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Activation of Phosphatidylinositol 3-Kinase and Extracellular Signal-Regulated Kinase Is Required for Glial Cell Line-Derived Neurotrophic Factor-Induced Migration and Invasion of Pancreatic Carcinoma Cells

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ABSTRACT

Pancreatic carcinoma cells exhibit a pronounced tendency to invade along and into intra- and extrapancreatic nerves, even at early stages of the disease. The neurotrophic factor glial cell line-derived neurotrophic factor (GDNF) has been shown to promote pancreatic cancer cell invasion. Here, we demonstrate that pancreatic carcinoma cell lines, such as PANC-1, expressed the RET and GDNF family receptor α receptor components for GDNF and that primary pancreatic tumor samples, derived from carcinomas with regional lymph node metastasis, exhibited marked expression of the mRNA encoding the RET51 isoform. Moreover, GDNF was an efficacious and potent chemoattractant for pancreatic carcinoma cells as examined in *in vitro* and *in vivo* model systems. Treatment of PANC-1 cells with GDNF resulted in activation of the monomeric GTPases N-Ras, Rac1, and RhoA, in activation of the mitogen-activated protein kinases extracellular signal-regulated kinase (ERK) and c-Jun NH_2-terminal kinase (JNK) and in activation of the phosphatidylinositol 3-kinase/Akt pathway. Both inhibition of the Ras-Raf-MEK (mitogen-activated protein/ERK kinase)-ERK cascade by either stable expression of dominant-negative H-Ras(N17) or addition of the MEK1 inhibitor PD98059 as well as inhibition of the phosphatidylinositol 3-kinase pathway by LY294002 prevented GDNF-induced migration and invasion of PANC-1 cells. These results demonstrate that pancreatic tumor cell migration and possibly perineural invasion in response to GDNF is critically controlled by activation of the Ras-Raf-MEK-ERK and the phosphatidylinositol 3-kinase pathway.

INTRODUCTION

Pancreatic adenocarcinoma is a very aggressive and destructive type of cancer, which is characterized by an extremely poor prognosis, pronounced invasiveness, and rapid progression (1). A common finding in pancreatic adenocarcinoma is invasion of the intrapancreatic perineural space and the extrapancreatic retroperitoneal nerve plexus (2, 3). Recent investigations led to the hypothesis that the specific perineural microenvironment supports tumor cell proliferation and dissemination. In this context, humoral factors or adhesion molecules, mainly expressed by intra- and/or extrapancreatic nerves, might be involved in tumor cell dissemination (2, 4). Expression of neurotrophins, such as nerve growth factor (NGF) or brain-derived nerve growth factor (BDNF), and their cognate Trk receptors has also been implicated in perineural invasion (5–8). Moreover, glial cell line-derived neurotrophic factor (GDNF) has been implicated in perineural invasion of pancreatic cancer cells (9).

The neurotrophic factor GDNF was identified in 1993 as a protein secreted by rat glial B49 cells that promotes survival and differentiation of dopaminergic neurons (10). Furthermore, GDNF is involved in the development of the enteric nervous system, in the morphogenesis of the kidney, and in the regulation of spermatogenesis (11). The GDNF family consists of four closely related members, GDNF, neurturin, persephin, and artemin (11), and all of the GDNF family members signal through an unique multicomponent receptor complex. The ligand-binding component is a glycosyl-phosphatidylinositol-anchored GDNF family receptor α (GFRα), which associates with the receptor tyrosine kinase RET after ligand binding (12, 13). Ligand specificity is determined by four different GFRα coreceptors. GDNF itself binds with high affinity to GFRα1 and with low affinity to GFRα2 (14).

The receptor tyrosine kinase RET was first described as an oncogene activated by DNA rearrangement during transfection (15). The human *ret* gene contains 21 exons, and alternative splicing at the 5′ and 3′ ends of the RET heterogenous nuclear RNA has been reported (16–18). The three RET isoforms, RET9, RET43, and RET51, which differ in 9, 43, and 51 amino acids at the COOH terminus, respectively, are produced by alternative splicing in the 3′ region of exon 19 (17, 18). All three of the RET transcripts are coexpressed in human adult tissues and most fetal tissues (16–18), and the isoforms seem to have different biological functions (19). Mutations in *ret* are detected in human neuroblastoma and medullary thyroid carcinoma and are the molecular basis of inherited cancer syndromes, such as human papillary thyroid carcinoma, multiple endocrine neoplasia type 2A and 2B, as well as Hirschsprung's disease, which is characterized by the congenital absence of enteric innervation (20).

The recent discovery of RET mRNA and protein expression in pancreatic carcinoma cell lines (9) in connection with GDNF-induced carcinoma cell migration reported here point toward an involvement of the GDNF/RET system in invasion of pancreatic carcinoma. It is currently unclear which molecular mechanisms are involved in GDNF-mediated pancreatic tumor cell migration and invasion. In this study, we have examined RET and GFRα expression in pancreatic carcinoma cells and have characterized the effect of GNDF on cell migration in *in vitro* and *in vivo* model systems. Furthermore, we have identified the signal transduction mechanisms initiated by GDNF in the pancreatic carcinoma cell line PANC-1. These results demonstrate that GDNF is an efficacious and potent chemoattractant for RET-expressing pancreatic carcinoma cells and that GDNF-induced tumor cell migration and invasion is dependent on activation of the Ras-Raf-MEK-ERK and the phosphatidylinositol 3-kinase (PI3k)/Akt signal transduction pathway.

MATERIALS AND METHODS

Materials. [γ-^{32}P]ATP (3000 Ci/mmol) was obtained from Amersham Biosciences (Freiburg, Germany); recombinant human GDNF, recombinant human BDNF, and recombinant human β-nerve growth factor were obtained from TEBU (Frankfurt am Main, Germany); and lysophosphatidic acid (LPA) and mitomycin C from Sigma (Taufkirchen, Germany). PD98059 was from New England Biolabs (Frankfurt am Main, Germany) and LY294002 was from Alexis Biochemicals (Grünberg, Germany). The following antibodies were used: ERK 2 (C-14), c-Jun NH_2-terminal kinase (JNK)1 (FL; Santa Cruz

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Biotechnology, Heidelberg, Germany); phospho-Akt^{Ser473} and Akt (New England Biolabs; Frankfurt am Main, Germany); human pancreatic polypeptide (DakoCytomation, Hamburg, Germany); Ret (C19; Santa Cruz Biotechnology); pan-Ras (Ab-3; Oncogene Sciences, Bad Soden, Germany); RhoA (26C4; Santa Cruz Biotechnology); and Rac1 and GDNFR-α (Transduction Laboratories; Heidelberg, Germany). Secondary antibodies were rabbit antigoat IgG (Sigma; Taufkirchen, Germany); peroxidase-conjugated goat antimouse IgG, mouse antigoat IgG, and goat antirabbit IgG (Sigma); and alkaline phosphatase-conjugated mouse antirabbit IgG (Dianova; Hamburg, Germany). Cell culture media and supplements were from Invitrogen (Groningen, The Netherlands).

Pancreatic Tissues, Cell Lines, and Culture Conditions. Pancreatic tissues from patients with chronic pancreatitis and with pancreatic adenocarcinoma were provided by the Department of Surgery at the University of Ulm and the Department of Surgery at the University of Homburg/Saar. All of the tissues were obtained after approval by the local ethics committees. Tissue samples from the resection margin obtained from patients with chronic pancreatitis that had undergone surgery were used as normal control pancreas. Human pancreatic carcinoma cell lines PANC-1 (CRL 1469), BxPC-3 (CRL 1687), MiaPaCa-2 (CRL 1420), AsPC-1 (CRL 1682), as well as HEK-293 (CRL 1573) and MDCK (CCL-34) were obtained from American Type Culture Collection (Manassas, VA). PaTu 8988t and PaTu 8988s were provided by Dr. Horst F. Kern (Marburg, Germany; Ref. 21), IMIM-PC1 and IMIM-PC2 were provided by Dr. Francisco X. Real (Barcelona, Spain; Ref. 22), and SK-N-SH (ATCC HTB-11) was provided by Dr. Juergen Engele (Leipzig, Germany). Cells were cultured in DMEM supplemented with 10% (v/v) FCS, L-glutamine (2 mM), and penicillin-streptomycin (100 IU/ml-0.1 mg/ml). PANC-1 cells stably expressing enhanced green fluorescent protein (EGFP) or EGFP-H-Ras(N17; Ref. 23) were incubated in growth medium supplemented with 1.5 mg/ml G418. Serum starvation was performed over night in DMEM without supplements or DMEM supplemented with 0.1% (w/v) BSA.

Reverse Transcription-PCR (RT-PCR) Analysis of RET and GFRα mRNA Expression. RNA from fresh-frozen pancreatic tissue was prepared as described previously (24). Total RNA from cell lines was extracted from cells grown to confluence using the RNeasy Midi kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions (1–2 $\times$ 10^7 cells/column). For RT-PCR, 10 μg of total RNA were reverse transcribed to first strand cDNA using oligodeoxythymidylic acid primers and the SuperScript preamplification system (Invitrogen). Human RET, GFRα, and β-actin cDNAs were amplified from single-stranded cDNA by PCR using 0.5 units of TaqDNA Polymerase (Sigma) and 0.4 μM of each primer. The amounts of single-stranded cDNAs used as templates were adjusted to similar levels according to the amount of single-stranded β-actin cDNA present in the sample. The RET PCR-primers were designed according to the human RET51 sequence (GenBank accession no. NM000323): 5′ primer (exon 16, nucleotides 2769–2788, numbering started at ^{1}ATG): 5′-TTTTGATCATACTACACCA-3′; RET51 3′ primer (exon 20, nucleotides 3322–3347): 5′-TGTTAACTATCAAACGTGTCCATTAA-3′. For amplification of cDNAs encoding the RET splice variants RET9 and RET43, the 3′ primers were designed according to the published sequences (17): RET9 3′ primer (nucleotides 3198–3216): 5′-GAATCTAGTAAATGCATGG-3′; RET43 3′ primer (nucleotides 3254–3272): 5′-GAAGTTACAGTGCTGACAA-3′. RET receptor fragments were amplified by 35 cycles: 94°C for 1 min, 52°C for 1 min (RET9 and RET43, 40°C for 1 min), 72°C for 1 min 30 s, and 72°C for 10 min. GFRα1 was amplified using PCR primers designed according to the human GFRα1 sequence (GenBank accession no. U95847): 5′ primer (exon 2, nucleotides 80–100): 5′-GGATTGCGTGAAAGCCAGTGA-3′; 3′ primer (exon 4, nucleotides 664–684): 5′-AGCACACAGGCACGATGGTCT-3′. The amplification protocol was: 35 cycles: 94°C for 1 min, 61°C for 1 min, 72°C for 3 min, and 72°C for 10 min. The human β-actin cDNA fragment (GenBank accession no.: XM037235) was amplified using the following PCR primers: 5′ primer (nucleotides 559–578): 5′-GACTACCTCATGAAGATCCT-3′; 3′ primer (nucleotides 851–870): 5′-GCGGATGTCCACGTCACACT-3′. The amplification protocol was: 25 cycles: 95°C for 45 s, 60°C for 1 min, 72°C for 1 min, and 72°C for 10 min. The PCR products were fractionated by [1.5% (w/v)] agarose gel electrophoresis and visualized by ethidium bromide staining. The sequences of the amplified cDNAs were verified by sequencing.

Activity of Monomeric GTPases. The GTP-bound forms of Ras, Rac1, and RhoA were recovered from cell lysates by affinity precipitation using glutathione *S*-transferase (GST)-fusion proteins carrying the GTPase-binding domains of GTPase-specific effector proteins as described previously (25, 26) and detected by SDS-PAGE and immunoblotting using the appropriate antibodies and the Enhanced Chemiluminescence Western Blotting Detection System (Amersham Biosciences).

ERK2 Activity Assay. Confluent cells (5 $\times$ 10^6 cells/10 cm dish) were incubated overnight in DMEM without supplements and treated with GDNF for the indicated periods of time. Activity of ERK2 was determined as described previously (25).

JNK Activity Assay. Cell lysates for determination of JNK activity were prepared as described for ERK2 activity assays. Immunoprecipitation of JNK was carried out with 1 μg of anti-JNK1, which was precoupled for 30 min at 4°C to 30 μl of protein A-agarose [50% slurry, Roche Diagnostics (Mannheim, Germany), washed, and equilibrated with MAPK-RIPA buffer]. JNK was immunoprecipitated for 2 h at 4°C by end-over-end rotation from 1 mg of cell lysate protein. The precipitate was washed twice with MAPK-RIPA buffer and twice with kinase buffer [25 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, and 2 mM DTT] and resuspended in 30 μl of kinase buffer. One-third of the precipitate (10 μl) was used to control for equal amounts of JNK in the samples by immunoblot analysis. The remainder (20 μl) was subjected to the kinase reaction, which was performed for 20 min at 30°C after addition of 1 μg of purified, recombinant GST-c-Jun, 2 μCi [γ-^{32}P]ATP (3000 Ci/mmol), and 20 μM ATP as JNK substrates. The reaction was terminated by the addition of 10 μl of 5 $\times$ SDS-gel loading buffer. Proteins were boiled and separated on 12.5% SDS-polyacrylamide gels. Phosphorylated products were visualized by autoradiography of wet gels. Recombinant GST-c-Jun was purified as described previously (27).

Akt Activity Assay. Confluent PANC-1 cells (5 $\times$ 10^6 cells/10 cm dish) were incubated overnight in DMEM without supplements and pretreated with inhibitors or solvent for 20 min. Treatment with GDNF was performed for 7 min. Cells were lysed in 500 μl of Akt-lysis buffer per dish [(Akt-lysis buffer: 20 mM Tris-HCl (pH 8.0), 2 mM EGTA, 10% (v/v) glycerol, 1% (v/v) NP40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 20 μM leupeptin; Ref. 28)]. Cleared cell lysates (50 μg of protein) were fractionated on 10% SDS-polyacrylamide gels, and proteins were subsequently transferred onto nitrocellulose membranes. Akt and phospho-Akt^{Ser473} were detected using specific antibodies and Enhanced Chemiluminescence Western Blotting Detection System.

Cell Migration Assays. Cell migration assays using the *trans*-well cell migration chambers (12-well inserts, 8-μm pore size; BD Biosciences, Heidelberg, Germany) and 5 $\times$ 10^4 cells per migration chamber were performed as described previously (26).

Chorioallantoic Membrane (CAM) Chemotaxis Assay. Fertilized chicken eggs were incubated at 37.8°C for 6 days and then prepared for implantation of tumor cells by removing the shell from the pointed end of the egg to obtain a round opening with a diameter of 2–3 cm. A silicone double-ring system consisting of two rings of 5-mm diameter connected by a 5-mm bridge was placed onto the CAM. PANC-1 cells (1 $\times$ 10^6 cells in 20 μl of DMEM) were seeded into one of the silicone rings. A dry nitrocellulose filter (2 $\times$ 4 mm) was placed into the other ring. Three μl of chemoattractant (1 ng/μl) or water were applied daily onto the nitrocellulose filter. The shell opening was covered with tape, and the eggs were incubated for another 6 days at 37.8°C, 60% relative humidity. The chorioallantoic tissue area containing the silicone double-ring was then isolated, fixed with 4% (v/v) formaldehyde for 24 h, drained, and embedded into paraffin. Sections (3–5 μm) were subjected to immunohistochemistry or stained with H&E as described previously (29). Mounted sections were examined with an Axioskop 2 plus microscope, and images were recorded using a CCD camera (AxioCam MRC) and the Axiovision 3.1 imaging system (Carl Zeiss, Oberkochen, Germany).

Miscellaneous. Protein concentrations were determined by the bicinchoninic acid assay (Pierce; Sankt Augustin, Germany) using BSA fraction V (2 mg/ml) as standard. All of the experiments were repeated at least three times. Similar results and identical trends were obtained each time. Data from representative experiments are shown.

RESULTS

Differential splicing at the 3′ end of the RET heterogenous nuclear RNA results in transcripts encoding three RET isoforms that differ in their COOH-terminal 51 (RET51), 43 (RET43), or 9 (RET9) amino acids (19). The RET51 and RET9 transcripts are much more abundant than the RET43 transcripts (17, 30, 31). Expression of RET51 mRNA in normal human pancreatic tissues, in pancreatic tissues of patients with chronic pancreatitis, and in pancreatic tissues of patients with pancreatic adenocarcinoma (stages I to IV) was determined by RT-PCR. To amplify a RET51 mRNA fragment, a common 5′ oligonucleotide binding to a sequence in exon 16 and a 3′ oligonucleotide binding to the 3′ end of exon 20 was used. Fig. 1*A* shows that no RET51 mRNA or only low levels of this mRNA were detected in samples from normal pancreas, from chronic pancreatitis, as well as from stage IA, IIA, III, and IV adenocarcinomas. In marked contrast, high or even very high levels of RET51 mRNA were detected in four of six samples from stage IIB adenocarcinomas. Specifically, the amount of the PCR product amplified from RET cDNA obtained for three of these samples was similar to the amount obtained for a sample from SK-N-SH neuroblastoma cells, which served as positive control (32). No RET mRNA was detected in the remaining two stage IIB adenocarcinoma samples. The percentage of tumor cells in the analyzed pancreatic carcinoma tissues was estimated histologically to range between 20% and 70%. The high level of RET51 mRNA expression in some of the stage IIB adenocarcinoma was not explained by a high percentage of tumor cells in these samples. To test the hypothesis that pancreatic carcinoma cells are the source of RET51 mRNA expression, the expression of this mRNA was determined by RT-PCR in nine different human pancreatic carcinoma cell lines. Fig. 1*B* shows that RET51 mRNA was detected in all nine of the cell lines. High levels were found in BxPC-1, MiaPaCa-2, IMIM-PC-1, PaTu8092, PaTu8988s, PaTu8988t, followed by PANC1, AsPC-1, and IMIM-PC-1 pancreatic carcinoma cells. No RET51 mRNA was detected in HEK293 embryonic kidney epithelial cells and MDCK kidney epithelial cells.

RET and GFRα1 Expression in PANC-1 Cells. The three RET transcripts generated by alternative splicing possess the same first 19 exons, followed by unique coding sequences generated by the alternative splicing process. Exon 19 is spliced to exon 20 in RET51 mRNA and to exon 21 in RET43 mRNA. In RET9 mRNA, the intron sequence downstream of exon 19 is transcribed (17, 18). RT-PCR analyses with a common 5′ oligonucleotide binding to a sequence in exon 16 and splice variant-specific 3′ oligonucleotides demonstrated that mRNAs encoding all three of the RET isoforms were expressed in PANC-1 pancreatic carcinoma cells (Fig. 2*A*). Because a number of malignancies is associated with mutational alterations within the coding sequence of the *c-ret* proto-oncogene (20), the nucleotide sequences of six overlapping RT-PCR fragments, each from four

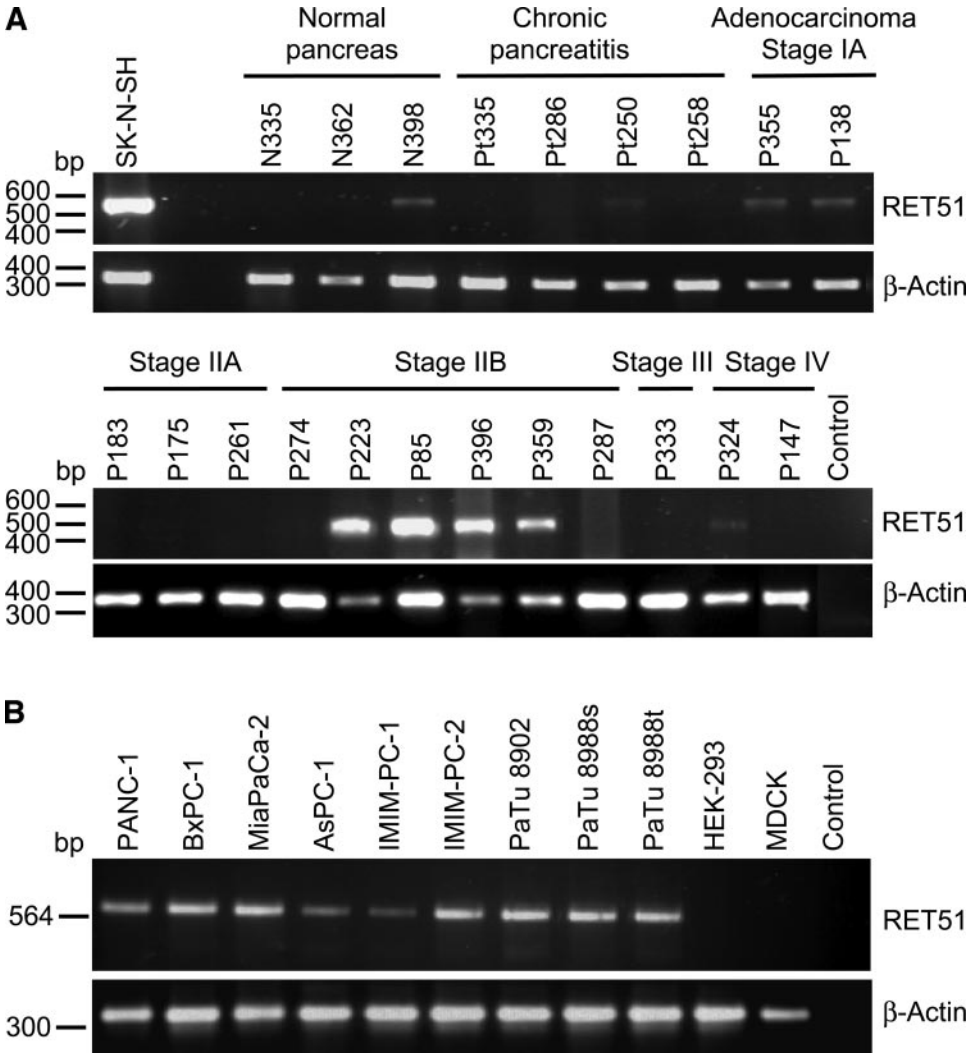

Fig. 1. Reverse transcription-PCR (RT-PCR) analysis of RET51 receptor mRNA expression in pancreatic tumor tissues and in pancreatic carcinoma cell lines. *A*, total RNA was prepared from normal human pancreatic tissues (*Normal pancreas*), from pancreatic tissues of patients with chronic pancreatitis (*Chronic pancreatitis*), and from pancreatic tissues of patients with pancreatic adenocarcinoma (*Adenocarcinoma, stages I* to *IV*), and used as a template for RT-PCR. The stage grouping of the pancreatic adenocarcinomas is specified in Table 1. A sample containing RNA prepared from SK-N-SH neuroblastoma cells (*SK-N-SH*) and a sample containing no template RNA (*Control*) served as controls. *B*, total RNA was prepared from the pancreatic carcinoma cell lines PANC-1, BxPC-3, MiaPaCa-2, AsPC-1, IMIM-PC1, IMIM-PC2, PaTu 8988s, and PaTu 8988t, as well as from the human embryonic kidney epithelial cell line HEK293, and the canine kidney epithelial cell line MDCK, and used as template for RT-PCR. A sample containing no template RNA served as control (*Control*). The amounts of cDNA used to amplify a 579-bp RET51 fragment (*top panels*) were adjusted to similar levels according to the amount of β-actin cDNA present in the samples (*bottom panels*). The positions of the RT-PCR products and the size standards are indicated.

Table 1 *Postsurgical histopathological pTNM[a] classification and stage grouping*

The postsurgical histophathological (pathological) pTNM classification and the stage grouping of the pancreatic adenocarcinomas analyzed in the experiment shown in Fig. 1 was done according to the guidelines established by the International Union Against Cancer (51). The classification is based on the evidence acquired before treatment, supplemented or modified by the additional evidence acquired from surgery and from pathological examination. The expression of RET mRNA was judged based on the intensity of the amplified RT-PCR product shown in Fig. 1.

Tissue	pTNM	Stage	RET expression
P355	pT1N0M0	IA	+
P138	pT1N0M0	IA	+
P183	pT3N0M0	IIA	−
P175	pT3N0M0	IIA	−
P261	pT3N0M0	IIA	−
P274	pT3N1MX	IIB	−
P223	pT3N1M0	IIB	+++
P85	pT1N1MX	IIB	+++
P396	pT3N1M0	IIB	+++
P359	pT3N1M0	IIB	++
P287	pT3N1M0	IIB	−
P333	pT4N1M0	III	−
P324	pT4N1M1	IV	+
P147	pT2N0M1	IV	−

[a] pTNM, pathological Tumor-Node-Metastasis; RT-PCR, reverse transcription-PCR; −, no expression of RET mRNA; +, low expression of RET mRNA; ++, high expression of RET mRNA; +++, very high expression of RET mRNA.

individual mRNA preparations from PANC-1 cells covering the entire coding region of the 21 exons of the *c-ret* proto-oncogene, were amplified by RT-PCR. No sequence alterations were detected when compared with the published RET sequence (GenBank accession no. NM000323), except for a silent mutation in codon 432 [GCG (Ala) → GCA (Ala)] in exon 7.

To investigate the expression of the RET protein(s) in PANC-1 cells, immunoprecipitation analyses were performed. Fig. 2*B* shows that the RET receptor that had been immunoprecipitated with a specific polyclonal RET (C-19) antiserum from SK-N-SH cells runs as a doublet on SDS-polyacrylamide gels, as reported by Takahashi *et al.* (33) for the differentially glycosylated 150 kDa and 170 kDa RET proteins. Using the same primary antiserum, only the 150 kDa component was immunoprecipitated from PANC-1 cell lysates. Thus, RET protein(s) are expressed in PANC-1 cells, but their post-translational glycosylation may be distinct from their modification in SK-N-SH cells.

Next, the expression of the mRNA of the GDNF coreceptor GFRα1 was examined by RT-PCR analysis (Fig. 2*C*). Our results clearly demonstrate GFRα1 mRNA expression in the pancreatic carcinoma cells PANC-1, BxPC-3, and AsPC-1. PANC-1 and AsPC-1 cells also expressed the GFRα1 protein, as demonstrated by immunoblot analysis of cell lysates using a GFRα1-specific monoclonal antibody (Fig. 2*D*). Human stomach lysates and rat glioma B49 cells served as positive and S*f*9 insect cells as negative controls. In conclusion, these results show that both components of the GDNF receptor complex, RET and GFRα1, are expressed in pancreatic adenocarcinoma cells at the mRNA and at the protein level.

Induction of PANC-1 Cell Migration by GDNF. To investigate whether the neurotrophic factor GDNF contributes to the development of the malignant phenotype of pancreatic carcinoma cells by stimulating proliferation or chemotaxis, the growth of PANC-1 cells was analyzed under serum-free conditions in the presence or absence of GDNF. Determination of cell number over a period of 10 days revealed that GDNF did not accelerate the proliferation of PANC-1 cells (data not shown). To examine whether GDNF was capable of

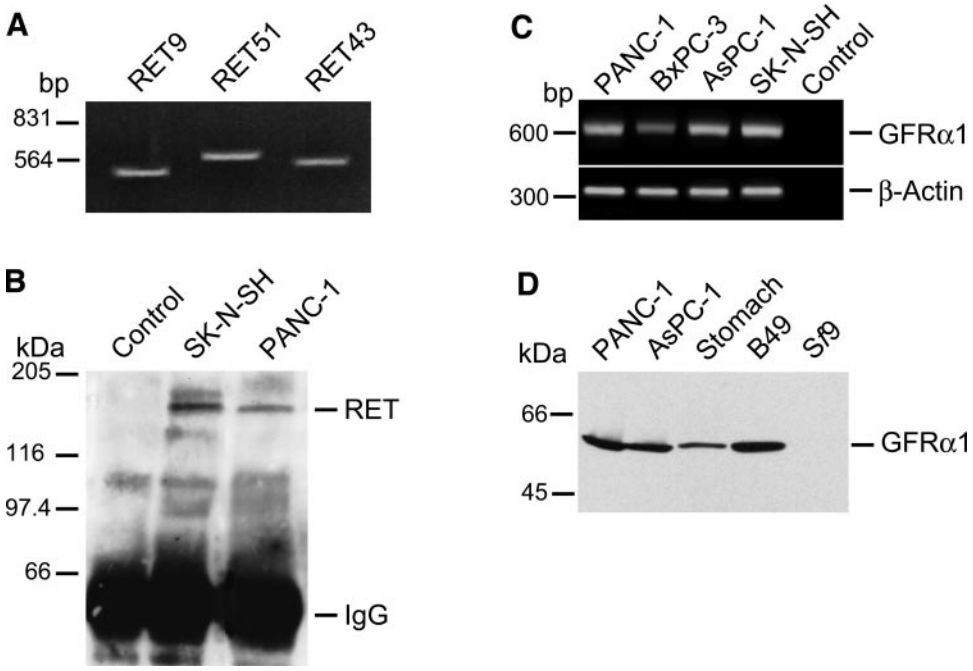

Fig. 2. Reverse transcription-PCR (RT-PCR) and immunoblot analysis of RET and GDNF family receptor α (*GFR*α) mRNA and protein expression in pancreatic carcinoma cells. *A*, expression of mRNA encoding the RET splice variants RET9, RET51, and RET43. Total RNA was prepared from human PANC-1 pancreatic carcinoma cells and used as template for RT-PCR. A common 5′ oligonucleotide and 3′ oligonucleotides specifically recognizing the cDNAs of RET9, RET51, or RET43 were used for RT-PCR. The positions of the size standards are indicated. *B*, expression of RET protein. SK-N-SH neuroblastoma cells and PANC-1 pancreatic carcinoma cells were lysed in radioimmunoprecipitation assay buffer. RET was immunoprecipitated from 2 mg (*SK-N-SH*) or 8 mg (*PANC-1*) protein using 4 μg IgG of goat anti-Ret (C19) antiserum and 10 μg IgG of rabbit antigoat antiserum. The control sample (*Control*) containing 8 mg of PANC-1 protein was treated with 10 μg of IgG of rabbit antigoat serum. The immunoprecipitated proteins were subjected to SDS-PAGE and immunoblotting using the anti-RET (C19) antiserum The positions of RET, the heavy chain of IgG, and the molecular mass standards are indicated. *C*, expression of GFRα1 mRNA. Total RNA from PANC-1, BxPC-3, and AsPC-1 pancreatic carcinoma cells, as well as from SK-N-SH neuroblastoma cells was used as template for RT-PCR. A sample containing no template RNA served as control (*Control*). The amounts of cDNA used to amplified a GFRα1 fragment (*top panel*) were adjusted to similar levels according to the amount of β-actin cDNA present in the samples (*bottom panel*). *D*, expression of GFRα1 protein. Homogenates (20 μg protein/sample) of PANC-1 and AsPC-1 pancreatic carcinoma cells, B49 glioma cells, S*f*9 insect cells, and human stomach tissue were subjected to SDS-PAGE and immunoblotting using an antiserum reactive against GFRα1. The positions of GFRα1 and the molecular mass standards are indicated.

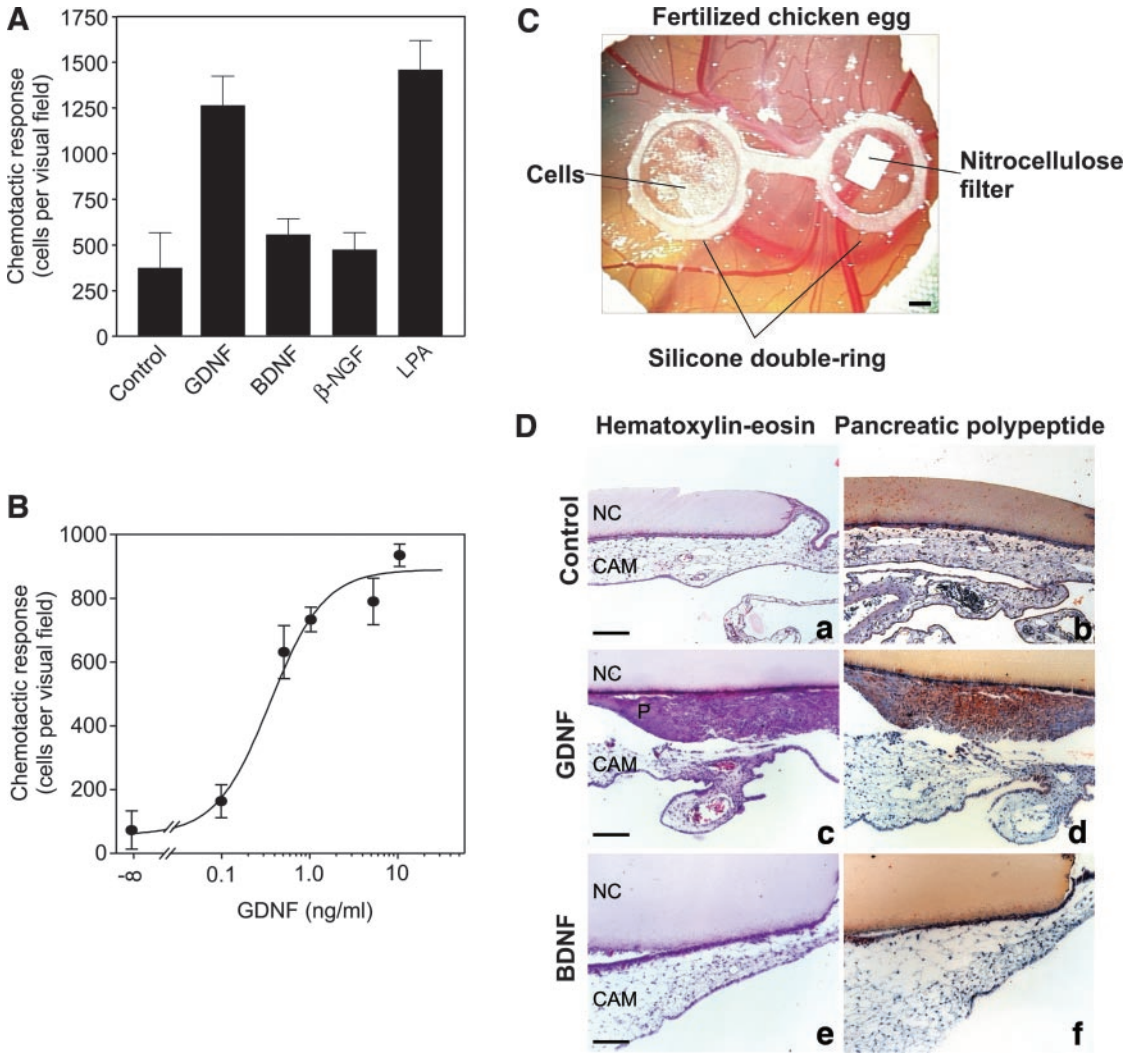

Fig. 3. Stimulation of pancreatic carcinoma cell migration and invasion by glial cell line-derived neurotrophic factor (*GDNF*). PANC-1 cells were seeded into *trans*-well cell migration chambers. In *A*, the upper chambers contained DMEM supplemented with 0.1% (w/v) BSA, the lower chambers contained DMEM supplemented with 0.1% (w/v) BSA (*Control*) or with 0.1% (w/v) BSA and 10 ng/ml GDNF, 10 ng/ml brain-derived nerve growth factor (*BDNF*), 10 ng/ml β-nerve growth factor (*β-NGF*), or 10 μM lysophosphatidic acid (*LPA*) as chemoattractant. Cells were incubated for 40 h, and the chemotactic response was calculated by counting the cells that had migrated to the bottom side of the filter in three randomly chosen visual fields per *trans*-well chamber (magnification, ×100). Means of the cell numbers determined in nine independent visual fields from three *trans*-well chambers are shown; *bars*, ±SD. In *B*, the lower chambers contained DMEM supplemented with 0.1% (w/v) BSA and increasing concentrations of GDNF as indicated at the abscissa. Each value represents the mean of the cell numbers determined in three independent visual fields; *bars*, ±SD. *C*, top view on the chorioallantoic membrane of a fertilized chicken egg. PANC-1 cells were seeded into the compartment formed by the left ring (*Cells*), and 3 μl of chemoattractant were applied onto the nitrocellulose filter (*Nitrocellulose filter*) in the compartment formed by the right ring. *Bar*, 1 mm. *D*, vertical cross-sections through the interface formed between the nitrocellulose filter and the chorioallantoic membrane. PANC-1 cells were seeded into the compartment formed by the left silicone ring and 3 μl of water (*Control*; *panels a* and *b*), water containing 1 ng/μl GDNF (*panels c* and *d*), or water containing 1 ng/μl BDNF (*panels e* and *f*) were applied daily onto the nitrocellulose filter in the right silicone ring. After 6 days, the chorioallantoic membranes were paraffin-embedded, cut into 3–5 μm vertical sections, and either stained with H&E (*left*) or subjected to immunostaining using antibodies reactive against pancreatic polypeptide (*right*). The positions of the nitrocellulose filters (*NC*), the chorioallantoic membrane (*CAM*), and the PANC-1 cells (*P*) are indicated. *Bars*, 100 μm.

stimulating PANC-1 chemotaxis, directed cell migration was studied using *trans*-well cell migration chambers. Fig. 3*A* shows that addition of 10 ng/ml GDNF to one compartment of the chambers resulted in an approximately 3–5-fold increase in PANC-1 cell migration, as compared with controls exposed to control medium. The neurotrophic factors BDNF and β-NGF had no effect. Note that 10 ng/ml GDNF was nearly as efficacious as a stimulator of PANC-1 cells as 10 μM LPA, which has been shown previously to be a powerful stimulator of PANC-1 cell chemotaxis (26). The effect of GDNF on PANC-1 cell chemotaxis was concentration-dependent (Fig. 3*B*) with half-maximal and maximal effects observed at ~0.4 ng/ml (35 pM) and 4 ng/ml (350 pM) GDNF, respectively.

Next, the ability of a local GDNF gradient to induce PANC-1 cell migration and invasion in a three-dimensional tissue such as the chorioallantoic membrane (CAM) of fertilized chicken eggs was investigated. The CAM represents an intermediate between *in vitro* and *in vivo* model systems and provides an excellent natural substrate for many types of tumor cells (29, 34, 35). To examine the effect of GDNF on PANC-1 cell chemotaxis in this system, we designed a gadget consisting of two silicon rings connected by a rigid, fixed-length bridge that allows for the application of the cells and the chemoattractant to the CAM at a defined distance to each other. Fig. 3*C* shows an image of the opened chicken egg equipped with the silicone double-ring system. The compartment formed by the left ring contained PANC-1 cells, whereas the compartment formed by the right ring contained a small piece of nitrocellulose filter that served as a reservoir for the chemoattractant. Three μl of GDNF (1 ng/μl), BDNF (1 ng/μl), or water were applied daily onto the nitrocellulose filter. After 6 days of incubation, vertical sections of the CAM were prepared and examined histologically. The CAM thickened around the

area of cell inoculation, and the cells formed compact microtumors on the outer chorioepithelial side of the CAM. Furthermore, tumor cells invaded through the chorio-epithelium into the underlying mesenchyme. Sections through the area underneath the nitrocellulose filter (Fig. 3*D*) demonstrated that application of GDNF resulted in a striking accumulation of PANC-1 cells underneath the nitrocellulose filter. The H&E-stained section in Fig. 3*D* (*panel c*) shows that PANC-1 cells formed solid tumor nests of closely packed cells underneath the destroyed chorio-epithelium within the mesenchymal tissue of the CAM. Fig. 3*D* (*panel d*) shows the corresponding section after specific immunostaining of the pancreas-specific protein pancreatic polypeptide. The fact that the cells accumulating under the nitrocellulose filters of the GDNF-treated sample were strongly immunoreactive confirms that the accumulating cells were indeed PANC-1 rather than CAM cells. No such accumulations were observed when water [images in Fig. 3*D* (*panel a*) and Fig. 3*D* (*panel b*)] or BDNF [images in Fig. 3*D* (*panel e*) and Fig. 3*D* (*panel f*)] had been applied to the nitrocellulose filters. These results confirmed that GDNF is an efficacious chemoattractant for PANC-1 cells.

Activation of the Mitogen-Activated Protein Kinases and Akt by GDNF. To characterize the signal transduction mechanisms involved in mediating the stimulatory effect of GDNF on chemotaxis, the effects of GDNF on the activity of the mitogen-activated protein kinases ERK and JNK were analyzed. Fig. 4*A* shows that addition of 100 ng/ml GDNF to serum-starved PANC-1 cells resulted in a time-dependent activation of ERK2. The activation of ERK2 was maximal after 10–15 min and returned to basal levels within 1 h. Additional experiments revealed that 10 ng/ml of GDNF were sufficient to induce the maximal, ~4-fold activation of ERK. Moreover, ERK activation was sensitive to MEK1 inhibition by PD98059, suggesting that the entire Ras-Raf-MEK-ERK cascade was activated by GDNF (data not shown). JNK activation was determined by *in vitro* phosphorylation assays. As depicted in Fig. 4*B*, treatment of serum-starved PANC-1 cells with 100 ng/ml GDNF resulted in a marked activation of JNK with a maximum after 15 min. GDNF-induced JNK activation was less pronounced than stress-induced kinase activation, such as observed after incubation of cells for 20 min at 42°C [Fig. 4*B* (*Co.*)]. A third pathway known to be activated by GDNF in neuronal cells (36) is the one involving PI3k and the protein kinase Akt. To detect a possible activation of this pathway by GDNF, the effect of GDNF on phosphorylation of Akt at serine 473 was determined. Fig. 4*C* shows that Ser^{473}-phosphorylated Akt migrated as a doublet upon fractionation of PANC-1 cell lysates by SDS-PAGE and, more importantly, that treatment of serum-starved PANC-1 cells with 25 ng/ml or 50 ng/ml GDNF resulted in a marked, ~3-fold increase in the level of Ser^{473}-phosphorylated Akt. Preincubation of the cells with the PI3k inhibitor LY294002 clearly reduced the stimulatory effect of GDNF on Akt phosphorylation, confirming that the effect of GDNF is mediated by PI3k activation.

Activation of Ras and Rho GTPases by GDNF. Ras and Rho GTPases are key regulators of the ERK and JNK pathway and are also involved in PI3k-mediated signaling. In the next set of experiments, the effect of GDNF on the activity of RhoA, Rac1, and Ras GTPases were determined in PANC-1 cells. Fig. 5 represents the time courses of the activity of the individual GTPases after treatment of serum-starved cells with GDNF. The levels of GTP-activated GTPases were measured *in vitro* by affinity precipitation using GST-fusion proteins carrying the GTPase-binding domains of the GTPase-specific effector proteins Rhotekin, PAK1b, and c-Raf1. Addition of 50 ng/ml GDNF to serum-starved PANC-1 cells induced a rapid and transient activation of RhoA (Fig. 5*A*, *top panel*), which was maximal at 1–3 min and was negligible at 5 min after addition of GDNF. Fig. 5*B* shows that Rac1 was also rapidly and transiently activated after the addition of 10 ng/ml GDNF and that the time course of this effect was similar to that observed for RhoA. Similar results were obtained using higher concentrations of GDNF, *e.g.*, 100 ng/ml (results not shown). Ras GTPases were also activated upon the addition of 50 ng/ml of GDNF. Note, however, that the time course of this activation was

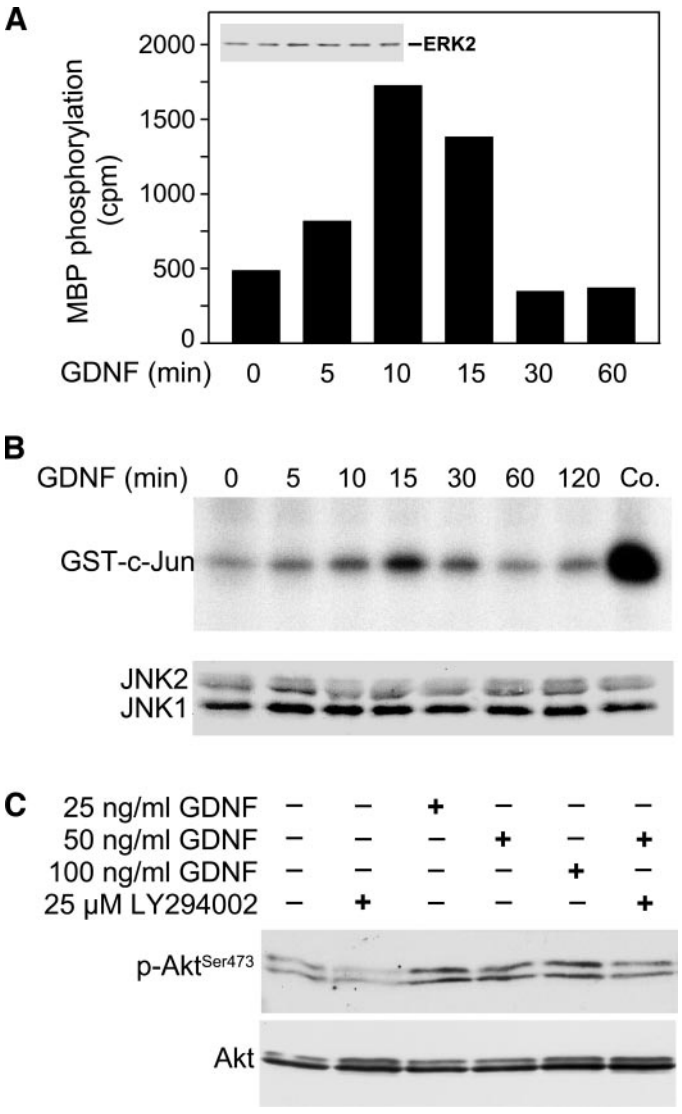

Fig. 4. Activation of mitogen-activated protein kinases by glial cell line-derived neurotrophic factor (*GDNF*). *A*, activation of extracellular signal-regulated kinase (ERK) 2. Confluent, serum-starved PANC-1 cells were treated for the indicated time periods with 100 ng/ml GDNF. ERK2 was immunoprecipitated from 1 mg of lysate protein, and the kinase activity was determined by its ability to [^{32}P]phosphorylate MBP peptide using [γ-^{32}P]ATP as substrate. The results of one experiment are given in cpm. *Inset*, one third of the sample containing the immunoprecipitated ERK2 used for the [^{32}P]phosphorylation reaction was subjected to SDS-PAGE and immunoblotting using anti-ERK2-antiserum. The position of ERK2 is indicated. *B*, activation of c-Jun NH_2-terminal kinase (*JNK*). PANC-1 cells were treated as described in *A*, and control cells (*Co.*) were incubated for 20 min at 42°C. JNK was immunoprecipitated from 1 mg of lysate protein. The activity of immunoprecipitated JNK was determined by its ability to [^{32}P]phosphorylate glutathione *S*-transferase (*GST*)-c-Jun using [γ-^{32}P]ATP as substrate. [^{32}P]phosphorylated GST-c-Jun was visualized by autoradiography of the wet SDS-polyacrylamide gel. The position of [^{32}P]GST-c-Jun is shown (*top panel*). One third of the sample containing the immunoprecipitated JNK used for the [^{32}P]phosphorylation reaction was subjected to SDS-PAGE and immunoblotting using anti-JNK1 antiserum. The positions of JNK1 and JNK2 are indicated. *C*, activation of Akt. Confluent, serum-starved PANC-1 cells were pretreated for 20 min with 25 μM LY294002 (+) or solvent (−). Afterwards, the cells were treated for 7 min in the absence (−) or presence (+) of the indicated concentrations of GDNF and LY294002. Aliquots containing 50 μg of protein were subjected to SDS-PAGE and immunoblotting, and phosphorylation of serine 473 of Akt was determined with phosphoamino-acid-specific anti-p-Akt^{Ser473} antiserum (*top panel*). The total amount of Akt in aliquots containing 50 μg of protein was determined using an antiserum reactive against Akt (*bottom panel*).

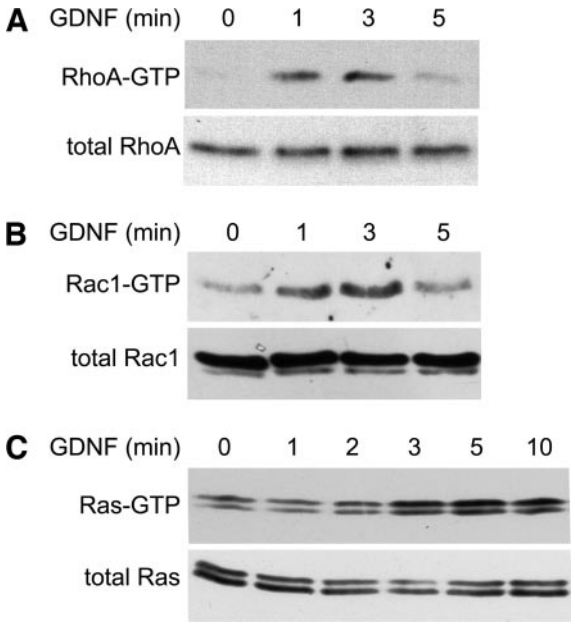

Fig. 5. Activation of the monomeric GTPases RhoA, Rac1, and Ras by glial cell line-derived neurotrophic factor (*GDNF*). Confluent, serum-starved PANC-1 cells were treated for the indicated time periods with GDNF [50 ng/ml for RhoA (*A*) and Ras (*C*), 10 ng/ml for Rac1 (*B*)]. GTP-bound GTPases were recovered from aliquots of the cell lysates containing 4 mg (*A*), 1 mg (*B*), and 0.5 mg (*C*) protein by affinity precipitation. To control for equal loading, aliquots of the samples containing 50 μg of protein were analyzed in parallel (*bottom panels*). The samples were subjected to SDS-PAGE and immunoblotting using antisera reactive against RhoA (*A*), Rac1 (*B*), or all Ras iso-forms (*C*).

much slower than time courses observed for RhoA and Rac1. Specifically, no stimulation was seen at 1 min, and maximal stimulation was seen only at 3–5 min, with little, if any decrease even at 10 min after addition of GDNF (Fig. 5*C*). Additional analyses revealed that the levels of GTP-bound Ras-GTPases returned to near-basal levels within the next 60 min. Ras-GTPases migrated as a doublet upon fractionation of PANC-1 cell lysates by SDS-PAGE. Using Ras isoform-specific antibodies, N-Ras was identified as the main Ras isoform activated by GDNF in PANC-1 cells (data not shown).

Signal Transduction Pathways Involved in Mediating the Effect of GDNF on Cell Migration. To assess whether activation of Ras is involved in mediating the stimulatory effect of GDNF on PANC-1 cell migration, the consequence of EGFP-H-Ras(N17) on this effect was determined in cells with stable expression of this dominant-interfering H-Ras mutant. Fig. 6*A* illustrates that EGFP-expressing control cells responded with an ~4-fold increase of cell migration upon addition of 10 ng/ml GDNF. Interestingly, stable expression of EGFP-H-Ras(N17) markedly reduced the stimulatory effects of both GDNF and LPA on PANC-1 cell migration by ~70%. In previous studies, we have documented that the Ras-Raf-MEK-ERK cascade is of considerable importance for directed PANC-1 cell migration (25, 26). Because treatment of PANC-1 cells with GDNF caused activation of ERK2 and PI3k, the relevance of these protein kinases to the chemotactic effect of GDNF was investigated by examining the influence of specific inhibitors of these kinases. Fig. 6*B* shows that addition of the MEK1 inhibitor PD98059 (*gray bars*) and of the PI3k inhibitor LY294002 (*black bars*) led to an almost complete or even complete loss of GDNF-stimulated PANC-1 cell chemotaxis, respectively. Note that each inhibitor also caused a slight reduction of the PANC-1 cell motility in the absence of chemotactic stimuli, indicating that these pathways are operative under basal conditions to stimulate cell migration.

The importance of the MEK-ERK pathway for the ability of GDNF to elicit directed cell migration was additionally analyzed by using the silicone double-ring system mounted on the CAM (Fig. 6*C*). Interestingly, inhibition of the MEK-ERK pathway by PD98059 caused a complete inhibition of the stimulatory effect of GDNF on chemotaxis of PANC-1 cells on the CAM. GDNF caused directed cell migration from the seeding area to the source of the chemotactic agent and formation of a solid tumor of PANC-1 cells in the mesenchymal tissue of the CAM [Fig. 6*C* (*panel b*)]. In the center of this large cell aggregate, a necrotic core was evident, indicating that the supply of oxygen and/or nutrients to this area was not sufficient to maintain cell survival. Importantly, PD98059-treated PANC-1 cells remained in the seeding area [Fig. 6*C* (*panel c*)] and barely moved into the surroundings. The region under the GDNF-soaked nitrocellulose filter was free of PANC-1 cells in the presence of the inhibitor [Fig. 6*C* (*panel d*)]. Immunostaining of these sections with antibodies reactive against pancreatic polypeptide confirmed that essentially no PANC-1 cells had migrated toward the GDNF source (data not shown). Collectively, these results show that activation of both the Ras-Raf-MEK-ERK and the PI3k pathway is essential for GDNF-induced movement of PANC-1 cells.

DISCUSSION

Perineural invasion is a characteristic feature and an important prognostic factor of pancreatic cancer (3, 7). In this report, we show that pancreatic carcinoma cells express RET and GFRα1, the components of the receptor for the neurotrophic factor GDNF. High levels of RET51 mRNA were detected in samples from stage IIB adenocarcinomas. The results in this study also show that the neurotrophic factor GDNF is a potent and efficacious chemoattractant for PANC-1 pancreatic carcinoma cells. GDNF-induced migration and invasion of pancreatic carcinoma cells is shown to be highly dependent on PI3k and Ras-Raf-MEK-ERK signaling. Thus, GDNF represents a chemotaxin for pancreatic carcinoma cells, which might facilitate perineural invasion.

The current study shows, for the first time, that expression of the mRNA for the RET51 isoform is up-regulated in pancreatic stage IIB tumors. The expression of RET51 mRNA was not enhanced in tissue samples from patients with chronic pancreatitis, which showed a similar degree and variation of inflammation and fibrosis as the pancreatic cancer tissues. Thus, it is likely that the tumor cells and not the surrounding inflammatory or stromal cells are the source of RET51 mRNA expression. This assumption is supported by the fact that all of the analyzed pancreatic carcinoma cell lines expressed high levels of RET51 mRNA, whereas nontumor-derived epithelial cells showed no RET51 mRNA expression. PANC-1 pancreatic carcinoma cells express transcripts of the three RET isoforms RET9, RET43, and RET51, and sequence analysis of RET RT-PCR fragments showed that none of these mRNAs contained mutations. This is worth mentioning, because *ret*-associated rearrangements and mutations have been found in a variety of inherited and sporadic cancer syndromes (20, 37). Most of the existing data on the biological effects of GDNF and on the mechanisms of GDNF-mediated signal transduction were obtained in neuronal cells (11, 37, 38), but the biological effects and signaling mechanisms are still elusive in non-neuronal cell systems with endogenous expression of the GDNF receptor system. In the present study, the biological effects of GDNF and the underlying signal transduction mechanisms initiated by GDNF in the human pancreatic carcinoma cell line PANC-1 were examined, and the impact of these mechanisms on the migratory and invasive behavior of these cells were investigated.

GDNF did not act as a mitogen for the PANC-1 cells, but did act as an efficacious and potent chemoattractant for these cells. Applica-

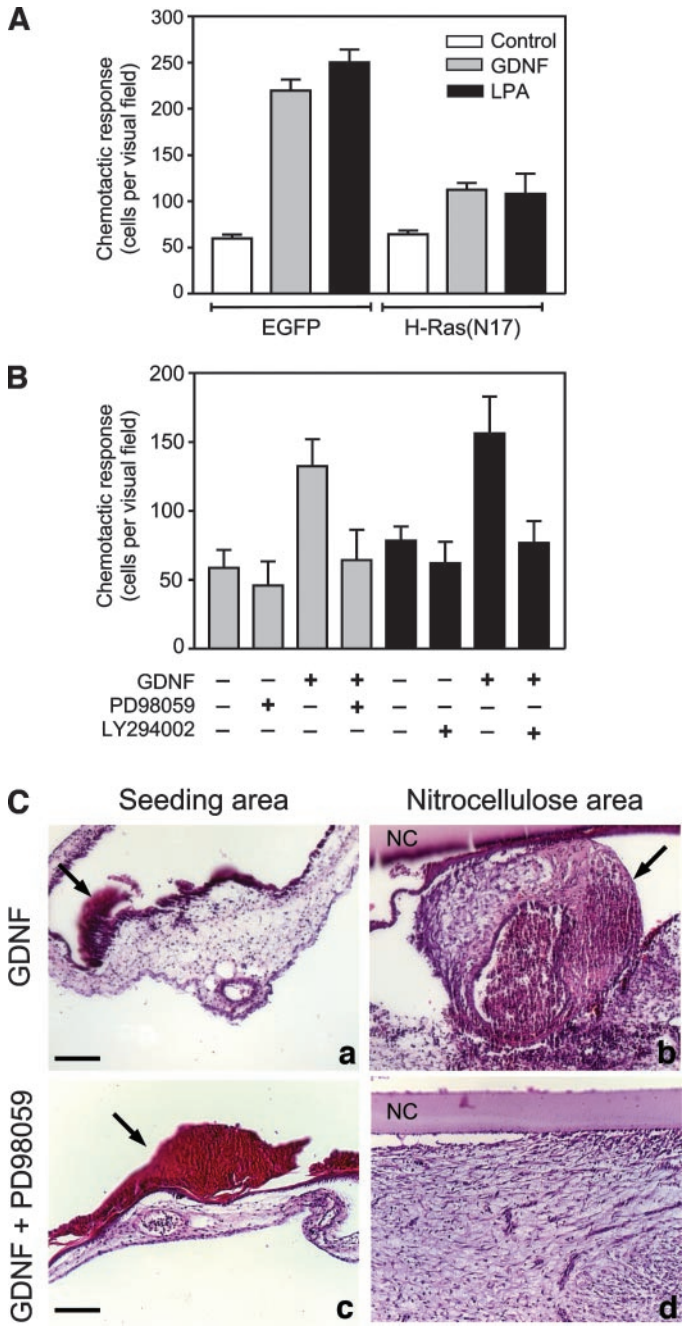

Fig. 6. Inhibition of glial cell line-derived neurotrophic factor (*GDNF*)-stimulated PANC-1 cell migration and invasion. *A*, inhibition of PANC-1 cell migration by enhanced green fluorescent protein (*EGFP*)-H-Ras(N17). PANC-1 cells stably expressing EGFP or dominant-negative EGFP-H-Ras(N17) were seeded into *trans*-well cell migration chambers. The upper chambers contained DMEM supplemented with 0.1% (w/v) BSA, the lower chambers contained DMEM supplemented with 0.1% (w/v) BSA (*white bars*; *Control*), with 0.1% (w/v) BSA and 10 ng/ml GDNF (*gray bars; GDNF*), or with 0.1% (w/v) BSA and 10 μM lysophosphatidic acid (*black bars; LPA*). Cells were incubated for 24 h, and the chemotactic response was calculated by counting the migrated cells in three randomly chosen visual fields per *trans*-well chamber (magnification, $\times$100). Means of the cell numbers determined in six independent visual fields from two *trans*-well chambers are shown; *bars*, $\pm$SD. *B*, inhibition of GDNF-stimulated PANC-1 cell migration by PD98059 and LY294002. PANC-1 cells were seeded into *trans*-well cell migration chambers. The upper chambers contained DMEM supplemented with 0.1% BSA, with 0.1% BSA and 25 μM PD98059, or with 0.1% BSA and 25 μM LY294002. The lower chambers contained DMEM supplemented with 0.1% BSA with or without 10 ng/ml GDNF, 25 μM PD98059, or 25 μM LY294002, as indicated at the abscissa (PD98059 experiments: *gray bars*; LY294002 experiments: *black bars*). Cells were incubated for 40 h, and the chemotactic response was calculated as described in *A*. Means are shown; *bars*, $\pm$SD. *C*, inhibition of GDNF-stimulated PANC-1 cell migration in the chorioallantoic membrane (CAM) by PD98059. PANC-1 cells were seeded into the compartment formed by the left silicone ring (*Seeding area*) and 3 μl of water containing 1 ng/μl GDNF (*GDNF*; *panels a* and *b*) were applied daily onto the nitrocellulose filter in the compartment formed by the right ring (*Nitrocellulose area*). Every second day, 3 μl of a solution containing PD98059 (25 μM, diluted in H_2O) were applied directly to the cells (*GDNF + PD98059*; *panels c* and *d*). Controls were treated with water (*GDNF*). After 6 days, the CAMs were paraffin-embedded and cut into 3–5 μm vertical sections. *Panels a* and *c* show photomicrographs of H&E-stained sections through the CAM in the seeding area. *Panels b* and *d* show H&E-stained sections through the CAM in the nitrocellulose area. PANC-1 cells are indicated by *arrows*. *Bars*, 100 μm.

tion of GDNF enhanced PANC-1 cell migration in a concentration-dependent manner, and GDNF was nearly as efficacious as LPA, a chemotaxin for pancreatic carcinoma cells (26). The chemotactic effect of GDNF was even more evident when the penetration of PANC-1 cells into and through a three-dimensional tissue was analyzed using the CAM model system adapted in this study to investigate chemotactic invasion. Thus, GDNF enhances the motility and the invasive potential of pancreatic carcinoma cells. In the peripheral nervous system, Schwann cells are a rich source of GDNF (39), and in human pancreatic cancer tissues, GDNF is localized to peripancreatic neural ganglia (9, 40). Thus, GDNF might function as an inductor of perineural invasion of pancreatic cancer cells.

Our previous observations that ligands of receptor tyrosine kinases and G protein-coupled receptors such as EGF and LPA, respectively, (25, 26) induce pancreatic carcinoma cell migration by activation of Ras-Raf-MEK-ERK signal transduction pathway prompted us to determine the signal transduction mechanisms involved in mediating the stimulatory effect of GDNF on migration and invasion. GDNF treatment resulted in transient activation of the monomeric GTPases N-Ras, Rac1, and RhoA and in activation of the mitogen-activated protein kinases ERK and JNK and the protein kinase Akt. Activation of the Ras-Raf-MEK-ERK pathway by the GDNF receptor is mediated by phosphorylation of Tyr1062 of the RET receptor tyrosine kinase, which is present in all three of the RET splice variants, RET9, RET43, and RET51 (41). Activation of N-Ras but not of K-Ras by GDNF resembles Ras isoform-specific activation by EGF and LPA in PANC-1 cells (25, 26). H-Ras is hardly expressed, and K-Ras is mutationally activated in PANC-1 cells (26). Thus, Ras-dependent signal transduction initiated by GDNF as well as other receptor ligands is mediated by wild-type N-Ras in these cells. In contrast to EGF and LPA (25, 26), GDNF induced a long-lasting Ras activation in PANC-1 cells, which emerged within 3 min after addition of the ligand and slowly declined within the next 30–60 min. Such long-lasting Ras activation was also observed in MG87 mouse fibroblasts transfected with wild-type RET51 and GFRα1 (41). How GDNF induces such a sustained, RET-dependent Ras activation is currently unknown.

GDNF-induced migration and invasion of PANC-1 cells is highly dependent on activation of the Ras-Raf-MEK-ERK pathway. This was demonstrated by inhibition of the Ras pathway by stable expression of dominant-negative EGFP-H-Ras(N17) or by application of the MEK1 inhibitor PD98059. In contrast to the complete abrogation of the stimulatory effect of GDNF on migration and invasion shown in this report, GDNF-stimulated migration of enteric neuronal crest cells was only partially dependent on activation of ERK (42), and activation of the Ras-ERK-pathway by GDNF in corneal epithelial cells was not associated with enhanced cell migration but with induction of gene transcription (43). Thus, the results obtained in this study establish a central role of the Ras-Raf-MEK-ERK pathway as a regulator of pancreatic carcinoma cell migration in response to GDNF. We have demonstrated recently that activation of ERK and translocation of the phosphorylated kinase to newly forming focal contact sites in the leading lamellae of PANC-1 cells migrating in response to LPA is crucial for the accurate organization of the actin cytoskeleton and the migratory response (26). If and how GDNF modifies focal contact

formation and the organization of the actin cytoskeleton in pancreatic carcinoma cells is currently not known, but the activation of Rac1 and RhoA by GDNF in PANC-1 cells point toward an impact of GDNF on actin filament reorganization. In neuroblastoma cells, both Rac1 and RhoA are involved in GDNF-induced actin reorganization and phosphorylation of focal adhesion kinase leading to lamellipodia formation and cell adhesion (44–46).

The second pathway discovered in this study to be crucially involved in mediating the stimulatory effects of GDNF on PANC-1 pancreatic carcinoma cell migration is the PI3k/Akt pathway. Pretreatment of PANC-1 cells with LY294002, a PI3k inhibitor, completely abolished the stimulatory effect of GDNF on cell motility. In other cells, activation of PI3k is mediated by GDNF-induced phosphorylation of Tyr1062 of RET and concomitant formation of a Shc-Gab1-Grb2 complex (47, 48). Moreover, the adapter protein Grb2 also binds to phosphorylated Tyr1096 in the long RET51 isoform (49), which results in activation of the PI3k/Akt signaling pathway (41). Thus, in PANC-1 cells expressing transcripts for RET9 and RET43 as well as the long RET51 isoforms, activation the PI3k/Akt signal transduction pathway could be mediated by phosphorylation of Tyr1062 and/or Tyr1096. Activation of PI3k in cultured primary neuronal cells and established neuronal cell lines primarily promotes cell survival and differentiation (41, 47, 50) but also supports migration of enteric nervous system progenitors during the development of the enteric nervous system in the gut (42). The importance of PI3k signaling for pancreatic carcinoma cell migration demonstrated in this study emphasizes the central role of this pathway for GDNF-induced, cell type-dependent cellular events. Moreover, the central role of the PI3k pathway in mediating pancreatic carcinoma cell migration and invasion is supported by a recent study performed by Okada *et al.* (40). According to their analysis, GDNF up-regulates the expression of matrix metalloproteinase 9 in a PI3k-dependent way and causes matrix metalloproteinase 9 activation in a Ras-Raf-MEK-ERK-dependent way in MiaPaCa-2 pancreatic carcinoma cells. Because matrix metalloproteinases degrade components of the extracellular matrix, this mechanism might support GDNF-induced invasion of pancreatic carcinoma cells.

In conclusion, we demonstrated that activation of both the Ras-Raf-MEK-ERK and the PI3k pathway is crucially involved in mediating the stimulatory effect of GDNF on cell migration and invasion of pancreatic carcinoma cells. The findings suggest that enhanced activity of these pathways might facilitate perineural invasion of pancreatic cancer.

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