Signaling through Ras Is Essential for ret Oncogene-induced Cell Differentiation in PC12 Cells*

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Specific germline mutations of the receptor tyrosine kinase, Ret, predispose to multiple endocrine neoplasia types 2A and 2B and familial medullary thyroid carcinoma. The mechanisms by which different Ret isoforms (Ret-2A and Ret-2B) cause distinct neoplastic diseases remain largely unknown. On the other hand, forced expression of these mutated versions of Ret induces the rat pheochromocytoma cell line, PC12, to differentiate. Here we used an inducible vector encoding a dominant-negative Ras (Ras p21N17) to investigate the contributions of the Ras pathway to the phenotype induced in PC12 cells by the expression of either Ret-2A or Ret-2B mutants. We show that the Ret-induced molecular and morphological changes are both mediated by Ras-dependent pathways. However, even though inhibition of Ras activity was sufficient to revert Ret-induced differentiation, the kinetics of morphological reversion of the Ret-2B was more rapid than the Ret-2A-transfected cells. Further, we show that in Ret-transfected cells the gua1-associated neurotrophic factor-induced tyrosine phosphorylation target, SNT, is chronically phosphorylated in tyrosine residues, and associates with the Sos substrate. These results indicate the activation of the Ras cascade as an essential pathway triggered by the chronic active Ret mutants in PC12 cells. Moreover, our data indicate SNT as a substrate for both Ret mutants, which might mediate the activation of this cascade.

Thus far, four ligands have been identified for the protein tyrosine kinase, Ret: the glial cell line-derived neurotrophic factor, neurturin, persenphil, and artemin. Ret association with either of these ligands is mediated by the presence of distinct glycosyl phosphatidylinositol-anchored proteins in the same molecular complex (1). In adult central and peripheral nervous systems, Ret is expressed in specific subsets of neuronal populations and participates in the neuronal reaction to axon injury (1–3).

Specific mutations of the ret gene are the causative genetic events for the inheritance of multiple endocrine neoplasia (MEN)1 type 2A and 2B syndromes and familial medullary thyroid carcinoma (4). MEN-2A and MEN-2B are distinct hereditary neoplastic syndromes both characterized by medullary thyroid carcinomas and pheochromocytomas. MEN-2A also features hyperplasia of parathyroid cells, whereas MEN-2B is a more severe disease, being associated with skeletal abnormalities, ganglioneuromas of the intestinal tract, and mucosal neuromas, and is also characterized by an earlier age of tumor onset (5). Mutations in cysteine residues of the extracellular domain are the most frequent causative genetic event of familial medullary thyroid carcinoma and MEN-2A syndromes (4). A single point mutation, which results in a Thr for Met substitution at codon 918 within the Ret catalytic domain, is responsible for the MEN-2B syndrome (4). Each of these mutations convert Ret into a dominant transforming protein (Ret-2A and Ret-2B oncogenes) and cause constitutive activation of its intrinsic tyrosine kinase activity (6–8).

In MEN-2 syndromes, the molecular mechanisms by which the mutated Ret alleles contribute to the development of neuroendocrine neoplasms remain largely unknown (9, 10). The inheritance of specific ret mutations causes distinct disease phenotypes, thus suggesting that some specific cell types undergo abnormal proliferation depending on the type of Ret activation (either via a MEN-2A or via a MEN-2B mutation) (4, 5). Indeed, Ret-2A and Ret-2B differ in their activation mechanisms. For Ret-2A, activation results from the formation of stable receptor homodimers linked by a disulfide bridge, whereas Ret-2B proteins do not constitutively dimerize and display altered substrate specificity (4, 8).

The effects of tyrosine kinase receptors are mediated by the concerted activation of several signaling pathways including those of phospholipase C-g, phosphatidylinositol 3-kinase, and the Ras/mitogen-activated protein (MAP) kinase (also known as ERK) (11). In the rat pheochromocytoma cell line, PC12,

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The abbreviations used are: MEN, multiple endocrine neoplasia; CAT, chloromphenicol acetyltransferase; FRS2, fibroblast growth factor receptor substrate 2; MAP kinase, mitogen-activated protein kinase; NGF, nerve growth factor; SNT, gua1-associated neurotrophic factor-induced tyrosine phosphorylation target; FGF, fibroblast growth factor; PAGE, polyacrylamide gel electrophoresis; ERK, extracellular signal-regulated kinase; PBS, phosphate-buffered saline.
activation of the Ras signaling cascade is a prerequisite for nerve growth factor (NGF)-induced cell differentiation (12, 13). Even though it has been shown that Ras is implicated in Ret-induced neuronal differentiation, little is known about the contribution of this pathway to the biological effects triggered by Ret mutants in neuroectodermal cells (14, 15). For instance, the expression of Ret active mutants and their causal function in neuroendocrine tumors, associated with MEN-2 syndromes, are difficult to reconcile with the dramatic differentiating effects observed when the same mutants are overexpressed either in PC12 or in human neuroblastoma cells (7, 14–16). Moreover, an understanding of how the different Ret mutations (either associated with MEN-2A or MEN-2B syndromes) lead to diverse tumor phenotypes is lacking (15, 17–20).

Ret codes for two alternative splice isoforms, Ret9 and Ret51, that differ in their carboxyl terminus for 9 and 51 amino acids, respectively (21). Consensus binding sites for Shc are present on both isoforms, whereas a potential site for the Src homology 2 domain of Grb2 is only present on the long isoform. Indeed, there is good evidence that either Retwt, or harboring a MEN-2 type mutation, may interact with Shc and Grb2; these interactions are likely to mediate the induction of Ras activity (18, 22–25). On the other hand, a number of receptor tyrosine kinases induce phosphorylation in tyrosine residues of multi-ple-docking site proteins as, for example, IRS-1, IRS-2, SNT, and FRS2 (FGF receptor substrate-2), which, in turn, recruit enzymes involved in specific signaling cascades (26–29). FRS2 is a recently identified docking protein, probably identical to the short isoform of the active Ret variants (Ret9C634Y and Ret9M918T, respectively) (30), as a model system. We took advantage of these cell lines to further study the biological consequences caused by the activation of the Ras signaling pathway in PC12 cells transfected either with pRet9C634Y or pRet9M918T.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—PC12 cells were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% heat-inactivated horse serum and 5% fetal calf serum. PC12/wt-C1.9, PC12/MEN2A-C1.3, and PC12/MEN2B-C1.7 (PC12 derivative cell lines, which expressed Retwt, Ret9C634Y, and Ret9M918T, respectively) were grown in RPMI 1640 (Life Technologies, Inc.) supplemented with 10% heat-inactivated horse serum, 5% fetal calf serum, and gpt selection medium as reported previously (30). For Ras p21N17 induction experiments, cells were treated with 6 h with 0.5 μM dexamethasone (Sigma), dissolved in 10 mM MeSO4. Untreated control cultures contained the same concentration of MeSO4 (vehicle). For NGF treatment, 100 ng/ml were added to the culture medium. Mouse 2.5 S NGF was purchased from Upstate Biotechnology. For morphological assays, 4 × 10^4 cells were plated in a 60-mm diameter tissue culture dish, and dexamethasone (0.5 μM) or 2-mercaptoethanol (0, 125, 250, and 500 μM and 1 mM) was added, twice per week, for the indicated time.

**Transfection Experiments**—The PMMrasDN plasmid is an inducible expression vector which encodes the dominant negative mutant of Ha-Ras, Ras p21N17 (kindly provided by S. Halegoua) (13). The pZipN17 contains the gene for Ras p21N17 inserted in pZipNeoSVK (31) (kindly provided by M. Karin). The pKrox-24-CAT (C4) contains sequences from −1150 to +200 bp from the permissive Krox-24 promoter transcrip-tional start site fused to the chloramphenicol acetyltransferase (CAT) gene (kindly provided by Moses V. Chao, Cornell University, New York, NY). The psgf-CAT contains the ubiquitin promoter, the 5'-noncoding and the first methionine of the CAT gene (32). The cDNA of Ha-Ras9α was inserted in the BamHI site of the pBabe Puro vector plasmid. Expression vectors for Ret9C634Y and Ret9M918T (pRet9C634Y and pRet9M918T, respectively) were described previously (8, 30). PC12/MEN2A-C1.3 and PC12/MEN2B-C1.7 were transfected with the PMMrasDN plasmid together with pWLneo using the Lipofectin reagent (Life Technologies, Inc.) as reported previously (30). The transfected cells were selected in presence of 4 mg/ml G418 (Life Technologies, Inc.) or dexamethasone (400 μg/ml) (Life Technologies, Inc.) and with increasing amounts of pZipN17. PC12/MEN2A-C1.9, R1, and PC12/MEN2B-C1.7–R2 cells, were transfected with 2 μg of psgf-CAT together with 8 μg of pBabe Ha-Ras either in presence or not of dexamethasone. In all experiments, total transfected DNA was kept constant by adding the empty vector. Transfection efficiency was checked for each experimental point by cotransfection with the pSV-Luc reporter plasmid, and measuring the luciferase activity.

**CAT Assays**—Cell extracts were prepared 72 h after transfection, and CAT activity was analyzed by thin layer chromatography with 95% chloroform, 5% methanol, as described previously (14). After running, the individual sections from thin layer chromatography plate, corresponding to acetylated and nonacylated chloramphenicol, were cut from the thin layer chromatography plate and counted in a scintillation counter to obtain the CAT activity for each independent experiment. The conversion of acetylated [14C]chloramphenicol was calculated and normalized for the transfection efficiency. Values from three independent experiments, each made in duplicate, were used to calculate standard deviation and plotted on an arbitrary scale as relative promoter induction.

**Northern Blot Analysis**—Total RNA was extracted with the RNeasy midi kit (Qiagen). 20 μg of total RNA were size-fractionated on a denaturing formaldehyde agarose gel and blotted onto nylon membranes (Hybond-N, Amersham Pharmacia Biotech). The Ha-ras probe was used. Hybridization and washing were carried out under stringent conditions: 0.1× SSC, 0.1% SDS, 60°C. Autoradiography was performed using X-AR films (Eastman Kodak Co.) at −80°C for 4–7 days with intensifying screens.

**Immunofluorescence**—Exponentially growing parental PC12 cells were transfected either with pRet9C634Y or pRet9M918T.

**Immunofluorescence**—Cells were seeded at low confluence on glass coverslips coated with poly-l-lysine (15 μg/ml) (Sigma). PC12 and PC12/MEN2 cells were either treated with 2-mercaptoethanol for 11 days or left untreated. PC12/MEN2 cells were treated with MeSO4 (vehicle) or NGF (400 μg/ml) for 11 days. Cells were fixed for 20 min in PBS containing 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 in PBS. After two washes with PBS, cells were stained with Hoechst-33258 (Sigma) for 5 min. After two washes with PBS, coverslips were mounted and analyzed with a Zeiss Axioshot epifluorescence microscope.

For phalloidin experiments, PC12 and PC12/MEN2 cells were
treated with 2-mercaptoethanol for 8 days or left untreated. PC12/
MEN2B
transfected cells were treated with 5 mM HEPES, pH 7.5, 1% (v/v) Triton X-100, 1% glycerol, 150 mM NaCl, 5 mM EGTA, 1.5 mM MgCl₂, 25 mM NaF, 20 mM sodium pyrophosphate, 10 mM sodium orthovanadate, 4 mM phenylmethylsulfonyl fluoride, 40 μg/mL aprotinin, and clarified by centrifugation at 10,000 x g for 15 min. Protein concentrations were estimated by a modifiedBradford assay (Bio-Rad). Equal amounts of proteins (2 μg) were incubated with rabbit anti-Ret polyclonal antibody, as indicated, for 16 h at 4 °C and subsequently incubated with protein A-Sepharose CL-4B (Amersham Pharmacia Biotech) for 1 h at 4 °C. Immunoprecipitates were subjected to SDS-PAGE (7.5% polyacrylamide) under reducing conditions and transferred to polyvinylidene difluoride (Millipore Co., Bedford, MA). Immunoblotting was carried out using either anti-Ret antibodies or anti-phosphotyrosine monoclonal antibodies (4G10, Upstate Biotechnology). Peroxidase-conjugated secondary antibodies were detected with the Anerambah ECL system. For co-immunoprecipitation experiments with anti-FRS-2 antibodies, cells were lysed in a buffer containing 50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 40 μg/mL aprotinin, 2 μg/mL pepstatin, 2 μg/mL leupeptin, 1 mM sodium orthovanadate. 2 mg of proteins were preclreated with rabbit IgG and protein G-plus agarose (Calbiochem), then centrifuged for 5 min at 2500 rpm; the supernatants were boiled in Laemmli buffer for 5 min before electrophoresis. Captured proteins were incubated with protein G-plus agarose (Calbiochem) for 1 h at 4 °C. Immunoprecipitates were subjected to SDS-PAGE (10% acrylamide) and blotted with anti-Ret (C-19, Santa Cruz) and anti-FRS-2 (H-91, Santa Cruz) antibodies.

RESULTS
Expression of Ras p21N17 in PC12/MEN2A and PC12/
MEN2B Cells—We have previously described the establishment of stable PC12 variant cell lines, PC12/MEN2A and PC12/MEN2B cell lines, which express the Ret9 active mutant, Ret-2A (Cys-634 → Tyr) and Ret-2B (Met-918 → Thr), respectively (30). We decided to utilize two cell clones, PC12/
MEN2A-c.3 and PC12/MEN2B-c.7 (hereafter named PC12/
MEN2A and PC12/MEN2B), which express comparable amounts of the Ret9 active RET-Ret-2A and RET-Ret-2B, respectively (see Fig. 2 of Ref. 30). Thus, we transfected both these cell lines with an inducible expression vector, coding for a dominant-negative mutant of Ras (Ras p21N17). Transfected cells (PC12/MEN2A-c.3 and PC12/MEN2B-c.l.7) were selected for resistance to G418, and individual clones were subsequently isolated and analyzed. Dexamethasone-inducible ras transcripts were detected both in PC12/MEN2A-c.3 and in PC12/MEN2B-c.l.7 cell clones, and the induction of ras transcripts was determined at 6 h of dexamethasone treatment (Fig. 1 and data not shown). For further analysis, we used two representative PC12/MEN2A-c.3 and PC12/MEN2B-c.l.7 cell clones. The magnitude of the Ras p21N17 mRNA induction largely varied among the clones analyzed. In the -R3 and -R6, the induction was between 8 and 10 times lower than in the -R1 and -R2 (Fig. 1). All PC12/MEN2A-c.3 and PC12/MEN2B-c.l.7 clones were morphologically indistinguishable from the parental cells (compare Fig. 3 to Fig. 4 and data not shown).

Expression of Ras p21N17 Reverts Ret-2A- and Ret-2B-
Induced Neuronal Differentiation in PC12 Cells—NGF Induction of PC12 cell differentiation involves the expression of a complex pattern of genes, including immediate-early (fos, Krox-24) and late response genes (vgf, SCG10, peripherin) (33). We have previously demonstrated that Ret-2A and Ret-2B are able to induce a differentiative expression program in PC12 cells (30). However, in these cells, differentiation is not terminal. Indeed, expression of the vgf and SCG10 genes is not associated to block of proliferation. To determine the possible involvement of Ras in the Ret-2A- and Ret-2B-induced neuronal differentiation, we analyzed the expression of Krox-24 (also known as NGF1-A, zif/268, Egr1, PC1, TIS8, d2) and vgf genes in PC12/MEN2A-c.3 and PC12/MEN2B-c.l.7 clones after stimulation with dexamethasone. Fig. 2A reports the results from representative clones (PC12/MEN2A-c.3 and PC12/MEN2B-c.l.7) compared with parental PC12/MEN2A and PC12/MEN2B. In Ras p21N17-transfected cells, the vgf but not the Krox-24 transcripts were present at lower levels than parental cells (Fig. 2A, compare lane 5 to lane 3 and lane 9 to lane 7), and similar results were obtained by the PC12/MEN2A-c.3 and the PC12/MEN2B-c.l.7 cell clones. (data not shown). We do not know whether the low basal levels of vgf transcripts, in Ras p21N17-transfected cells, reflect a clonal difference in the cell lines or the presence of low, non-induced levels of dominant-negative Ras (see below). On the other hand, in PC12/MEN2A-c.3 and PC12/MEN2B-c.l.7 cell clones, stimulation with dexamethasone resulted in inhibition of both Krox-24 and vgf genes expression (Fig. 2A, lanes 6 and 10). Consistently, the activity of

Fig. 1. Ras expression induced by dexamethasone in PC12/
MEN2A-c.3 and PC12/MEN2B-c.l.7 cell lines. Northern blot analysis of total cellular RNA (20 μg/lane) obtained from PC12 and PC12 variant cells as indicated: parental PC12, PC12/MEN2A-c.3 (MEN2A), PC12/MEN2A-c.l.7 (MEN2A-c.3), PC12/MEN2B-c.7 (MEN2B), and PC12/MEN2B-c.l.7 (MEN2B-c.l.7). Cells were treated for 6 h either with vehicle (−) or with dexamethasone (0.5 μM) (+), as indicated (DEX). Filters were hybridized with a ras probe and equal gel loading was confirmed by the hybridization of the same filter with a ribosomal 18 S probe. The relative amounts of Ret protein in all cell lines were determined in Western blot with anti-Ret antibodies and normalized with anti-tubulin antibodies. The amount of Ret in PC12/MEN2A-c.3 was arbitrarily taken as 1. The resulting relative amounts were: PC12/MEN2A-c.3 = 1; PC12/MEN2A-c.l.7 = 1.3; PC12/MEN2B-c.7 = 1.1; PC12/MEN2B-c.l.7 = 2; PC12 parental = undetectable; PC12/MEN2A-pool = 0.5.
FIG. 2. Ras is required for expression of Krox-24 and vgf genes and for ERK kinase activity. A, Northern blot analysis of total cellular RNA (20 μg/lane) obtained from PC12 and PC12 variant cells as indicated: parental PC12, either untreated or stimulated for 5 h with NGF (100 ng/ml) as indicated; PC12/MEN2A-cl.3 (MEN2A); PC12/MEN2A N17-cl.1 (MEN2A N17); PC12/MEN2B-cl.7 (MEN2B); PC12/MEN2B N17-cl.2 (MEN2B N17) were treated for 6 h either with vehicle (−) or with dexamethasone (0.5 μM) (+), as indicated (DEX). The same filters were hybridized with either a Krox-24-specific or vgf-specific probe as indicated, and equal gel loading was confirmed with a ribosomal 18 S probe. B, the cells and the treatment were the same as in A (except for NGF stimulation that lasted for 10 min). The cell lysates were immunoprecipitated with anti-ERK-1 antibody and assayed for kinase activity as described under “Experimental Procedures.” In each experiment we verified that the same amount of ERK was used in the kinase assay. Proteins from each of the immunoprecipitates were separated on 11% SDS-PAGE and immunblotted with the same anti-ERK-1 antibody. Immunoblots confirmed that the same amount of ERK was used in each kinase assay (data not shown). The results shown were typical and representative of two independent experiments. C, PC12 cells were transfected with pKrox-24-CAT (2 μg) together with pRet9 C634Y (0.6 μg) or pRet9 M918T (0.6 μg). Where indicated, cells were also cotransfected with increasing amount of the pZipN17 expressing the Ras p21N17 mutant. The inhibition, calculated as the difference between the CAT activity in the absence and in the presence of pZipN17, is plotted as percentage relative to the induction observed in the absence of pZipN17. Results were from a representative experiment, and they were confirmed in two independent experiments each performed in duplicate. D, PC12 variant cells were transfected with pvgf-CAT (2 μg) together with either the pBabe Ha-Ras or the pBabe vector (8 μg), as indicated. Where indicated, dexamethasone was added 5 h after transfection. Results are expressed as percentage of conversion to acetylated [14C]chloramphenicol.
the downstream Ras effector, ERK-1, was depressed to basal levels (Fig. 2B, compare lane 1 to lane 6 and lane 7 to lane 12).

To further confirm these expression data, we performed CAT-based assays in the parental PC12 cells. We have previously shown that activated forms of Ret induce the transcription of a reporter plasmid containing the CAT gene fused to a fragment of the Krox-24 promoter (Krox-24-CAT) (14, 30). Thus, to evaluate the ability of a dominant inhibitory mutant of Ha-ras (Ras p21N17) to inhibit the expression of Krox-24-CAT, we transfected the PC12 cells with an expression vector for each active Ret mutants (pRet9C634Y or pRet9M918T), either alone or together with increasing amounts of an expression vector containing the Ras p21N17 (pZipN17). As shown in Fig. 2C, Ras p21N17 expression inhibited the transcription driven by the Krox-24 promoter to similar extents, when induced by the Ret-2A or by the Ret-2B mutants (reaching more than 80% of inhibition with 4 μg of pZipN17).

Further, to confirm that, in PC12/MEN2AN17 and PC12/MEN2BN17 cell clones, the blocked vgf expression was the consequence of the inhibited Ret activity, we performed transient transfection using a vgf-CAT construct as reporter gene. Expression of vgf-CAT was highly depressed upon stimulation with dexamethasone, and rescued by forced expression of the Ha-raswt (Fig. 2D).

Ret and Ras Activities Are Required for Maintenance of the Flat-adherent Cell Phenotype—We next studied whether the signal triggered by the Ret mutants and conveyed through Ras was necessary to determine the flat, substrate-adherent phenotype that is characteristic of both the PC12/MEN2A and PC12/MEN2B cells. We first addressed the question of whether the flat morphology depended on the continuous Ret activity. To accomplish this we took advantage of the fact that the C634Y mutation causes stable disulfide-linked homodimers, which activate the tyrosine kinase activity. Thus, in agreement with recent reports, treatment of PC12/MEN2A cells with a reducing agent should inhibit RεC634Y dimer formation and abrogate their activity (34). On the other hand, since the M918T mutation causes the Ret tyrosine kinase activation by a distinct mechanism that does not involve the formation of stable dimers, the PC12/MEN2B cells should be insensitive to reducing agents, and thus can be used as a negative control. We treated both the PC12/MEN2A and PC12/MEN2B cells with increasing amounts of 2-mercaptoethanol (0, 125, 250, and 500 μM and 1 mM). Mercaptoethanol had no toxic effect on Ret-transfected PC12 cells up to 500 μM (data not shown). As shown in Fig. 3, continuous treatment with 2-mercaptoethanol, at 250 (data not shown) and 500 μM, caused a dramatic change in morphology, clearly visible at 6 days (data not shown), and up to 11 days of treatment (Fig. 3A) and converting a large fraction of PC12/MEN2A, but not of PC12/MEN2B, cells into a small and round-shaped phenotype with little substrate adherence. These cells behave morphologically similar to parental PC12 and formed aggregates overgrowing on a layer of flat cells, morphologically similar to the untreated PC12/MEN2A controls. Nuclear integrity of cells treated with 2-mercaptoethanol was assessed by staining with Hoechst dye (Fig. 3B). Moreover, to further confirm morphological reversion, cells from PC12, PC12/MEN2A aggregates, or PC12/MEN2B were picked up, dissociated, plated again on sterile microscope coverglass for 24 h, and stained with phalloidin. As shown in Fig. 3C, the PC12/MEN2A aggregates were constituted of small round-shaped cells, similar to the control parental PC12 cells. On the other hand, when replated, cells from PC12/MEN2B behave as flat-adherent and similar to the untreated controls (Fig. 3C and data not shown). Morphological reversion of the PC12/MEN2A was associated with a clear reduction in phosphorytrosine content of the mature 170-kDa Ret product, but not of the 150-kDa isoform, which is not exposed on the cell membrane (Fig. 3D, upper panel, lanes 1 and 2). In the PC12/MEN2B cells, no appreciable reduction in phosphorytrosine content of Ret was observed (Fig. 3D, upper panel, lanes 3 and 4), in agreement with the absence of morphological reversion (Fig. 3, A–C, right panels). Moreover, in PC12/MEN2A cells, Ret tyrosine phosphorylation was not completely abrogated, in good agreement with the observation that morphological reversion was only partial even upon 11 days of treatment.

To determine whether the morphology, characteristic of the Ret-transfected cells, was the consequence of the chronic stimulation of Ras activity by Ret mutants, we used the PC12/MEN2AN17 and PC12/MEN2BN17 cell clones. A phenotypic reversion to small rounded cells was evident at 5 days in PC12/MEN2BN17-R2 (Fig. 4A, middle panel), and at 8 days of treatment with dexamethasone in PC12/MEN2AN17-R1 (data not shown), and increased in both cell lines up to 11 days (Fig. 4A, lower panel). Similar results were obtained using the PC12/MEN2AN17-R3 and the PC12/MEN2BN17-R6 cells, in which Ras p21N17 transcripts were inducible to lower extents. In these cell lines reversion was observed at 9 days in the -R6, and at 12 days in the -R3 cells (data not shown). As a control, we treated the parental cells (PC12/MEN2A and PC12/MEN2B) with the same amounts of dexamethasone, and no changes in cell shape were observed for up to 11 days (data not shown). Morphological reversion was thus confirmed as in Fig. 3C by replating cells from either PC12/MEN2AN17 or PC12/MEN2BN17 aggregates followed by staining with phalloidin (Fig. 4B). The actin cytoskeleton was similar to that of the parental PC12 cells, and neurites were generally no more visible (compare Fig. 4B to Fig. 3C). The results are consistent with previous reports, which indicate that expression of Ras p21N17 inhibits differentiation of PC12 cells but not proliferation (35, 36).

Ret Mutants Induce FRS2/SNT Tyrosine Phosphorylation and Association with Sos—Although the biochemical functions for SNT still remain to be determined, compelling evidence suggests that SNT, and SNT-like proteins, provide docking sites for signaling molecules involved in Ras activation (28, 29).

Thus, we tried to determine whether the chronic stimulation of Ret tyrosine kinase was capable of triggering the persistent tyrosine phosphorylation of SNT. As already reported, SNT was not phosphorylated in tyrosine residues in parental PC12 cells, and become phosphorylated upon NGF stimulation (Fig. 5A, upper panel). In contrast, both in the PC12/MEN2A and PC12/MEN2B cells, SNT was constitutively phosphorylated at high levels, comparable to those reached in the NGF-stimulated parental PC12 cells (Fig. 5A, upper panel, lanes 3 and 7). Consistent with a previous report (27), Ret-induced SNT-tyrosine phosphorylation was not affected when Ras activity was inhibited (Fig. 5A, upper panel, lanes 6 and 10).

Upon NGF stimulation the Suc1-SNT complex is found associated to a number of proteins, some of which are phosphorylated on tyrosine residues, including the NGF receptor, TrkA, and Sos1 (27, 28). Consistent with this, we observed a pattern of tyrosine phosphorylated products, in both Ret-transfected cell lines (Fig. 5A, upper panel). In the PC12/MEN2AN2A and PC12/MEN2BN2B cells, the Suc1-SNT complex seems to be associated to the same phosphorylated products as in the NGF-stimulated PC12 parental cells. We next examined the association of Sos to the Suc1-SNT complex. Fig. 5A (lower panel) shows an immunoblot of suc1-captured proteins with antibodies directed against Sos1. A band corresponding to a protein

2 M. Billaud, personal communication.
doublet of 180 kDa was clearly detected in p13 suc1-agarose precipitates from PC12 cells stimulated with NGF, but only barely in precipitated from untreated cells. Sos1 was also easily detected in suc1-captured proteins from PC12/MEN2A and PC12/MEN2B cells; this association was upstream and independent of Ras activity. According to previous findings, Sos association with the Suc1-SNT complex is ligand-dependent in parental PC12 cells and correlates with tyrosine phosphorylation of SNT (28, 29). SNT has recently been shown to be highly related, or identical, to the FGF receptor signaling molecule, FRS2 (29). Thus, because of the absence of SNT-specific antibodies, we used anti-FRS2 antibodies to demonstrate that endogenous FRS2/SNT co-immunoprecipitate with the Ret active oncoproteins in PC12/MEN2A and PC12/MEN2B (Fig. 5B).

DISCUSSION

Here we have demonstrated that, in PC12 cells, chronic Ret-stimulation of Ras is required to maintain differentiation. In fact, both expression of neuronal specific genes and cell morphology were dependent on Ras activity. Moreover, we propose SNT tyrosine phosphorylation as an additional Ras-independent component of the transducing machinery triggered by Ret.

Nerve growth factor-induced differentiation in PC12 cells requires signaling by Ras and MAP kinase. Indeed, persistent stimulation of this signaling cascade has been implicated in PC12 cell terminal differentiation, and the forced expression of an interfering Ras mutant blocks the NGF from inducing neurite outgrowth and expression of neuron-specific genes (12, 13,
The expression of Ret active variants, Ret9 or the Ret51 isoforms, induces the PC12 cells to differentiate, and, in most cases, differentiation is terminal being associated with growth arrest (7, 14, 15, 30). Consistently, recent reports indicate that Ret signaling requires the stimulation of the Ras cascade (14, 15). On the other hand, activated Ret causes medullary thyroid carcinomas, and frequently pheochromocytomas in MEN-2 syndromes (5). As possible explanation for this apparent discrepancy (terminal differentiation in PC12 cells versus proliferation and terminal differentiation (15). Accordingly, expression in PC12 cells of the active Ret mutants at low levels results in isolation of stable cell lines, which reproduce in vitro some of the biological events caused by Ret in human tumors, including unlimited growth (15, 30, 41).

These PC12 cell lines, which express the Ret9 isoform of active Ret mutants (PC12/MEN2A and PC12/MEN2B cell lines), are partially differentiated. Indeed, important molecular markers characteristic of the PC12 neuronal differentiation are expressed, but differentiation is not terminal and proliferation is not blocked (30). Because of the strong implication of Ras in determining terminal differentiation of PC12 cells, the question arises as to whether or not Ras activity is still required for maintenance of the differentiated phenotype in these cells that actively proliferate.

Our results show that expression of an inducible Ras p21N17 mutant in PC12/MEN2A and PC12/MEN2B cells is sufficient to abrogate the expression of genes belonging to the early and late neuronal response (Krox-24 and vgf, respectively). Moreover, even though basal, non-induced levels of Ras p21N17 were hardly detectable, they were sufficient enough to severely im...

FIG. 4. Reversion of PC12/MEN2N17 cells morphology induced by the expression of Ras p21N17. A, phase contrast microphotograph of PC12/MEN2N17-R1 and MEN2BN17-R2 cells. PC12/MEN2N17-R1 and PC12/MEN2BN17-R2 cells were grown up to 11 days either in the absence (vehicle) or in presence of dexamethasone (0.5 μM) as indicated. The microphotographs are representative of three independent experiments. B, cells were grown as in A, picked up, trypsinized, and plated on coverslips. 24 h later, cells were fixed and stained with phalloidin. PC12/MEN2N17-1 (MEN2A1) and PC12/MEN2BN17-1 (MEN2B1) cells were either from random fields (vehicle) or aggregates (dexamethasone). The figure is representative of at least three fields for each cell line, and confirmed in three independent experiments.
pair the vgf gene expression, but not to revert the flat cell shape. This indicates that, despite the chronic activity of Ret, the extent of Ras stimulation is low enough to allow the basal Ras p21^N17 to partially interfere with its downstream signaling. Stronger induction of Ras p21^N17 was needed to revert from flat to PC12-like small-rounded cell morphology and to completely repress vgf and Krox-24 gene expression. Thus, in these cells, the Ras signaling cascade seems to be stimulated at intermediate levels, which are enough to induce neuronal gene expression, but still compatible with cell proliferation (41). Even though it seems likely that Ret, and Ret mutants, trigger the activation of various independent pathways (39, 40) that may contribute to the choice between cell proliferation (as observed in tumors) and terminal differentiation (as frequently observed in vitro), these results indicate that signaling through Ras is strictly required for Ret-induced differentiation.

In contrast to the more frequent MEN-2A-like mutations, located in the extracellular domain, the MEN-2B mutation does not provoke constitutive dimerization of the Ret protein (6, 8). Indeed, despite the fact that compelling experimental evidence which indicates that induction of SNT tyrosine phosphorylation is necessary to mediate Ret signaling is presently unknown and will be the focus of further research.

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