different experiments and are presented relative to control cells that were not treated with GDNF. *C*, lysates from the indicated cell lines treated with GDNF (50 ng/ml) were analyzed by Western blotting (*IB*) using anti-P-Akt antibodies (P-Akt, *upper panel*) and reprobed with anti-total Akt antibodies (*lower panel*).

Akt was completely eliminated in the Y1062F/Y1096F double mutant (Fig. 3*C*), demonstrating the participation of Tyr-1096 in the activation of the PI3K/Akt pathway by c-Ret.

PI3K may be activated through the recruitment of the $p85^{PI3K}$ regulatory subunit to activated receptors. We examined the role of $p85^{PI3K}$ in the activation of PI3K by c-Ret using pull-down assays with a recombinant GST-$p85^{PI3K}$ fusion protein followed by c-Ret immunoblotting. GST-$p85^{PI3K}$ brought down wild type c-Ret in a ligand-dependent manner (Fig. 4*A*), suggesting a capacity of the $p85^{PI3K}$ subunit for interacting with the activated receptor. In agreement with our previous results, we observed a diminished but significant interaction of GST-$p85^{PI3K}$ with Y1062F c-Ret but not with the Y1062F/Y1096F double mutant (Fig. 4*A*), underscoring the role of both these tyrosine residues in the recruitment of $p85^{PI3K}$ to the activated receptor. GST-$p85^{PI3K}$ was also able to pull-down the Y981F mutant in a ligand-dependent manner (Fig. 4*A*), indicating that this residue does not play a significant role in the activation of PI3K by c-Ret. This was also confirmed by the ability of this mutant to induce normal phosphorylation of Akt upon ligand stimulation (data not shown). In addition to these fibroblast cell lines, GST-$p85^{PI3K}$ could also precipitate c-Ret in a ligand-dependent manner from cells of neuronal origin expressing endogenous c-Ret receptors, including the motorneuron cell line MN1 and the neuroblastoma Neuro2A-α1 (Fig. 4*B*).

The Adaptors Grb2 and Gab2 Link PI3K and SHP-2 to the Activated c-Ret Receptor in Neuronal Cells—Despite its ability to precipitate c-Ret from lysates of cells stimulated with GDNF, GST-$p85^{PI3K}$ failed to recognize the activated receptor in Far Western overlay assays of c-Ret immunoprecipitates (data not shown), although several other interactors could be identified by this method (see Fig. 6). This suggested that $p85^{PI3K}$ associated only indirectly with c-Ret after GDNF stimulation, and we therefore set out to identify the cellular component(s) mediating this interaction.

For these experiments, we turned to the motorneuron cell line MN1. This cell line, derived by immortalization of primary motorneurons by cell fusion (34), expresses endogenous GFRα1 and c-Ret receptors (2, 8) and displays survival and differentiation responses to GDNF treatment (2).[2] Immunoprecipitation of $p85^{PI3K}$ from lysates of MN1 cells followed by Tyr(P)-immunoblotting revealed that association of several tyrosine-phosphorylated proteins could be stimulated by treatment with GDNF (Fig. 5*A*). Re-probing of $p85^{PI3K}$ immunoprecipitates with specific antibodies demonstrated the ligand-stimulated incorporation of Grb2, the tyrosine phosphatase SHP-2, and Shc to this complex (Fig. 5*A*). A similar result was obtained by

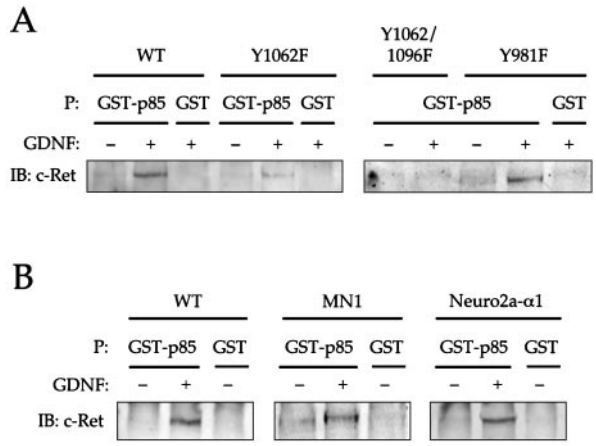

FIG. 4. **Binding of p85^{PI3K} to wild type and mutant c-Ret.** *A* and *B*, lysates from cell lines expressing wild type (*WT*) or mutated forms of c-Ret treated with GDNF (50 ng/ml) for 15 min were incubated with GST-p85^{PI3K} or GST alone and then blotted with anti-c-Ret antibodies. *P*, precipitation; IB, immunoblot.

precipitation of cell lysates with GST-p85^{PI3K} (Fig. 5*A*), confirming the presence of c-Ret, Shc, Grb2, and SHP-2 in the same complex. Immunoprecipitation of SHP-2 brought down a similar complement of tyrosine-phosphorylated proteins in MN1 cells stimulated with GDNF (Fig. 5*B*), and reprobing experiments confirmed that association of Grb2, p85^{PI3K}, and SHP-2 in the same complex is stimulated by ligand treatment (Fig. 5*B*).

A prominent 100-kDa phosphoprotein could be detected in both p85^{PI3K} and SHP-2 immunoprecipitates of GDNF-stimulated cells (Figs. 5, *A* and *B*). Subsequent immunoprecipitation/Western assays in MN1 cells identified this protein as the adaptor Gab2. This protein was found to co-immunoprecipitate with either p85^{PI3K}, SHP-2, or Grb2 in MN1 cells; however, only the interactions with p85^{PI3K} and SHP-2 were stimulated by ligand treatment (Fig. 5*C*). Of note, proline-rich regions in Gab proteins have been shown to bind to the SH3 domain of Grb2 (38), an interaction that appears not to be regulated by extracellular signals. GDNF treatment induced tyrosine phosphorylation of Gab2 in MN1 cells (Fig. 5*D*). Immunoprecipitation of Gab2 from MN1 cell lysates showed increased levels of Shc, SHP-2, and p85^{PI3K} associated with Gab2 after GDNF stimulation (Fig. 5*D*). Although clearly stimulated by ligand, there also appeared to be a degree of constitutive interaction of Gab2 with Shc and p85^{PI3K} in MN1 cells. On the other hand, the interaction between Gab2 and SHP-2 appeared to be truly ligand-dependent (Fig. 5*D*). Gab1, a better characterized Gab homologue, could not be detected in MN1 cells, although it was found to form part of an analogous protein complex in c-Ret-expressing fibroblasts (data not shown).

Taken together, these results suggested that GDNF stimulation of neuronal cells induced the formation of a large protein complex that included c-Ret, Shc, Grb2, Gab2, p85^{PI3K}, and SHP-2, but they did not establish the chain of direct interactions linking these components. We addressed this issue in Far Western overlay assays using recombinant GST-p85^{PI3K} and GST-Grb2 fusion proteins to probe Gab2, Grb2, SHP-2, and p85^{PI3K} immunoprecipitates from MN1 cells treated with GDNF. Gab2 was the principal protein recognized by the GST-p85^{PI3K} probe in Gab2, Grb2, and SHP-2 immunoprecipitates (Fig. 6*A*), suggesting a direct interaction between Gab2 and p85^{PI3K}. GDNF treatment stimulated the binding of GST-p85^{PI3K} to Gab2 (Fig. 6*A*), probably as a result of the ligand-dependent tyrosine phosphorylation of the adaptor. In agreement with this, a GST fusion protein with only the N-terminal SH2 domain of p85^{PI3K} was equally efficient at recognizing Gab2 in these immunoprecipitates (data not shown), confirming that the interaction between p85^{PI3K} and Gab2 is dependent upon phosphorylation of Gab2.

The GST-Grb2 probe also recognized Gab2 in p85^{PI3K}, SHP-2, and Gab2 immunoprecipitates (Fig. 6*B*). Equal levels of Gab2 were detected by GST-Grb2 in Gab2 immunoprecipitates from treated and untreated cells (Fig. 6*B*), in agreement with the constitutive interaction between these two adaptor proteins. The elevated levels of Gab2 detected in the p85^{PI3K} and SHP-2 immunoprecipitates after ligand treatment indicated increased association of these proteins with the Gab2 adaptor in stimulated cells (Fig. 6*B*). The second most prominent band detected by the GST-Grb2 probe was Shc (Fig. 6*B*). This interaction was stimulated by GDNF treatment, reflecting its dependence upon tyrosine phosphorylation of Shc by c-Ret. Taken together, the results from Far Western experiments indicated that, in the PI3K complex activated by GDNF and c-Ret, Grb2 interacted directly with Shc through its SH2 domain and with Gab2 through its SH3 domains, whereas p85^{PI3K} interacted directly with Gab2 via its SH2 domain.

Ras Is Not Required for GDNF-dependent Survival of SCG Neurons—Our results in fibroblasts and immortalized motorneurons indicated the existence of at least two Ras-independent routes available to c-Ret for activation of the PI3K pathway, suggesting that Ras may have a secondary role in mediating biological activities of GDNF that are dependent on PI3K activation. Survival of SCG neurons was found to be completely dependent upon PI3K activity (Fig. 7*A*), which prompted us to test the requirement of Ras for sympathetic neuron survival in response to GDNF. To this purpose, we microinjected Ras-blocking antibodies into newborn rat SCG neurons in culture and assessed the survival of microinjected cells in the presence of GDNF or NGF. Using these same antibodies (32) or adenoviruses expressing dominant negative Ras mutants (33), previous studies show that, in SCG neurons, Ras contributes to approximately 50% of the stimulation of PI3K activity and neuron survival in response to NGF. In agreement with these data, we observed a 60% reduction in neuron survival in the presence of NGF after microinjection of Ras-blocking antibodies compared with control IgG (Fig. 7*B*). The same treatment, however, had no effect on neuron survival in response to GDNF (Fig. 7*B*), indicating that activation of Ras is not necessary for GDNF-dependent survival of sympathetic neurons.

DISCUSSION

In this study, we have investigated proximal signaling events following activation of the GDNF receptor tyrosine kinase c-Ret. We have focused in elucidating the protein complexes that interact with the receptor, leading to activation of the Ras/Erk and PI3K/Akt pathways. Our results confirm the importance of phosphorylated Tyr-1062 in the cytoplasmic tail of c-Ret for the activation of these two pathways by GDNF, as suggested by previous studies using oncogenic forms of this receptor (18, 20, 25).

In contrast to Tyr-1062, phosphorylation of Tyr-1096 appears to contribute only to activation of the PI3K pathway, with little or no role in activation of Ras and Erk. This was surprising, as Grb2, which binds to phosphorylated Tyr-1096, is a well known link between receptor tyrosine kinases and the Ras pathway. This adaptor can be present in a pre-associated complex with different downstream components, including the Ras activator protein Sos and the Gab1/2 adaptors. The Grb2/Sos and Grb2/Gab complexes are stabilized via interaction between the SH3 domains in Grb2 and proline-rich regions in Sos and Gab. It is possible that the Grb2/Sos complex, although capable of interacting with phosphorylated Shc, may be steri-

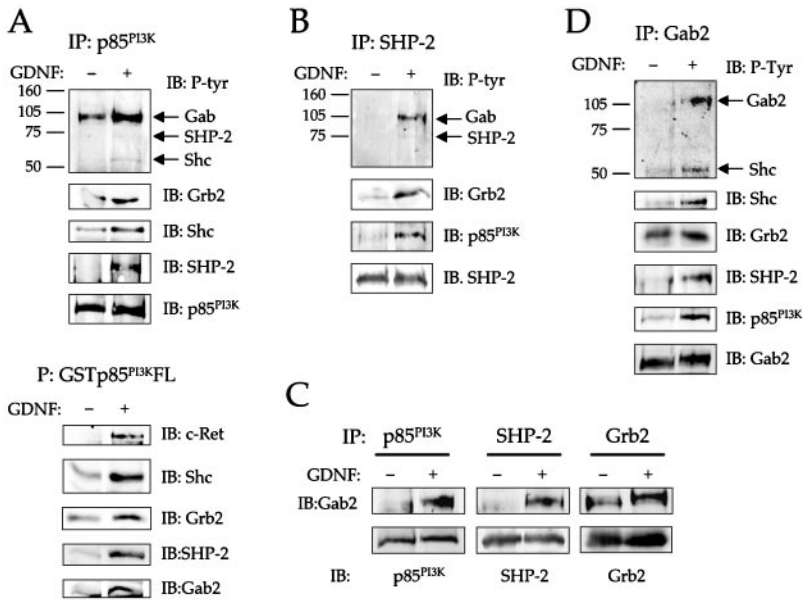

FIG. 5. **GDNF induces the formation of a large macromolecular complex containing c-Ret, Shc, Grb2, Gab2, p85^{PI3K} and SHP-2 in immortalized motorneurons.** Cell lysates from MN1 cells treated for 15 min with 50 ng/ml GDNF as indicated were immunoprecipitated with the indicated antibodies (*IP*) or GST fusion proteins (*P*) and then blotted with antibodies to different signaling components as indicated. Molecular mass markers are indicated in kDa. *A*, immunoprecipitation with anti-p85^{PI3K} (*top*) and pull-down with GST-p85^{PI3K} (*bottom*). *IB*, immunoblot; *P-tyr*, phosphotyrosine. *B*, immunoprecipitation with anti-SHP-2. *C*, p85^{PI3K}, SHP-2 and Grb2 immunoprecipitates probed with anti-Gab-2 antibodies. *D*, immunoprecipitation with anti-Gab2 antibodies.

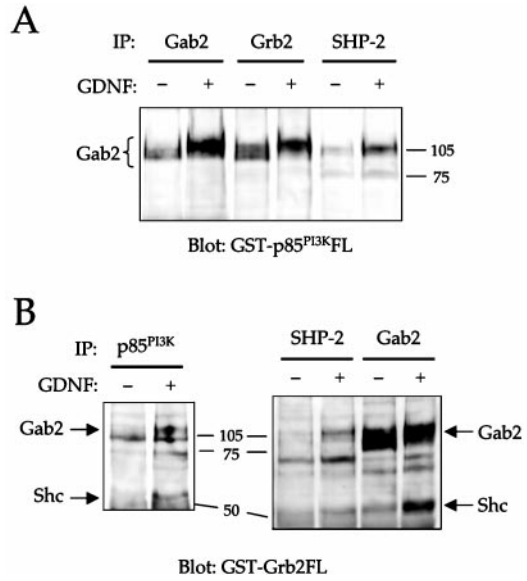

FIG. 6. **Direct ligand-dependent association of Gab2 with p85^{PI3K}, SHP-2, and Grb2.** Cell lysates from MN1 cells treated for 15 min with 50 ng/ml GDNF as indicated were immunoprecipitated (*IP*) with the indicated antibodies and then blotted with GST-p85^{PI3K}FL (*A*) or GST-Grb2FL (*B*) fusion proteins. Molecular mass markers are indicated in kDa.

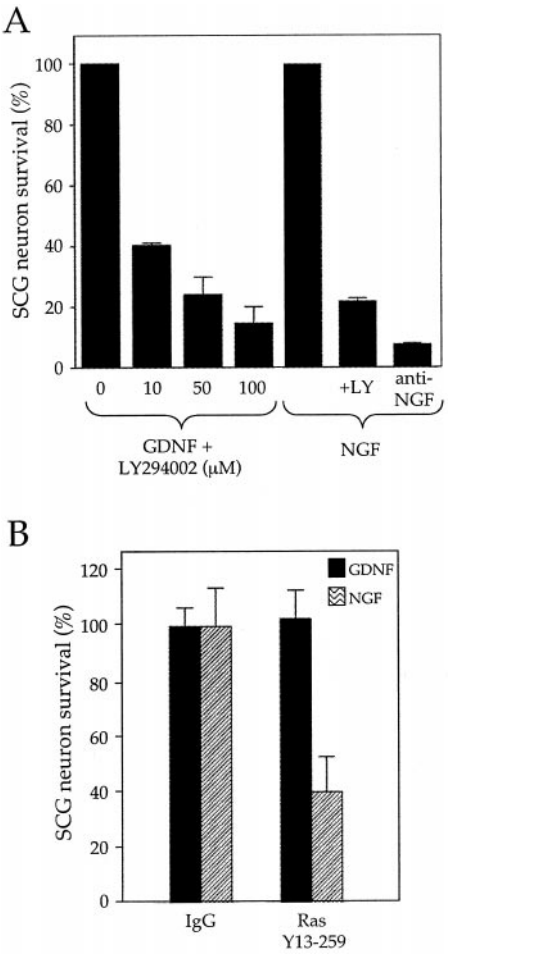

FIG. 7. **Survival of sympathetic neurons induced by GDNF is dependent on PI3K activation but is not affected by microinjection of blocking anti-Ras antibodies.** *A*, neurons from P0 rat SCG were cultured with GDNF (100 ng/ml) or NGF (10 ng/ml) in the presence of the indicated concentrations of the PI3K inhibitor LY294002. With NGF, LY294002 (*LY*) was used at 50 μM. Results are the mean of three experiments ± S.E. performed in triplicate. *B*, neurons from P0 rat SCG microinjected with the indicated antibodies were cultured in the presence of NGF (*hatched bars*) or GDNF (*solid bars*) and scored for survival 24 h after microinjection. Results are the mean of three experiments ± S.E. performed in triplicate.

cally hindered to bind to phosphorylated Tyr-1096. In agreement with this hypothesis, this residue does not appear to be crucial for the Ras-dependent-transforming activity of either MEN2A or MEN2B Ret oncogenic forms (39). On the other hand, our results indicate that the Grb2·Gab complex binds readily to phosphorylated Tyr-1096 and Shc, linking both Tyr-1062 and -1096 of c-Ret to activation of PI3K through the recruitment of p85^{PI3K}. The lack of residual PI3K activation in the Y1062F mutant of the short isoform of the MEN2A Ret oncoprotein (25) could have been due to the absence of Tyr-1096 in this receptor form and suggests differences in the mechanisms of PI3K activation by the two c-Ret isoforms. Alternatively, and because Grb2 appears to also interact directly with the short c-Ret isoform (14), the inability of Y1062F MEN2A to stimulate PI3K activity could indicate differences in the signaling mechanisms of oncogenic *versus* ligand-activated Ret receptors.

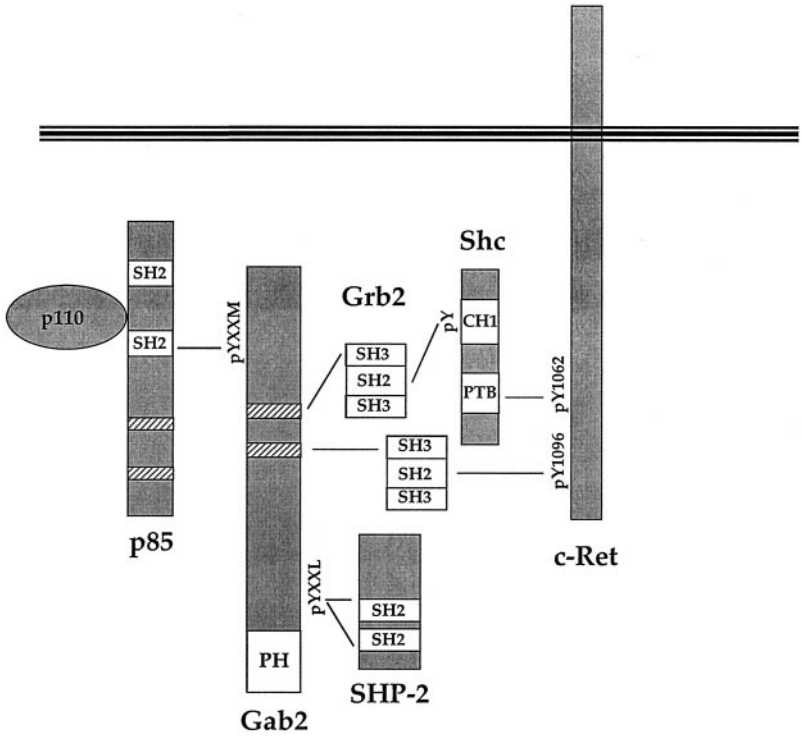

FIG. 8. **Macromolecular complexes leading to PI3K activation by c-Ret.** The scheme shows protein interactions involved in the complex formed between PI3K and c-Ret upon GDNF stimulation of MN1 cells. For SH2-mediated associations, the consensus binding sites, as reported in the literature, are indicated. Proline-rich motifs are indicated by *hatched boxes*.

The PI3K/Akt pathway can be activated by at least two distinct mechanisms: (i) binding of the regulatory subunit $p85^{PI3K}$ to tyrosine phosphorylated residues on the receptor and on docking proteins and ii) binding of the catalytic subunit $p110^{PI3K}$ to Ras (reviewed in Ref. 40). We have shown that a recombinant GST-$p85^{PI3K}$ associates with c-Ret and that this association as well as Akt phosphorylation is lost in the Y1062F/Y1096F double mutant, linking both Tyr-1062 and -1096 of c-Ret to activation of PI3K through the recruitment of $p85^{PI3K}$. Although Tyr-981 of c-Ret is present in a consensus sequence (*i.e.* Y*XX*M), preferentially recognized by the SH2 domains of $p85^{PI3K}$, we and others (25) did not find any evidence of the participation of this residue in the activation of the PI3K/Akt pathway by c-Ret.

By pull-down and co-immunoprecipitations experiments, we have shown that PI3K is linked to c-Ret through a complex containing Gab1/2, Grb2, Shp-2, and Shc. These results are summarized in the scheme shown in Fig. 8. As evidenced by Far Western blot experiments, Gab interacts directly with $p85^{PI3K}$ and Grb2. Although the interaction of $p85^{PI3K}$ with Gab was found to be stimulated by GDNF-induced phosphorylation, Grb2 binding to Gab was constitutive. Gab proteins possess several proline-rich regions (*i.e.* P*XX*P) susceptible to bind the SH3 domain of Grb2. Although such motifs are also present in $p85^{PI3K}$, we found no evidence for direct binding of Grb2 to $p85^{PI3K}$ by Far Western blot analysis (data not shown). Gab proteins possess several consensus sites for the SH2 domains of SHP-2 (41). Although $p85^{PI3K}$ could also in principle interact directly with SHP-2 through the Y*XX*M motifs present on the phosphatase, the results of our Far Western experiments indicate that, after stimulation with GDNF, the majority of SHP-2 in the $p85^{PI3K}$ complex is bound to Gab2.

Our observations implicate for the first time the adaptor protein Gab2 in receptor tyrosine kinase signaling in neuronal cells. This protein had originally been isolated as a main signaling component downstream of cytokine and antigen receptors in hematopoietic cells (41–43). Similar to its better-characterized homologue Gab1, Gab2 has been reported to bind Grb2 and $p85^{PI3K}$ as well as SHP-2 and to potentiate activation of Erk (41–43). Unlike Gab1, however, Gab2 is unable to interact directly with the c-Met receptor tyrosine kinase (38), one of the main upstream activators of Gab1, suggesting distinct functions for the two proteins. Gab2 is more abundantly expressed in the nervous system than Gab1 (43), indicating functions in neuronal cells. Gab1 had been found to mediate NGF-induced survival, neurite outgrowth, and DNA synthesis in PC12 cells, where its overexpression induced activation of Akt and Erk kinases (44, 45). Although we found that Gab1 plays a similar role to that of Gab2 in GDNF signaling in fibroblast cells,[3] we were unable to detect this adaptor protein in MN1 cells.

GDNF promotes survival of newborn rat SCG neurons *in vitro* (31, 35, 46) and is required for their survival *in vivo* (47–49). GDNF induces PI3K activity in SCG neurons (31), and we have shown that neuron survival in response to GDNF is dependent upon activation of this enzyme. Our results indicate that activation of PI3K by GDNF can be mediated independently of Ras by recruitment of $p85^{PI3K}$ to the activated c-Ret receptor. In agreement with this, we show that survival of sympathetic neurons in response to GDNF is dependent upon PI3K activation but is not affected by inhibition of Ras. This is in contrast to NGF, where Ras activity contributes to both PI3K activation and neuron survival in sympathetic as well as sensory neurons (this study and Refs. 33 and 50). Interestingly, survival of serum-deprived PC12 cells in the presence of NGF does not appear to require Ras activity (51), indicating that the same receptor system may also work differently in different cell types. An important difference between NGF and GDNF signaling is that, whereas in the NGF receptor TrkA both Ras and PI3K pathways depend upon the binding of Shc to a single phosphotyrosine residue (*i.e.* Tyr-490) (52, 53), at least two different residues in c-Ret (*i.e.* Tyr-1062 and -1096) can mediate PI3K activation (Fig. 8). Thus, it may be that Ras plays only a minor contribution in PI3K activation by GDNF or, alternatively, that PI3K activity in GDNF-treated cells is not limiting,

[3] V. Besset, unpublished observations.

so that elimination of the Ras component does not reduce it below the threshold level required to maintain maximal neuronal survival. In agreement with the former possibility, inhibition of Ras activity in the neuroectoderm-derived cell line SKF5 had no significant effect on Akt activation and lamellipodium formation induced by an EGFR/c-Ret chimeric receptor (11).

In conclusion, our results indicate that c-Ret can activate the PI3K/Akt pathway via Tyr-1062 and-1096, whereas only Tyr-1062 appears to contribute to activation of the Ras/Erk pathway. Activation of PI3K by GDNF is mediated by the assembly of a large protein complex onto the c-Ret receptor in which the adaptor proteins Shc, SHP-2, Grb2, and Gab2 play a crucial role. Finally, although GDNF-induced survival of SCG neurons depends on PI3K activity, in contrast to NGF, it is independent of Ras. Because sympathetic and other neurons are responsive to both GDNF and NGF (or other neurotrophins), differences in the requirements of signaling components for the biological activities of these ligands may form a molecular basis for the synergistic cooperation of different neurotrophic factors in neuronal survival and differentiation.

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