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Glial Cell Line-derived Neurotrophic Factor-stimulated Phosphatidylinositol 3-Kinase and Akt Activities Exert Opposing Effects on the ERK Pathway

IMPORTANCE FOR THE RESCUE OF NEUROECTODERMIC CELLS*

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Glial cell line-derived neurotrophic factor (GDNF) plays a crucial role in rescuing neural crest cells from apoptosis during their migration in the foregut. This survival factor binds to the heterodimer GDNF family receptor α1/Ret, inducing the Ret tyrosine kinase activity. *ret* loss-of-function mutations result in Hirschsprung's disease, a frequent developmental defect of the enteric nervous system. Although critical to enteric nervous system development, the intracellular signaling cascades activated by GDNF and their importance in neuroectodermic cell survival still remain elusive. Using the neuroectodermic SK-N-MC cell line, we found that the Ret tyrosine kinase activity is essential for GDNF to induce phosphatidylinositol 3-kinase (PI3K)/Akt and ERK pathways as well as cell rescue. We demonstrate that activation of PI3K is mandatory for GDNF-induced cell survival. In addition, evidence is provided for a critical up-regulation of the ERK pathway by PI3K at the level of Raf-1. Conversely, Akt inhibits the ERK pathway. Thus, both PI3K and Akt act in concert to finely regulate the level of ERK. We found that Akt activation is indispensable for counteracting the apoptotic signal on mitochondria, whereas ERK is partially involved in precluding procaspase-3 cleavage. Altogether, these findings underscore the importance of the Ret/PI3K/Akt pathway in GDNF-induced neuroectodermic cell survival.

Embryonic development of the enteric nervous system (ENS)[1] requires proliferation, migration, differentiation, and survival of the neural crest cells. The glial cell line-derived neurotrophic factor (GDNF), produced by the enteric mesenchyme, has emerged as a key molecule for this process (1–3). The action of GDNF is mediated by a multicomponent receptor complex composed of the Ret tyrosine kinase (TK) and the glycosyl phosphatidyl inositol-linked protein, GDNF family receptor α1 (GFRα1) (4, 5), both expressed by the crest-derived precursors in the developing ENS (6). Compelling evidence has helped to determine that signals by GDNF-activated Ret are essential for the *survival* of neural crest cells that colonize the gut. Indeed, germ line loss-of-function *ret* mutations that impair either its expression or its intrinsic TK are involved in the Hirschsprung's disease, a frequent development defect (1 in 5000 births) characterized by the loss of the enteric ganglia (for a review, see Ref. 7). Consistently, gene-targeted deletion in mice of either GDNF (1, 3) or *ret* (8, 9) results in intestinal aganglionosis. The notion that Ret is absolutely required for rescue of neural crest cells came from the recent demonstration that *ret*-deficient mice develop an aganglionic phenotype because of the apoptosis of crest-derived precursors as they enter the foregut (10).

Although critical to the normal ENS development, the intracellular signaling cascades used by the GDNF-induced Ret activity to block neuroectodermic cell apoptosis still remain elusive. In several neuronal cell lines, it has been observed that GDNF induces the activation of phospholipase C-γ, c-Src, phosphatidylinositol 3-kinase (PI3K), and extracellular signal-regulated kinase (ERK; also referred as mitogen-activated protein kinase) pathways (11–14). Of these, PI3K and ERK are likely candidates for mediating GDNF-induced cell survival. Indeed, it is well known that interleukin-2 (15), interleukin-3, nerve growth factor (16, 17), and insulin-like growth factor I (18, 19) promote cell survival by activating PI3K. In contrast, activation of ERK is associated with antiapoptotic signaling of cardiotrophin-1 (20), fibroblast growth factor-2 (21), PACAP-38 (22), and endothelin-1 (23). Likewise, it has been reported that BDNF rescues cerebellar granule neurons from apoptosis via

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[1] The abbreviations used are: ENS, enteric nervous system; ERK, extracellular signal-regulated kinase; anti-P Akt, anti-Ser^{473}-phosphorylated Akt; anti-phospho-ERK, anti-Thr^{202}/Tyr^{204}-phosphorylated ERK; GDNF, glial cell line-derived neurotrophic factor; GFRα1, GDNF family receptor α1; Ly, Ly294002; PI3K, phosphatidylinositol 3-kinase; Ret^{WT}, wild type Ret; Ret^{H13}, Ser^{765} → Pro Ret; SK-Ret^{WT} cells, Ret^{WT}-transfected SK-N-MC cells; TK, tyrosine kinase; WCL, whole cell lysate(s); Ψ_M, mitochondrial transmembrane potential; WT, wild type; PI, phosphatidylinositol; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling; PAGE, polyacrylamide gel electrophoresis.

both ERK and PI3K (24) and motoneurons via PI3K (25). Altogether, this illustrates that the implication of ERK and/or PI3K pathways in the mediation of cell survival is critically dependent of the cytokines but also of the biological systems examined. Three recent studies have suggested the involvement of PI3K in the GDNF-induced survival of dopaminergic, sympathetic, and spinal cord motor neurons (26–28), although the PI3K targets and their role in the mediation of this anti-apoptotic effect are still unknown. However, the fact that the development of the enteric neurons and not of these GDNF-responsive neurons is impaired in *ret*-deficient mice supports the idea that the mechanisms underlying the survival of the crest-derived cells might be different from those of other origins.

This prompted us to use in this study the human neuroectodermic SK-N-MC cell line, because it presents the advantage of expressing constitutively GFRα1 but not Ret (13). In these conditions, SK-N-MC cells could be stably transfected with either the wild type (WT) or the kinase-dead H13 *ret* cDNA. This model is suitable to dissect the signaling events induced by GDNF and to address their involvement in the survival of crest-derived cells. We demonstrate that the Ret-dependent activation of PI3K/Akt is essential for SK-N-MC cell rescue. Interestingly, we found that ERK is finely regulated by PI3K and Akt and that it does contribute to the ability of GDNF to prevent procaspase-3 cleavage. Altogether, our data underscore the critical role played by the PI3K/Akt pathway in tight control of ERK signal and GDNF-induced cell rescue. Implications of these findings in the understanding of how GDNF supports ENS development are discussed.

EXPERIMENTAL PROCEDURES

Plasmids

The full-length *ret* cDNA coding for the short isoform (Ret9; 1072 amino acids) was cloned into the pAlter vector (Promega), and site-directed mutagenesis of Ser^{765} by Pro (H13 mutation, TCC → **C**CC) was performed as already described (29). Both the WT and the H13 mutated *ret* cDNAs were then subcloned into the *Xba*I site of the pRc/CMV expression vector (Invitrogen). The plasmids for the following cDNA constructs have been described previously: $\Delta p85^{PI3K}$ (a deletion mutant of $p85^{PI3K}$, which is unable to bind to $p110^{PI3K}$, thus preventing specifically its activation (30)), $p110^{PI3K+}$ (a dominant positive mutant of $p110^{PI3K}$ (31)), ST^{-} AKT (a dominant negative mutant of Akt in which the sites of phosphorylation Thr^{308} and Ser^{473} are replaced with Ala (32)), K → A AKT (the kinase-dead construct of Akt in which the Lys^{179} is replaced with Ala (32)), GAG-AKT (a dominant positive mutant of Akt in which Gag protein is fused in frame with the N terminus of Akt, corresponding to the retroviral AKT 8 oncogene (32, 33)), RAC^{N17} (a dominant negative mutant of Rac in which Thr^{17} is replaced by Asn (34)), GST-Raf-1 RBD (a glutathione *S*-transferase fusion protein containing the Ras-binding domain of Raf-1 (35)).

Cell Culture and Transfections

The human neuroectodermic SK-N-MC cell line (13) was cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (Life Technologies, Inc.), 2 mM pyruvate, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (complete medium). To generate SK-N-MC cell lines expressing either the WT or the H13 mutated Ret, 150-mm diameter plates of SK-N-MC cells were stably transfected with 10 μg of corresponding *ret* plasmid using the polyethyleneimine (Sigma) precipitation method. 48 h later, the transfected cells were selected in complete medium containing 0.8 mg/ml G418 (Geneticin; Life Technologies). After 2 weeks, at least 100 G418-resistant clones were picked, expanded, and assayed for Ret expression by anti-Ret Western blotting (C19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The SK-N-MC cell lines that express either the WT or the H13 mutated Ret are referred throughout as $SK\text{-}Ret^{WT}$ and $SK\text{-}Ret^{H13}$ cells, respectively.

Cell Treatments

For all experiments, cells were grown to 70% confluence, serum-starved for 6–16 h in fresh Dulbecco's modified Eagle's medium supplemented with 0.1% bovine serum albumin (A7030; Sigma), and stimulated with GDNF (+ in Figs. 1, 2*B*, 3–10; 100 ng/ml; Genentech). When either wortmannin (PI3K inhibitor (36), 100 nM; Sigma), Ly294002 (stable PI3K inhibitor (37), 10 μM; Sigma), or Park Davis inhibitor PD98059 (MEK-specific inhibitor (38), 50 μM; Biomol Research Laboratories) was used, it was added to the starvation medium (for 60 and 90 min, respectively) prior to GDNF activation. As controls, cells were incubated with Me_2SO (excipient; 1:5000) or left untreated (− in Figs. 1, 3, and 5–10).

Cell Lysis and Immunoprecipitation

Cells were washed with PBS and solubilized in Nonidet P-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM Na_3VO_4, 10 mM β-glycerophosphate, 10 mM sodium fluoride, 2 mM EDTA, 1 μM aprotinin, 25 μM leupeptin, 1 μM pepstatin, 2 mM phenylmethylsulfonyl fluoride). 1 mg of precleared whole cell lysates (WCL) was immunoprecipitated with 2 μg of either anti-Ret, anti-phosphotyrosine (Tyr(P); Upstate Biotechnology, Inc.), anti-Shc (Upstate Biotechnology), or anti-$p85^{PI3K}$ (Upstate Biotechnology) antibody, for either 4 h (kinase assays) or 16 h at 4 °C, as described (39).

Western Blotting

WCL (50 μg) or immunoprecipitates were separated on 9% SDS-PAGE, electroblotted onto a polyvinylidene fluoride membrane (Immobilon-P; Millipore Corp.) and analyzed by Western blotting with either anti-Ret (1:2000), biotinylated anti-Tyr(P) (Upstate Biotechnology; 1:10000), anti-phospho-Ser^{473} Akt (anti-P Akt; 1:1000; New England Biolabs) or the indicated monoclonal or polyclonal specific antibodies, as described (39). Where indicated, the immunoblots were stripped by 30-min incubation at 50 °C in 67 mM Tris-HCl, pH 6.7, 2% SDS, 100 mM β mercaptoethanol and reprobed.

Analysis of ERK Activation

Analysis by Western Blotting—Western blots were generated as described above but developed with an affinity-purified rabbit polyclonal antibody that specifically recognizes the dually Thr^{202}/Tyr^{204}-phosphorylated, active form of ERK kinases (anti-phospho-ERK; 1:2000; New England Biolabs). Equal loadings of proteins were verified by reprobing the blot with Erk1 (Santa Cruz Biotechnology) antibody.

Analysis by Elk-dependent Gene Transcription—Once phosphorylated, ERKs translocate to the nucleus, where they phosphorylate and activate the transcription factor Elk-1 (reviewed in Ref. 40). Hence, to assess the activation of the ERK pathway, we also measured the Elk-1-driven transcription of luciferase reporter gene using the PathDetect Elk-1 trans-reporting system (Stratagene) following the manufacturer's instructions. Briefly, $SK\text{-}Ret^{WT}$ cells (0.8 10^6/six-well dish) were transiently transfected using the polyethyleneimine reagent with 1 μg of firefly luciferase reporter pFR-LUC plasmid and 0.1 μg of pFA2-ELK-1 trans-activator plasmid. The contribution of signaling molecules to the activation of the ERK pathway was then tested by cotransfection with 0.5 μg of vectors encoding either dominant negative or positive mutants. Transfection efficiencies were normalized by measuring the activity of a cotransfected pRL-TK plasmid (0.01 μg) that expresses *Renilla* luciferase (dual luciferase reporter assay system; Promega). Following transfection, cells were left for 24 h in serum-free medium, and GDNF (100 ng/ml) was added for 18 h. 48 h after transfection, dual luciferase activities (firefly and *Renilla*) were measured in the same sample with a Berthold luminometer. The firefly luciferase activity was adjusted by dividing with the corresponding *Renilla* luciferase activity.

Pull-down Experiment to Detect Activated Ras

Ras activity was measured by a nonradioactive method based on the capability of the active GTP-bound Ras to bind to the Ras binding domain of Raf-1 (35). Briefly, $SK\text{-}Ret^{WT}$ cells were stimulated with GDNF for 20 min and lysed in buffer containing 1% Triton X-100 and 1% *N*-octyl-D-glucopyranoside. 15 μg of recombinant GST-Raf-1 Ras binding domain previously coupled to glutathione-Sepharose (Amersham Pharmacia Biotech) were added to ~750 μg of protein extract. Protein complexes were allowed to form for 2 h at 4 °C. Precipitates were washed three times with lysis buffer with *N*-octyl-D-glucopyranoside and once with PBS and analyzed by Western blotting with an anti-pan-Ras antibody (Calbiochem), as described above.

Raf-1 Kinase Assay

Cells were stimulated with GDNF for 20 min, lysed (in Nonidet P-40 buffer), and immunoprecipitated with 2 μg of anti-Raf-1 (Upstate Biotechnology) antibodies for 4 h. The Raf-1 kinase activity in

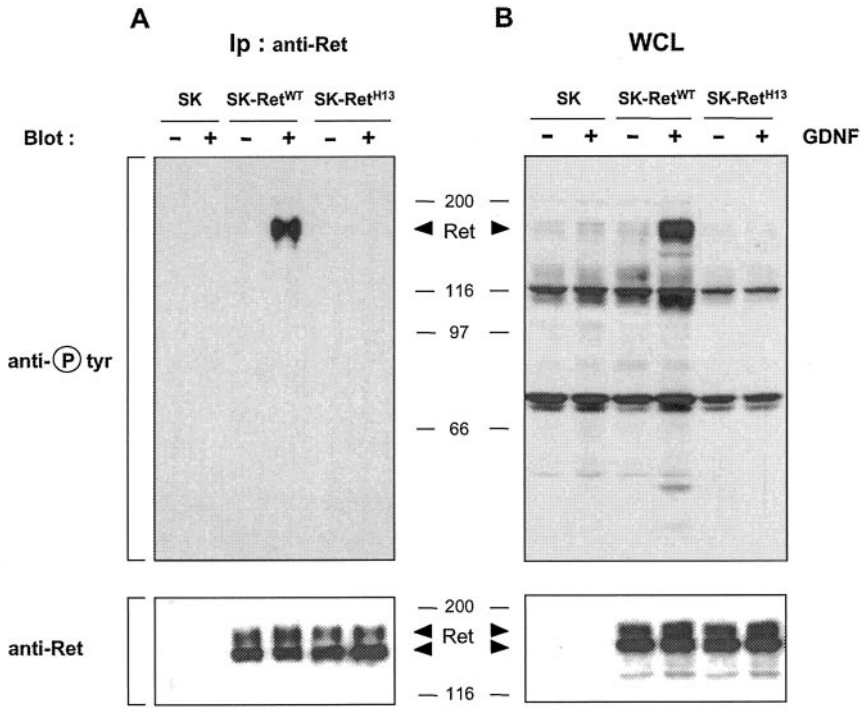


FIG. 1. **GDNF signaling in WT- and H13 Ret-transfected SK-N-MC cells.** The human neuroectodermic SK-N-MC cells were stably transfected with the WT (SK-Ret^{WT} cells) or the H13 mutated *ret* cDNA (SK-Ret^{H13} cells). The indicated cell lines were incubated in serum-free medium in the absence (−) or the presence of GDNF (+; 100 ng/ml) for 15 min prior to cells lysis. Anti-Ret immunocomplexes (*A*, *Ip*) or whole cell lysates (*B*, WCL) were separated on reducing 9% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Upon incubation with biotin anti-Tyr(P) antibodies, tyrosine-phosphorylated proteins were detected, as described under "Experimental Procedures." After stripping, Ret expression was analyzed by reprobing the same blots with anti-Ret (*lower panels*). The *black arrowheads* indicate the positions of Ret proteins.

immunoprecipitates was measured *in vitro* by a coupled kinase assay. In this assay, the ERK cascade was reconstituted *in vitro*; the activity of the immunoprecipitated Raf-1 was measured by its ability to stimulate a recombinant nonphosphorylated MEK1 (400 ng), the activation of which was then revealed by phosphorylation of recombinant p42^{ERK2} (Raf-1 kinase cascade assay; Upstate Biotechnology). After a 30-min incubation at 30 °C in the presence of Mg^{2+} and [γ-^{32}P]ATP (Amersham Pharmacia Biotech), kinase reactions were stopped by the addition of reduced Laemmli buffer, resolved onto 12.5% SDS-PAGE, and visualized by autoradiography. Immunoprecipitation of equal amounts of Raf-1 was verified by immunoblotting. Control reactions, in which either MEK1 or Raf-1 immunoprecipitates were omitted, showed no ERK phosphorylation.

PI Kinase Assay

Cells were treated with GDNF or left unstimulated for 15 min prior to Nonidet P-40 cell lysis. The PI kinase assay was then carried out on anti-Tyr(P) (Upstate Biotechnology) immunoprecipitates with 10 μCi of [γ-^{32}P]ATP and freshly prepared phosphatidylinositol (PI; Sigma) as substrate, as previously described (41). The radiolabeled phosphatidylinositol phosphate obtained was analyzed by thin layer chromatography using $CHCl_3/CH_3OH/NH_4OH/H_2O$ (8.6:7.6:1:1.4, v/v/v/v) as migration buffer. In the case of the anti-Tyr(P) immunoprecipitates from SK-Ret^{WT} cells, PI kinase analysis was also performed in the presence of 100 nM wortmannin.

Induction and Detection of Apoptosis

Cells were treated with GDNF for 1 h before being either incubated with anisomycin (10 μg/ml; Sigma) or camptothecin (1 μM; Sigma) or exposed to ultraviolet B irradiation (200 mJ/cm^2). 2–4 h after the induction of apoptosis, both adherent cells (recovered after trypsinization) and nonadherent cells (present in the culture medium) were combined and washed in PBS. Then three different parameters describing characteristic features of apoptotic cells were measured as discussed below.

Breakdown of Mitochondrial Transmembrane Potential (Ψ_M)—Early in apoptosis, the Ψ_M decreases because of the opening of mitochondrial pores through which cytochrome C and apoptosis-inducing factor are released into the cytoplasm. To discriminate between living cells (high Ψ_M) and apoptotic cells (low Ψ_M), the above cell preparations were stained with $DiOC_6$ (3,3′-dihexyloxadicarbocyanine; 40 ng/ml; Molecular Probes, Inc., Eugene, OR), a lipophilic cationic fluorochrome that accumulates in mitochondria with an uptake directly proportional to Ψ_M. Propidium iodide (5 μg/ml, Sigma) was then added to stain the cells with damaged plasma membrane (late apoptotic and necrotic cells) before the analysis by flow cytometry (FACScan Becton Dickinson apparatus). Hence, the cell population with low fluorescence intensity with both $DiOC_6$ (green fluorescence) and propidium iodide (red fluorescence) was the early apoptotic cells and scored among 10,000 gated events (using CellQuest software (Becton Dickinson)).

Procaspase-3 Cleavage—Caspase-3 (Cpp32) is a cysteine protease that plays a key role in the execution of the apoptotic program (42). It is synthesized as an inactive proenzyme of 32 kDa that is cleaved in cells undergoing apoptosis. Therefore, procaspase-3 cleavage was analyzed by Western blotting of WCL using an anti-procaspase-3 antibody (1:2000; Transduction Laboratories, Lexington, KY).

DNA Degradation—The degradation of genomic DNA into small oligonucleosomal fragments is a late hallmark of cells that succumb to apoptosis but not to necrosis. Since they possess a 3′-OH termini, these fragments can be labeled in cells by the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) method. Briefly, the above cell preparations were fixed with 4% paraformaldehyde for 30 min at 4 °C and permeabilized with 70% ethanol at −20 °C for at least 30 min. After washing, the presence in apoptotic cells of ladder fragments was detected by labeling their termini with bromo-conjugated dUTP and exogenous terminal deoxynucleotidyltransferase (APO-BrDu kit; Pharmingen) for 1 h at 37 °C. Subsequently, the reaction was blocked, and anti-bromo-dUTP fluorescein isothiocyanate was added for 30 min at room temperature before the analysis by flow cytometry.

RESULTS

Tyrosine Phosphorylation Pattern Activated by GDNF in Neuroectodermic Cells—Neuroectodermic SK-N-MC cells, which express both GFRα1 and GFRα2 (data not shown (43)), were stably transfected with either the WT or the H13 mutated *ret* cDNA (referred as SK-Ret^{WT} and SK-Ret^{H13} cells, respectively). This latter cDNA carries the Ser^{765} → Pro substitution in exon 13, found in a Hirschsprung's disease-affected patient, which abolishes the Ret TK activity (29). As shown in Fig. 1 (*lower panels*), both transfected SK-Ret^{WT} and SK-Ret^{H13} cells expressed correctly the mature p170^{ret} as well as the incompletely glycosylated p150^{ret} precursor. The different cell lines were first assessed for their ability to respond to GDNF in terms of tyrosine phosphorylation. Analysis of anti-Ret immunoprecipitates by anti-phosphotyrosine (anti-Tyr(P)) Western blotting (Fig. 1*A*) revealed that the addition of GDNF induced a marked increase in p170^{ret} autophosphorylation level in SK-Ret^{WT} but not in SK-Ret^{H13} cells. Consistently, GDNF stimu-

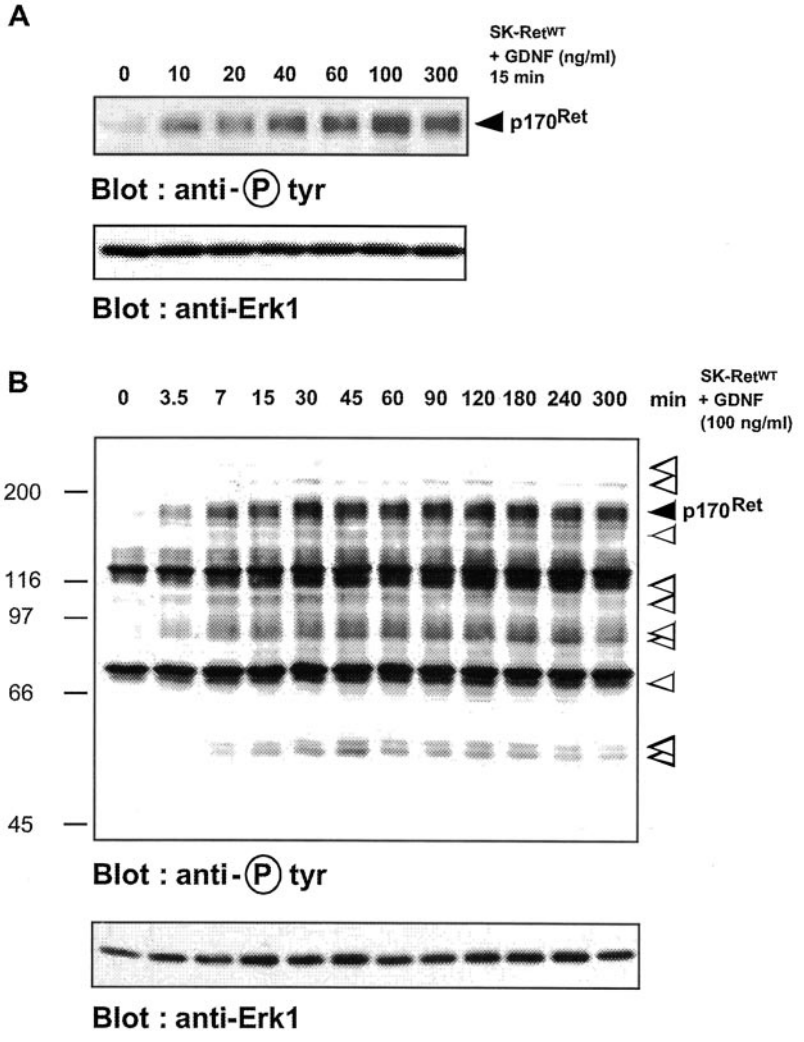


FIG. 2. **Dose response and time course of GDNF-induced tyrosine phosphorylation in SK-Ret^{WT} cells.** *A*, dose response of GDNF-induced Ret tyrosine phosphorylation. SK-Ret^{WT} cells were incubated in the presence of the indicated concentration of GDNF for 15 min prior to cells lysis. *B*, kinetic of GDNF-induced tyrosine phosphorylation. SK-Ret^{WT} cells were stimulated with 100 ng/ml of GDNF and lysed at the indicated time. Tyrosine-phosphorylated proteins were detected by Western blotting of WCL, as described in the legend to Fig. 1. After stripping, loading of equal amounts of proteins was verified by reprobing the same blots with anti-Erk1 (*lower panels*). The positions of the phosphorylated p170^{ret} and cellular substrates are indicated by *arrows*.

lation of SK-Ret^{WT} cells induced tyrosine phosphorylation of several cellular proteins of 120, 52, and 42 kDa, while the phosphorylation profile in the SK-Ret^{H13} cells was similar to that observed in the parental SK-N-MC cells (Fig. 1*B*). This demonstrates that the expression of a functional Ret TK was required to trigger GDNF-induced substrate tyrosine phosphorylations. We verified that tyrosine phosphorylation of p170^{ret} was dose-dependent with a maximal effect at 100 ng/ml (Fig. 2*A*). Moreover, the time course showed that phosphorylation of cellular substrates started at 3.5 min of GDNF treatment to reach a plateau value by 15 min and lasted 3 h before slowly declining by 5 h (Fig. 2*B*). This sustained profile distinguishes neuroectodermic cells from the other GDNF-sensitive cellular models (sympathetic, sensory, or motor neurons) used so far (44, 45).

Activation of PI3K → Akt Pathway in GDNF-stimulated SK-Ret^{WT} Cells—The PI3K/Akt pathway has been shown to play an essential role in the protection of neuronal cells from apoptosis (46). We therefore verified whether GDNF triggered the activation of PI3K activity in the neuroectodermic SK-N-MC cells. As shown in Fig. 3*A*, a strong PI kinase activity was detectable in anti-Tyr(P) immunoprecipitates from GDNF-stimulated but not from control SK-Ret^{WT} cells. The total inhibition of this activity with the PI3K inhibitor, wortmannin, supports the idea that it corresponded to PI3K. In contrast, no PI kinase activity was detected in precipitates from SK-Ret^{H13} or from untransfected cells, indicating the close relationship between the GDNF-induced Ret TK and PI3K activation.

Wortmannin-sensitive PI3Ks consist of a 110-kDa catalytic subunit (p110^{PI3K}) and an 85-kDa regulatory subunit (p85^{PI3K}) that binds to tyrosine-phosphorylated proteins via its SH_2 domains (47). To determine whether GDNF induced the recruitment of p85^{PI3K} to activated Ret, SK-Ret^{WT} cell lysates were subjected to immunoprecipitation with anti-p85^{PI3K} antibodies followed by anti-Tyr(P) immunoblotting (Fig. 3*B*). Exposure of SK-Ret^{WT} cells with GDNF resulted in the coimmunoprecipitation of p85^{PI3K} with a phosphoprotein of 170 kDa, which was further identified as p170^{ret} by anti-Ret Western blotting.

Once activated, PI3K catalyzes the production of PI 3,4-bisphosphate, which acts as membrane docking site for Akt (also known as protein kinase B), the activation of which has been shown to rescue cells from apoptosis (18, 48). To investigate the ability of GDNF to stimulate Akt, cells were incubated with GDNF and lysed, and Akt activation was studied by Western blotting using anti-P Akt. As shown in Fig. 3*C*, GDNF stimulation induced a rapid and sustained phosphorylation of Akt in SK-Ret^{WT} cells, which was detectable in neither SK-Ret^{H13} cells nor untransfected SK-N-MC cells (Fig. 3*D*). Maximal Akt phosphorylation lasted 15–45 min after GDNF treatment before slowly declining to the basal level by 5 h. Consistent with a control of Akt activation by PI3K, pretreatment of SK-Ret^{WT} cells with wortmannin abolished GDNF-induced Akt phosphorylation (Fig. 3*D*).

PI3K Activity Is Required but Not Sufficient for the Activation of ERK Pathway in SK-Ret^{WT} Cells—ERK-1 and ERK-2 activities are candidates that have been involved in cell sur-

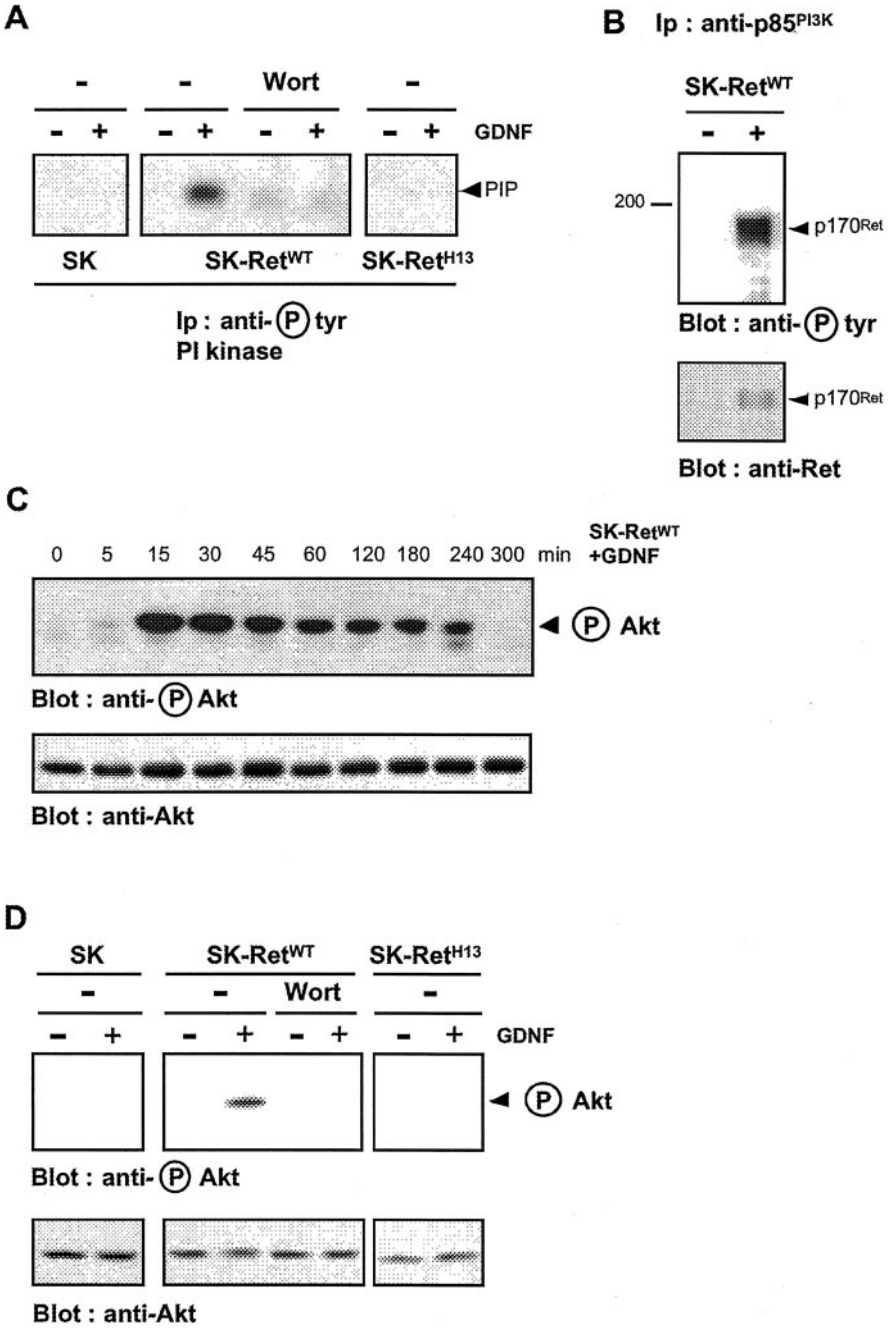


FIG. 3. **Activation of PI3K/Akt pathway in GDNF-stimulated SK-Ret^{WT} cells.** *A*, recruitment and activation of PI3K in GDNF-stimulated SK-Ret^{WT} cells. The indicated cells were treated with GDNF (+, 100 ng/ml) or left unstimulated (−) for 15 min prior to cell lysis (*A* and *B*). The PI kinase assay was carried out on anti-Tyr(P) immunoprecipitates, with or without the addition of wortmannin (100 nM; *Wort*), as described under "Experimental Procedures." *B*, coimmunoprecipitation of p85^{PI3K} with the autophosphorylated p170^{ret} in GDNF-stimulated SK-Ret^{WT} cells. Anti-p85^{PI3K} immunoprecipitates from unstimulated and GDNF-stimulated SK-Ret^{WT} cell lysates were analyzed by anti-Tyr(P) Western blotting. After stripping, the presence of p170^{ret} was detected by reprobing the same blot with anti-Ret (*lower panel*). *C*, activation of Akt in GDNF-stimulated SK-Ret^{WT} cells. SK-Ret^{WT} cells were stimulated with GDNF (100 ng/ml) and lysed at the indicated time. Akt activation was then analyzed by Western blotting of WCL with anti-P Akt antibodies. *D*, PI3K-dependent activation of Akt in GDNF-stimulated SK-Ret^{WT} cells. Cells were pretreated for 60 min with wortmannin before the addition of GDNF. After 15 min, the cells were lysed, and Akt activation was analyzed by Western blotting with anti-P Akt. Loading of equal amounts of Akt was verified by reprobing the same blots with anti-Akt (*lower panels*). The positions of the phosphorylated Akt and of p170^{ret} are shown.

vival. To gain insight into their possible involvement in the mediation of the GDNF antiapoptotic effect in the neuroectodermic cells, activation of ERKs was assessed by Western blotting with anti-phospho-ERK. GDNF induced the activation of both p42^{ERK2} and p44^{ERK1} in SK-Ret^{WT} (Fig. 4) but not in SK-Ret^{H13} or untransfected SK-N-MC cells (Fig. 5*A*, *lower panels*). ERK activation started at 15 min, peaked at 45 min, and lasted up to 5 h (Fig. 4), reminiscent of the kinetic profile observed for Akt. This prompted us to investigate a possible control by the PI3K/Akt pathway of the GDNF-induced ERK activation. Abrogation of PI3K by pretreatment of SK-Ret^{WT} cells with wortmannin significantly inhibited the GDNF-induced phosphorylation of ERKs (Fig. 5*A*). Similar results were obtained with Ly294002, a stable PI3K inhibitor, over the entire time span examined (Fig. 4, *lower panels*), underscoring the positive contribution of PI3K to both the early and sustained activation of ERKs.

On their phosphorylated activated state, ERKs translocate to the nucleus, where they phosphorylate and activate several transcription factors such as Elk-1 (reviewed in Ref. 40). We observed that GDNF stimulation of SK-Ret^{WT} cells resulted in a 100–145-fold increase of Elk-1-driven transcription of the Gal-4 luciferase reporter gene (Fig. 5*B*). Induction of the luciferase activity by GDNF was completely abrogated by the MEK-specific inhibitor PD98059, indicating that the GDNF-induced Elk-1 activity was totally dependent on the activation of ERK pathway. To further explore the relationship between PI3K and ERK, we then tested the effect of Δp85, a dominant negative mutant of PI3K. In agreement with the above data, expression of Δp85 reduced by 84% the GDNF-stimulated Elk-1 transactivation. However, we did not find a constitutive increase of Elk transactivation in SK-Ret^{WT} cells transfected with an activated form of p110^{PI3K} (p110^{PI3K+}), although these cells presented constitutive high levels of phosphorylated Akt (data not

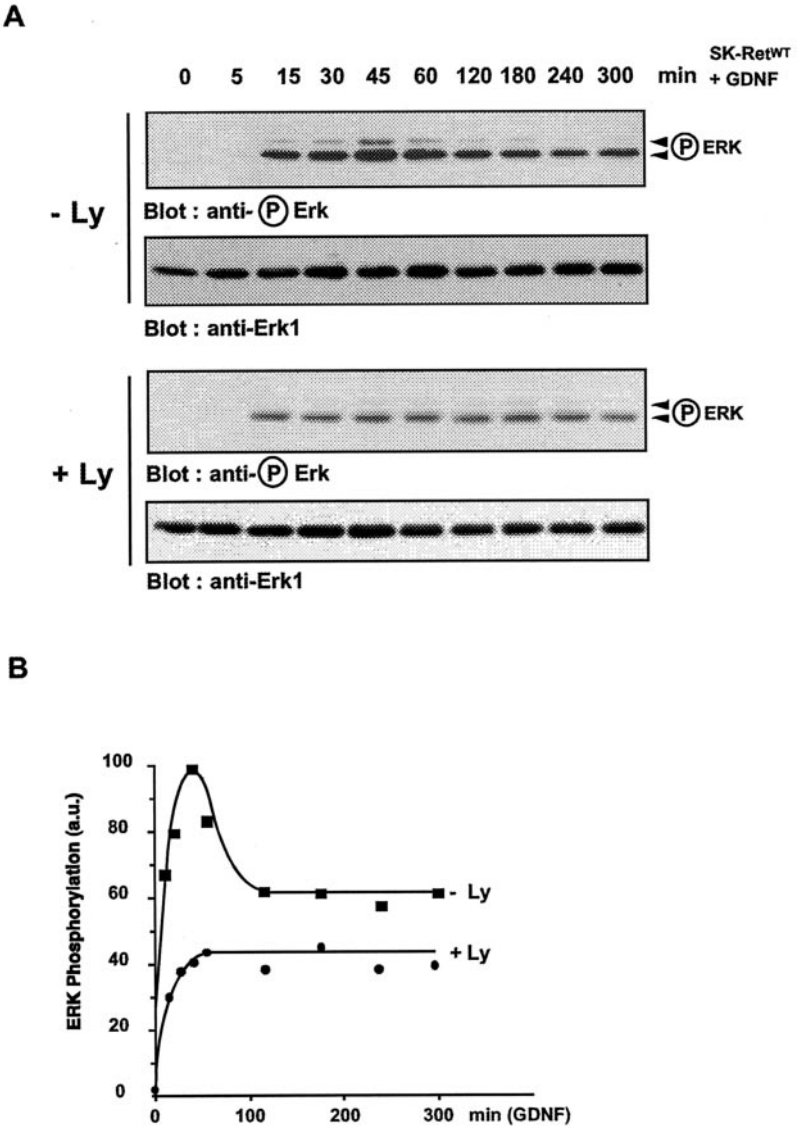


FIG. 4. **PI3K-dependent activation of ERK pathway in GDNF-stimulated SK-Ret^{WT} cells.** The SK-Ret^{WT} cells were pretreated with Ly (10 μM) or Me_2SO (−) for 60 min before the addition of GDNF (100 ng/ml). At the indicated time, cells were lysed, and ERK activation was assessed by Western blotting of WCL with anti-phospho-Thr^{202}/Tyr^{204} ERK (*anti-P ERK*) antibody. After stripping, loading of equal amounts of Erk1 was verified by reprobing the same blots with anti-Erk1 (*lower panels*). The positions of the phosphorylated p42^{ERK2} and p44^{ERK1} are shown. *B*, densitometric scannings (UltroScan laser densitometer) of p42^{ERK2} phosphorylation shown in *A*.

shown). Taken together, these pharmacological and genetic approaches support the idea that activation of PI3K was required but not sufficient for the activation of the GDNF-induced ERK activity in neuroectodermic cells.

PI3K Inhibition Does Not Prevent GDNF-induced Ret and Shc Tyrosine Phosphorylation or Ras Activation—We therefore sought to identify at which step PI3K controlled the GDNF-induced ERK pathway. Anti-Tyr(P) Western blotting of WCL and Shc immunoprecipitates (Fig. 6*A*, *right* and *left panels*, respectively) evidenced that the GDNF-induced tyrosine phosphorylation of p170^{ret} and of the three isoforms of Shc (p46, p52, and p66) was not significantly affected by wortmannin or Ly, indicating that the PI3K inhibitors did not act on the Ret TK activity itself but rather on a downstream signaling component that is required for ERK activation. Given the ability of PI3K to bind and activate Ras (49), we next investigated whether the GDNF-induced Ras activation was under the control of PI3K. In this purpose, the active Ras were specifically detected by a pull-down assay. As shown in Fig. 6*B*, GDNF markedly induced Ras activation that was not affected by a treatment with wortmannin or Ly, indicating that PI3K acted downstream of Ras.

The GDNF-induced PI3K Acts on Raf-1 Activation—To test whether the activation of the Ras target, Raf-1, was dependent on the GDNF-activated PI3K, Raf-1 activity was measured *in vitro* after GDNF stimulation by a coupled kinase assay. As shown in Fig. 6*C*, GDNF stimulation of SK-Ret^{WT} cells dramatically induced the Raf-1 activity, which was almost completely inhibited by wortmannin and Ly pretreatment, indicating that PI3K exerted its activation effect at the level of Raf-1.

GDNF-induced Akt Activity Down-regulates ERK Pathway—We next addressed whether Rac and Akt, two targets situated downstream of PI3K, were involved in the modulation of the ERK pathway. As shown in Fig. 7, expression of the dominant negative Rac (RAC^{N17}) inhibited by 70% the GDNF-stimulated Elk-1-driven transcription, indicating the positive contribution of Rac in the mediation of the GDNF-activated ERK pathway. Surprisingly, the expression of dominant positive Akt (GAG-AKT) did not by itself elicit Elk but instead significantly decreased the GDNF-induced Elk activity. These observations indicate that Akt failed to induce the ERK pathway and rather caused its inhibition. This was strengthened by the findings that expression of two dominant negative mutants of AKT (ST^{-} AKT or K → A AKT) led to an increased induction of Elk activation upon GDNF treatment. These results point to the new notion that the steady state level of ERK pathway is achieved by the opposing effects of both PI3K and Akt in the GDNF-stimulated cells.

Antiapoptotic Signaling by GDNF Depends on Ret TK Activity—After dissecting the specific signaling events induced by

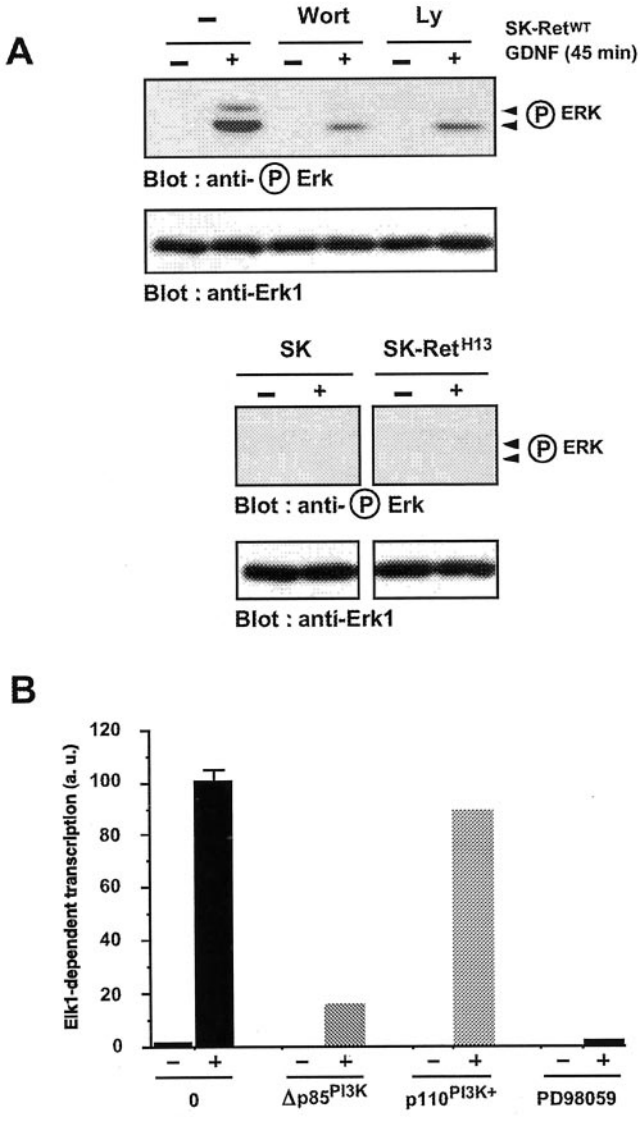


FIG. 5. **PI3K activity is required but not sufficient for the activation of ERK pathway in SK-Ret^{WT} cells.** *A*, the indicated cells were pretreated with either Ly (10 μM), wortmannin (*Wort*; 100 nM), or Me_2SO (−) for 60 min before the addition of GDNF (100 ng/ml). At the indicated time, cells were lysed, and ERK activation was assessed by anti-phospho-ERK Western blotting of WCL, as described in Fig. 4. After stripping, loading of equal amounts of Erk1 was verified by reprobing the same blots with anti-Erk1 (*lower panels*). The positions of the phosphorylated p42^{ERK2} and p44^{ERK1} are shown. *B*, inhibition of the Elk-dependent gene transcription by Δp85^{PI3K} in GDNF-stimulated SK-Ret^{WT} cells. SK-Ret^{WT} cells were transiently cotransfected with either the dominant negative (Δp85^{PI3K}) or positive (p110^{PI3K+}) mutants of PI3K, and their effects on the activation of the ERK pathway were measured by the Elk-1-dependent transcription of luciferase reporter plasmids. The following day, GDNF was added (100 ng/ml). Where indicated, cells were treated with the MEK-specific inhibitor PD98059 (50 μM) for 90 min before the addition of GDNF. Luciferase activities were measured 48 h after transfection. The results are expressed relative to the level of luciferase activity in GDNF-stimulated cells, which was given an arbitrary value of 100. Samples represent an $n = 3$, and error bars represent the S.D.

GDNF, we aimed to delineate the pathways required for GDNF-induced neuroectodermic cell survival. We first tested the ability of GDNF to protect the SK-Ret^{WT} cells from apoptosis. As shown in Fig. 8, within 3 h after the addition of anisomycin, the SK-Ret^{WT} cells underwent massive programmed cell death as measured by the proportion of cells exhibiting low mitochondrial transmembrane potential (Ψ_M; Fig. 8*A*, 64.5 *versus* 7.2%), full proteolytic activation of procaspase-3 (Fig. 8*B*), and DNA fragmentation into oligonucleosomal fragments (Fig. 8*C*, 28 *versus* 4.4%). Preincubation with GDNF prevented anisomycin-induced Ψ_M decrease (14.5%), procaspase-3 cleavage, and DNA fragmentation (6.1%). In contrast, untransfected SK-N-MC and SK-Ret^{H13} cells underwent apoptosis upon anisomycin treatment regardless of the presence of GDNF. Taken together, these findings clearly indicate that the rescuing effect of GDNF was correlated with its ability to trigger Ret TK activity.

Activation of PI3K/Akt Pathway Is Essential for All GDNF-rescuing Effects, while the ERK Pathway Partially Inhibits Procaspase-3 Cleavage—Given the well recognized involvement of PI3K in the antiapoptotic signaling of several cytokines (46), we next investigated the role of this kinase in GDNF-induced SK-Ret^{WT} cell survival. Inhibition of PI3K activity by wortmannin abrogated all protective effects of GDNF on anisomycin-induced decrease of Ψ_M (Fig. 9*A*, 64 *versus* 13%), cleavage of procaspase-3 (Fig. 9*B*), and DNA fragmentation (Fig. 9*C*, 87 *versus* 15.1%), indicating that the antiapoptotic activity of GDNF was mediated via PI3K. To evaluate the respective contribution of ERK and Akt, to the GDNF protective effects, SK-Ret^{WT} cells were pretreated with specific inhibitors. As shown in Fig. 9, pretreatment with PD98059 significantly reduced the ability of GDNF to prevent anisomycin-induced procaspase cleavage (*B*), strongly suggesting that the ERK pathway did participate in this effect. However, most of the protective activity of GDNF did not depend on ERK, since PD98059 did not override the preventive effects of GDNF on either anisomycin-induced Ψ_M collapse (*A*) or DNA fragmentation (*C*). By contrast, we found that the PI3K-dependent activation of Akt was essential for these GDNF antiapoptotic effects, since expression of the dominant negative mutant of Akt (ST^{-} AKT) in Ret-expressing SK-N-MC cells barely induced apoptosis by itself (13.7%) but abrogated the protective effects of GDNF on anisomycin-induced Ψ_M collapse (Fig. 10, 93.1 *versus* 23%). Furthermore, expression of the activated Gag-Akt construct was sufficient to protect SK-Ret^{WT} cells from apoptosis without GDNF stimulation (15 *versus* 92%). Similar results were obtained when apoptosis was monitored by TUNEL labeling of genomic DNA fragments (data not shown). Taken together, these data underscore that activation of Ret, PI3K, Akt, and, at a lesser level, ERK is necessary for mediating the GDNF protective effect.

DISCUSSION

It has been recently established that GDNF plays a crucial role in promoting the survival of neural crest-derived precursors during the ENS development (1–3). This antiapoptotic effect requires the activation of a receptor complex composed of the Ret TK and the GPI-linked GFRα1 protein (4, 5). The importance of a functional Ret TK in the mediation of GDNF-induced neural crest cell survival has recently been evidenced by the apoptosis of enteric crest-derived precursors in *ret*-deficient mice (10). Nevertheless, several puzzling observations have suggested that the protective effects of GDNF might not require Ret. Indeed, outside the ENS, GFRα1 is often expressed in the absence of Ret (50–52), and GDNF has been recently shown to evoke survival and signaling *independently of Ret* (14). The aims of this study were thus to ascertain the requirement of Ret for the mediation of GDNF-induced survival *in neuroectodermic cells* and to dissect the signaling pathways involved in this process. To address this issue, we used the human neuroectodermic SK-N-MC cell line as a cellular model, since it presents the advantage of expressing constitutively GFRα1 but not Ret (13). In these conditions, SK-N-MC cells were transfected with either the WT or the kinase-dead H13 *ret* cDNA.

We provide here several lines of evidence that the activity of the Ret TK is essential for the GDNF-antiapoptotic signaling.

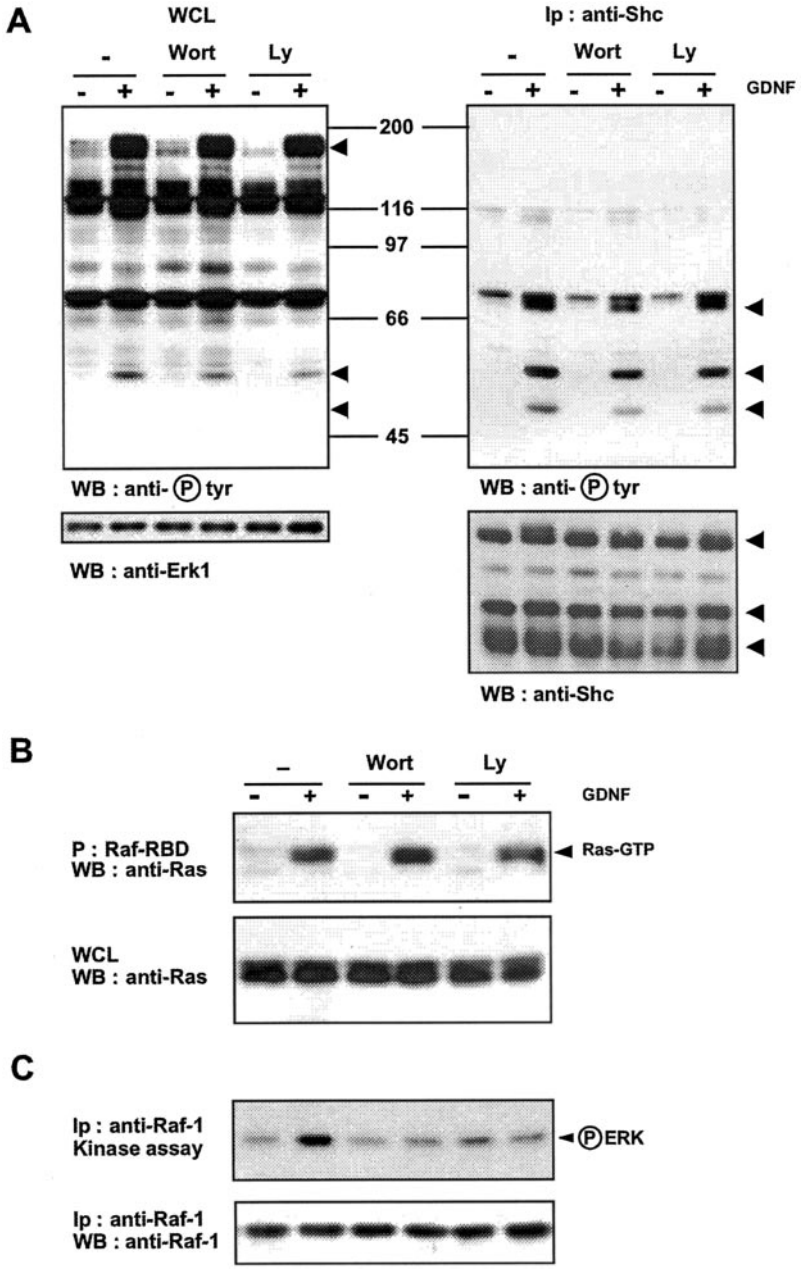


FIG. 6. **The step in the Ret → Shc → Ras → ERK pathway, regulated by PI3K, lies between Ras and Raf-1.** Serum-starved SK-Ret^{WT} cells, pretreated with either wortmannin (*Wort*), Ly, or vehicle, were stimulated with GDNF (100 ng/ml) for 20 min and lysed. *A*, PI3K inhibition does not prevent GDNF-induced Ret and Shc tyrosine phosphorylations. WCL (*right panel*) and Shc immunoprecipitates (*left panel*) were analyzed by anti-Tyr(P) Western blotting. After stripping, Erk1 and Shc expressions were verified by reprobing the same blots with Erk1 and Shc antibodies (*lower panels*). The positions of Ret and of the three Shc isoforms are shown. *B*, GDNF-induced Ras activation is independent of PI3K. Active GTP-bound Ras was precipitated (*P*) by binding to GST-Raf-Ras binding domain (*GST-Raf RBD*) precoupled to glutathione-Sepharose beads followed by anti-Ras Western blotting. *Upper panel*, Ras-GTP; *lower panel*, total amount of Ras in 10% of the extract. *C*, GDNF-induced Raf-1 activity is dramatically reduced by the PI3K inhibitors. The Raf-1 kinase activity in immunoprecipitates was measured *in vitro* by its ability to stimulate the activity of a recombinant MEK-1; the activation of MEK-1 is then detected by the phosphorylation of recombinant p42^{ERK2} (coupled MEK-ERK protein kinase assay). After a 30-min incubation at 30 °C in the presence of [γ-^{32}P]ATP, kinase reactions were analyzed by SDS-PAGE and autoradiography. Immunoprecipitation of equal amounts of Raf-1 was verified by anti-Raf-1 Western blotting (*lower panels*).

Indeed, we show that GDNF did rescue the neuroectodermic SK-N-MC cells that coexpressed Ret and GFRα1 but not the cells that harbored GFRα1 alone or together with the Ret^{H13} mutant. Instead, expression of this mutant was associated with an increased sensitivity to anisomycin-induced apoptosis, as was previously reported for a kinase-dead mutant of the insulin-like growth factor I receptor (19). Consistently, we found that GDNF stimulation led to Ret autophosphorylation, assembly of phosphotyrosine-dependent signaling complexes, and the subsequent activation of ERK and PI3K pathways over several hours in SK-Ret^{WT} cells. By contrast, binding of GDNF to GFRα1 or to Ret^{H13}-GFRα1 complex was unable to induce signal transduction. Therefore, we conclude that the absence of a functional Ret TK abrogates the ability of GDNF to trigger both signaling and survival of neuroectodermic cells. Conversely, recent studies have evidenced the ability of GDNF to promote cell survival (14) and Src and ERK activations independently of Ret (14, 53). These differences might explain why germ line loss-of-function Ret mutations specifically affect the enteric ganglia and highlight the importance of studying the

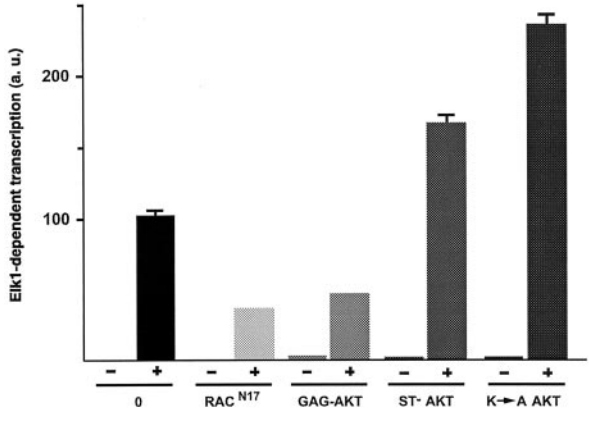
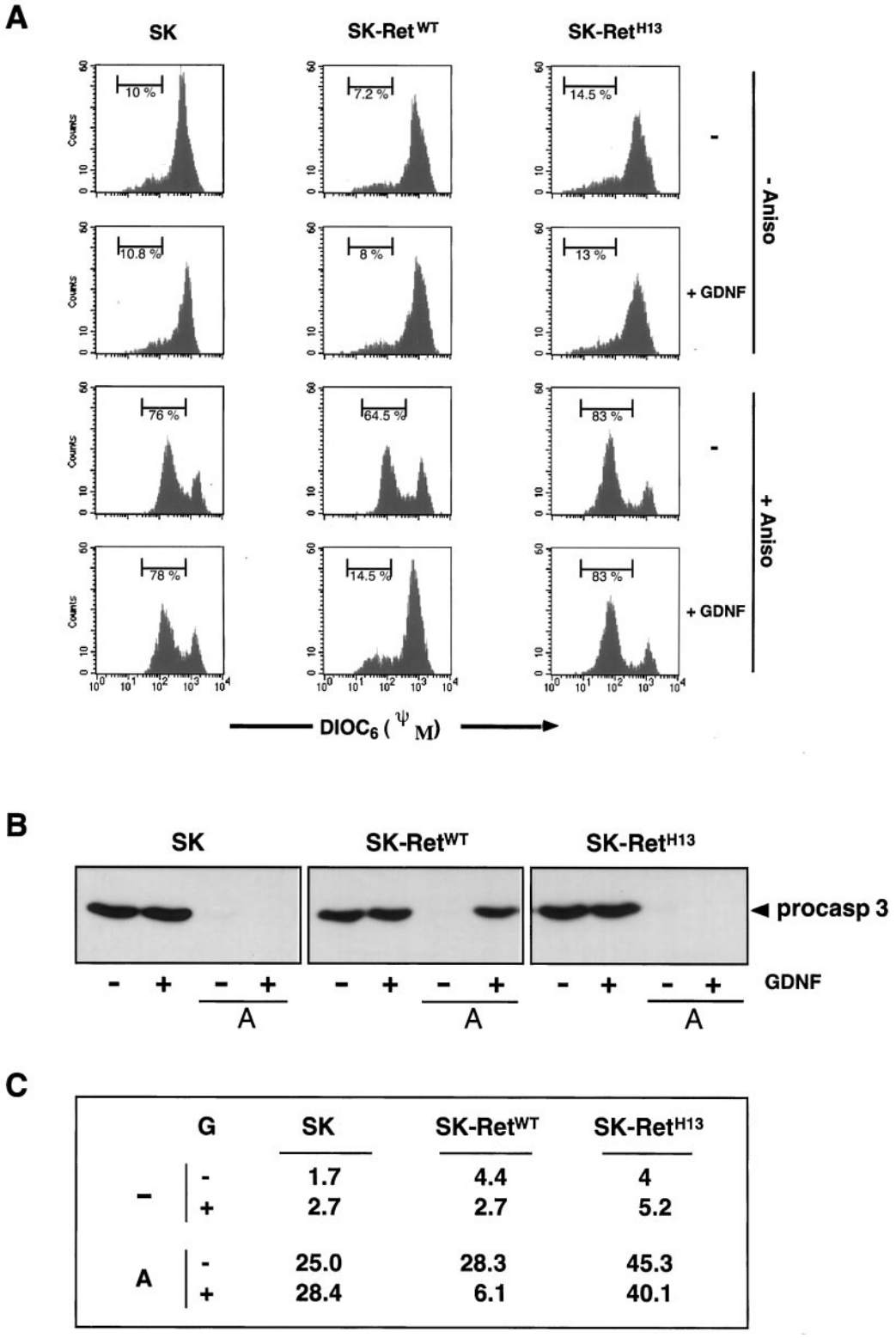


FIG. 7. **Involvement of Akt in the down-regulation of the GDNF-induced ERK pathway.** The effects on the activation of ERK pathway of either the dominant negative (RAC^{N17}) mutant of RAC or the dominant positive (GAG-AKT) or negative (ST^{-} AKT or K → A AKT) mutants of AKT were investigated as described in the legend to Fig. 5.

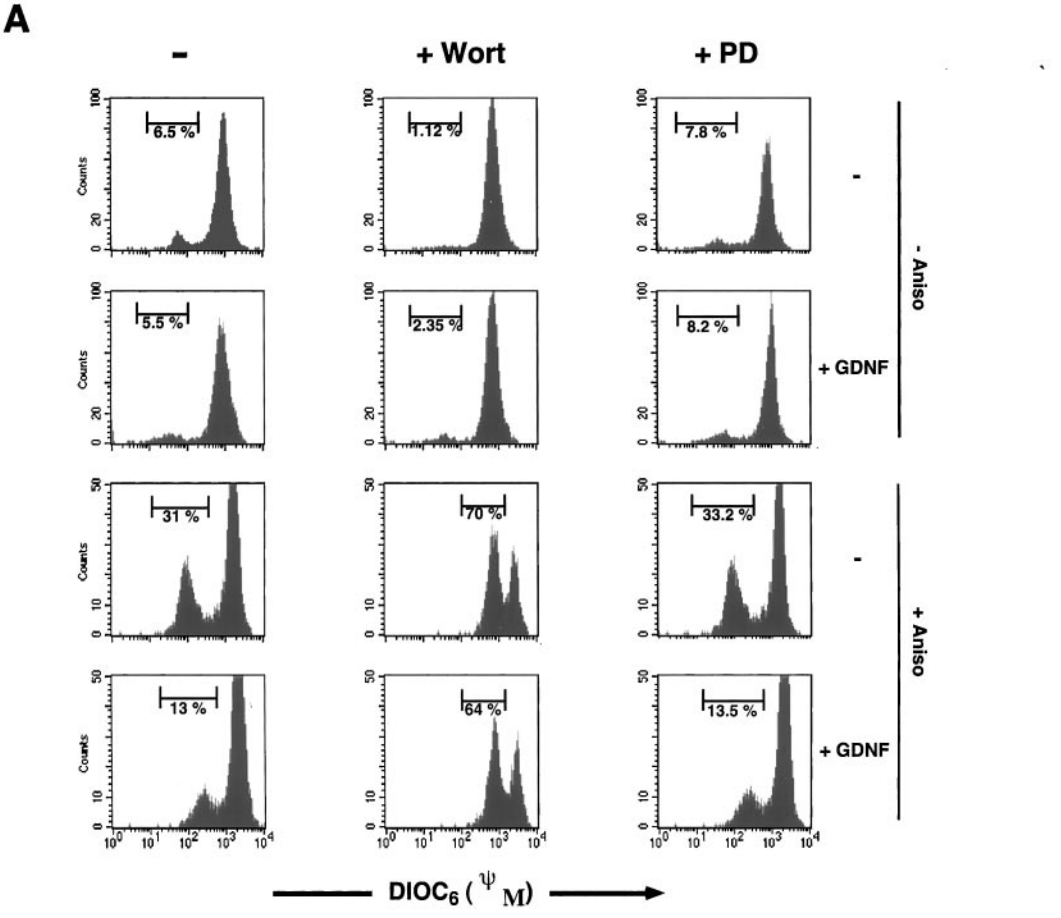


	G	SK	SK-Ret^{WT}	SK-Ret^{H13}
−	-	1.7	4.4	4
	+	2.7	2.7	5.2
A	-	25.0	28.3	45.3
	+	28.4	6.1	40.1

FIG. 8. **GDNF-induced antiapoptotic signaling depends on Ret TK activity.** SK-Ret^{WT}, SK-Ret^{H13}, and untransfected SK-N-MC cells were treated with GDNF (+, 100 ng/ml) or left unstimulated (−) for 1 h before being incubated with anisomycin (10 μg/ml). 3 h later, both adherent (recovered after trypsinization) and nonadherent cells (present in the culture medium) were pooled. Apoptosis was then measured as described under "Experimental Procedures" by analyzing the collapse of transmembrane mitochondrial potential Ψ_M (by flow cytometry) (*A*), the cleavage of procaspase-3 (anti-caspase-3 Western blotting of WCL) (*B*), and fragmentation of genomic DNA into oligonucleosomal fragments (by flow cytometry) (*C*). The percentage of apoptotic cells with a lower fluorescence with the mitochondrial $DiOC_6$ probe (*A*) or positive after TUNEL labeling (*C*) is indicated in each *panel*.

signaling pathways critical for GDNF-induced neural crest cell survival in a relevant neuroectodermic model.

We found that GDNF stimulated the activity of PI3K and association of autophosphorylated p170^{ret} with p85^{PI3K} in SK-Ret^{WT} cells. The cytoplasmic domain of Ret contains one tyrosine (Tyr^{981}) that matches the consensus Y*XX*M (Y^{981}RLM) motif that can accommodate the SH_2 domains of p85^{PI3K}. However, several recent reports have identified Tyr^{1062} on Ret as



C

	G	-	+Wort	+PD
-	-	5.3	6.2	6.7
	+	6.3	4.5	5.8
A	-	81.8	82	58
	+	15.1	87	17

FIG. 9. **PI3K is essential for all GDNF protective effects, whereas ERK is involved in preventing procaspase-3 cleavage.** SK-Ret^{WT} cells were treated with either wortmannin (*Wort*; 100 nM) or PD98059 (50 μM) for 1 h, before being incubated with GDNF (100 ng/ml). 1 h later, anisomycin (10 μg/ml) was added. Apoptosis was measured, as described in the legend to Fig. 8, by analyzing the decrease of Ψ_M (*A*), the cleavage of procaspase-3 (*B*), and the DNA fragmentation (*C*). Pretreatment with wortmannin or PD98059 had no significant effect on basal SK-Ret^{WT} cell survival.

the tyrosine critical for PI3K activation and recruitment. Interestingly, Tyr^{1062} is the Ret binding site for Shc that nucleates the multiprotein complex (Ret-Shc-Gab-PI3K) (27, 43, 54). In agreement with previous reports (14, 26–28), inhibition of PI3K activity by wortmannin pretreatment of SK-N-MC cells abolished both the ability of GDNF to trigger Akt and to prevent apoptosis, underscoring the essential role played by PI3K in the mediation of the GDNF-induced cell survival. We provide

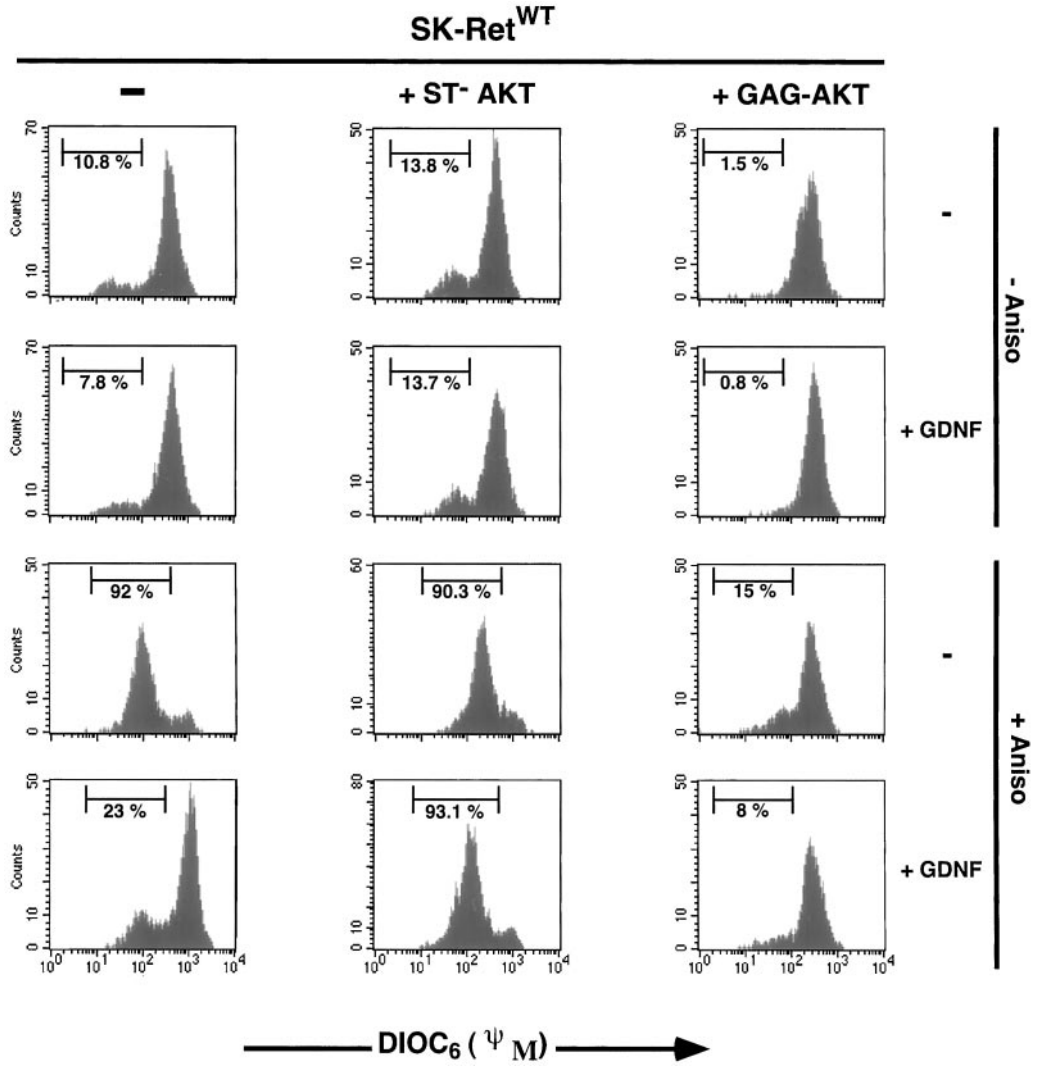


FIG. 10. **Activation of Akt is essential for the GDNF antiapoptotic function.** SK-N-MC cells were cotransfected with Ret^{WT} and either the constitutive activated (GAG-AKT) or the negative (ST^{-} AKT) mutant of AKT. Cells were then pretreated with GDNF (100 ng/ml) for 1 h, and anisomycin was added. After an additional 3 h, apoptosis was measured, as described in Fig. 8, by analyzing the decrease of Ψ_M.

here the first direct demonstration that Akt activation is mandatory for GDNF-induced cell survival as expression of a dominant negative form of Akt blocked GDNF-induced survival. This notion was strengthened by the fact that expression of the activated Gag-Akt construct was sufficient by itself to protect cells from anisomycin-induced apoptosis. Growing evidence is accumulating showing that Akt promotes cell survival by phosphorylating and inactivating components of the apoptotic machinery such as BAD (55) and caspase-9 (56) or by inducing the expression of Bcl-2 (57) resulting in the prevention of the cytochrome *c* release from mitochondria (58). Akt also prevents the expression of genes that are critical for cell death by phosphorylating the transcription factor FKHRL1 (59). Of these, the possible antiapoptotic mechanisms of downstream Akt in GDNF-stimulated cells are the protection of mitochondrial integrity, the ensuing inhibition of procaspase cleavage (as we have shown here), the up-regulation of Bcl-2 (28), and the inactivation of the transcription factor FKHRL1 (60).

Other kinases that can be considered in the propagation of survival signal are the mitogen-activated protein kinase ERKs (24, 61). Indeed, it has been reported that inhibition of their activities by growth factor deprivation or PD98059 causes apoptosis (61, 62). Furthermore, ERKs are required for cell survival in response to fibroblast growth factor (21), epidermal growth factor (63, 64), BDNF (24), and insulin-like growth factor I (65). It has been shown that Ret is linked to the ERK pathway via the adapter Shc, and point mutation of the tyrosine that binds Shc is associated with the development of Hirschsprung's disease (66). These data suggest that the ERK pathway may contribute, like the PI3K/Akt pathway, to the GDNF-induced neuroectodermic cell survival. However, it has been demonstrated that inhibition of PI3K but not of ERK abrogates GDNF-induced survival of dopaminergic, sensory, and spinal cord motor neurons (26–28). These findings clearly indicate that PI3K is necessary and sufficient to mediate the GDNF-antiapoptotic effect. However, they do not rule out the possibility that ERK may cooperate with PI3K/Akt pathway to fully protect cells against apoptosis. Indeed, it has been recently demonstrated that activation of the ERK pathway by an oncogenic form of Ret is responsible for the rescue of PC12, a cell line of neural crest origin (54). Thus, the possibility that ERK could participate to the GDNF-induced neuroectodermic cell survival still remains open.

We found that GDNF stimulation of $SK\text{-}Ret^{WT}$ cells resulted in a sustained ERK activation with a kinetic paralleling that of Akt. Moreover, GDNF-induced survival and Akt and ERK activations were wortmannin-sensitive. We therefore sought to determine whether ERK could behave as the downstream target that propagates the PI3K/Akt-dependent survival signals. Inhibition of GDNF-induced ERK activation by PI3K inhibitors (Δp85, wortmannin, and Ly294002) supported this possibility.

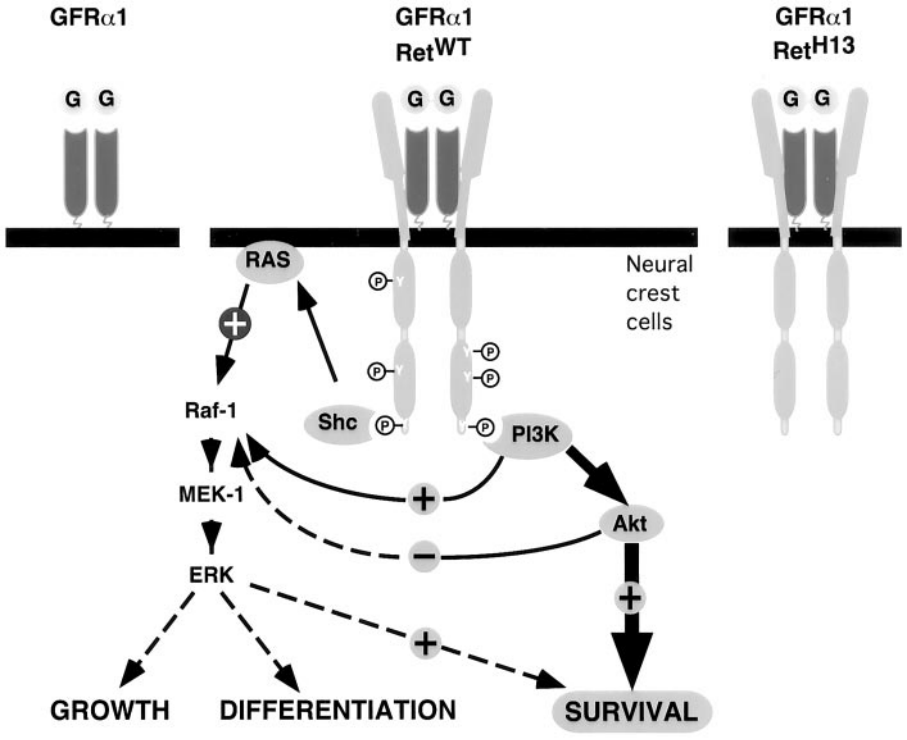


FIG. 11. **Roles of PI3K/Akt in GDNF-induced crest cell responses.** Results from this work uncover the fact that GDNF promotes neuroectodermic cell survival by activating the Ret → PI3K → Akt pathway and, at a lesser level, ERK. Akt and ERK interfere with the apoptotic signal at different stages; Akt targets the mitochondria, where it prevents Ψ_M collapse, ensuing caspase activation, and DNA fragmentation, whereas ERK may safely keep the activation of caspase-3 in check, ensuring via the PI3K/Akt pathway cell survival. Concomitantly, PI3K further amplifies the GDNF-induced ERK pathway by increasing the activation of Raf-1. Interestingly, activation of Akt in turn down-regulates the ERK pathway. Given the demonstration that Akt inhibits Raf-1 by phosphorylation of Ser^{259} (75, 76), it is reasonable to assume that Raf-1 is the point of convergence for multiple Ret-triggered positive (Ras and PI3K) and negative (Akt) signals. Hence, both PI3K and Akt are required to reach a threshold of ERK activity that may be critical in the final cell decision between growth or differentiation. *Arrows* or interactions do not imply a direct but rather a deduced or presumed (*dashed lines*) order of different signaling components.

Along this line, PI3K inhibitors did not modify the level of tyrosine phosphorylation of Ret and Shc, nor did they impair the GDNF-induced Ras activation, indicating that GDNF did activate the classical Shc/Grb2/SOS → Ras → ERK pathway in a PI3K-independent manner. However, we observed that, in SK-Ret^{WT} cells, GDNF exerted a potent PI3K-dependent activating effect on Raf-1 activity, in accord with previous data obtained with insulin-like growth factor I, interleukin-8, or integrin in myoblasts, neutrophils, or COS-7 cells (67–69). Our findings demonstrate that the Shc/Grb2/SOS → Ras and the PI3K pathways are both involved in the activation of Raf-1 and thus of ERK in GDNF-stimulated neuroectodermic cells.

The molecular mechanism responsible for the PI3K activation of Raf-1 remains unknown. One possibility would be that PI3K promotes the endocytosis of activated Ret-Ras complex, to bring it in contact with Raf, as suggested by York *et al.* (70). PI3K may also produce a phosphoinositide mediator that could directly act on Raf-1 activity, as suggested by its ability to bind lipids (71, 72). Alternatively, a downstream PI3K target might relay an activating signal to Raf-1. In this regard, Akt and Rac appear as possible candidates. Rac is a downstream target of PI3K, known to modulate Raf activity via the serine/threonine kinase PAK (73). By the use of a dominant negative Rac mutant, we could demonstrate that Rac contributes to the activating PI3K effect on ERK. Due to the crucial effect played by Akt in the mediation of the GDNF rescuing effect, one might expect that Akt could also intervene in the mediation of the PI3K up-regulation of the ERK pathway. Surprisingly, expression of activated Gag-Akt construct failed to induce Elk, as reported in fibroblasts (32, 74), and instead significantly decreased the GDNF-induced Elk activity. This raised the possibility that, although GDNF activated via PI3K both Akt and ERK activities, Akt attenuated the ERK pathway. This was supported by the fact that inhibition of the endogenous Akt, by the expression of two dominant negative Akt constructs (ST^- Akt or K → A Akt), resulted in a significant increase of the GDNF-induced Elk activity. While this work was in progress, it was shown that Akt inhibits the insulin-like growth factor I-induced ERK activation in differentiated myotubes by the phosphorylation of Raf-1 on Ser^{259}, which is in agreement with our results (75, 76). However, an important distinction is that in this latter model PI3K also inhibits the ERK pathway. Intriguingly, in their myoblast precursors, PI3K is (as described herein) required for the efficient ERK activation (67), while Akt has no effect (75). These puzzling discrepancies may be a reflection of the differentiation states of the cell system examined, as suggested by Rommel *et al.* (75). Recently, Akt has also been reported to inhibit B-Raf (77). To our knowledge, our study provides the first evidence that Akt and PI3K could play opposite roles in the control of the ERK pathway in the same cellular context. Such a tight control of ERK led us to believe that the steady state level of ERK is critical for GDNF-induced neuroectodermic cell survival. Consistently, a significant reduction of the GDNF protective effect against anisomycin-induced procaspase-3 cleavage was observed upon PD98059 pretreatment. But at odds with these data, PD98059 did not prevent GDNF from rescuing SK-Ret^{WT} cells from apoptosis induced by different types of cell death inducers (anisomycin, camptothecin, or ultraviolet B (data not shown)).

Altogether, these findings demonstrate that the activation of the Ret → PI3K → Akt pathway was mandatory for GDNF-induced survival of neuroectodermic cells (Fig. 11). In addition, activation of the ERK pathway participated in the GDNF-protective effect by blocking procaspase-3. Interestingly, while PI3K amplified the ERK pathway by increasing the activation of Raf-1, activation of Akt in turn attenuated ERK. In this regard, it seems that both PI3K and Akt function in concert to finely regulate the level of the ERK activity. Considering the multiple steps intervening during the colonization of the foregut by crest-derived precursors, this sophisticated ERK homeostasis might have important consequences on the GDNF-induced cell fate, inasmuch as the duration and the extent of ERK activation have been shown to be crucial in the final cell decision between growth and differentiation (reviewed in Refs. 78 and 79). In support of this hypothesis, it has been recently reported that inhibition of PI3K and ERK activities blocks GDNF-induced neuronal differentiation (28, 80). This novel antagonistic effect of Akt and PI3K on the ERK pathway should have widespread implications, since these three signal-

ing molecules are simultaneously activated in response to all growth and differentiation factors studied so far. Further studies of the GDNF signaling in neuroectodermic cells should provide important insights into the understanding of how subtle quantitative differences between PI3K, Akt, and ERK activities could lead to distinct cell responses.

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