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A potential pathogenetic mechanism for multiple endocrine neoplasia type 2 syndromes involves *ret*-induced impairment of terminal differentiation of neuroepithelial cells

(neuroendocrine/nerve growth factor/signal transduction/PC12/TRK)

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ABSTRACT **Germ-line missense mutations of the receptor-like tyrosine kinase *ret* are the causative genetic event of the multiple endocrine neoplasia (MEN) type 2A and type 2B syndromes and of the familial medullary thyroid carcinoma. We have used the rat pheochromocytoma cell line, PC12, as a model system to investigate the mechanism or mechanisms by which expression of activated *ret* alleles contributes to the neoplastic phenotype in neuroendocrine cells. Here we show that stable expression of *ret* mutants (MEN2A and MEN2B alleles) in PC12 cells causes a dramatic conversion from a round to a flat morphology, accompanied by the induction of genes belonging to the early as well as the delayed response to nerve growth factor. However, in the transfected PC12 cells, the continuous expression of neuronal specific genes is not associated with the suppression of cell proliferation. Furthermore, expression of *ret* mutants renders PC12 cells unresponsive to nerve growth factor-induced inhibition of proliferation. These results suggest that induction of an aberrant pattern of differentiation, accompanied by unresponsiveness to growth-inhibitory physiological signals, may be part of the mechanism of action of activated *ret* alleles in the pathogenesis of neuroendocrine tumors associated with MEN2 syndromes.**

Multiple endocrine neoplasia (MEN) types 2A and 2B are hereditary neoplastic syndromes characterized by the presence of medullary thyroid carcinomas and pheochromocytomas. MEN2B is also associated with skeletal abnormalities, ganglioneuromas of the intestinal tract, and mucosal neuromas. Familial medullary thyroid carcinoma is a related cancer disorder characterized by medullary thyroid carcinoma in the absence of pheochromocytoma (1, 2). Mutations in one of five cysteine residues in the extracellular domain of the *ret* gene, which encodes a ligand-orphan receptor-like tyrosine kinase (3), are the genetic cause of familial medullary thyroid carcinoma and MEN2A syndromes (4, 5). A single point mutation within the *ret* catalytic domain, which results in a Thr for Met substitution at codon 918, is responsible for the MEN2B syndrome (6, 7). These mutations convert *ret* into a dominant transforming gene (retMEN2A and retMEN2B alleles) by causing constitutive activation of the intrinsic tyrosine kinase activity of its product (8, 9). However, retMEN2A and retMEN2B differ in their mechanism of activation. In the case of retMEN2A, activation likely results from constitutive receptor dimerization, whereas retMEN2B proteins do not dimerize, but display altered substrate specificity (9, 10).

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The biological activity of retMEN2 alleles has so far been studied in NIH 3T3 cells, a system prone to "one-hit" transformation. The molecular mechanisms by which *ret* alleles contribute to the development of neuroendocrine cancer syndromes remain to be elucidated. In MEN2 syndromes, the first step toward neoplastic progression is a generalized hyperplasia of the entire population of thyroid C-cells and of the adrenal medulla chromaffin cells (11). Hyperplasia is then followed by the development of a multifocal malignancy. The inheritance of mutated *ret* alleles seems to implicate them in the pathogenesis of the first "hyperplastic" step. To gain insight into this issue, we studied the effects of retMEN2A and retMEN2B expression in the rat pheochromocytoma cell line PC12 (12). This cell line, albeit of tumor origin, retains many characteristics of normal adrenal medulla chromaffin cells, including the ability to undergo terminal differentiation upon nerve growth factor (NGF) treatment (12).

Expression of retMEN2A and retMEN2B in PC12 cells resulted in a morphological conversion to a flat phenotype and caused a neuronal pattern of gene expression. An altered control of cell proliferation was, in addition, observed in that transfected PC12 cells continued to proliferate and were insensitive to growth arrest induced by NGF.

MATERIALS AND METHODS

Cell Culture and Transfection Experiments. PC12 cells were grown in RPMI 1640 medium (GIBCO), supplemented with 10% horse serum and 5% fetal calf serum (12). Cells transfected with the retMEN2A, retMEN2B, wild-type *ret* (ret-wt), or LTR-3 plasmids were selected for their ability to grow in the presence of mycophenolic acid. The transfected cells were selected in *gpt* selection medium for 3 weeks, and individual cell colonies were isolated and expanded. Where indicated, 2.5S NGF (100 ng/ml; Upstate Biotechnology, Lake Placid, NY) was added to the culture medium.

For transient transfection assays, cells were plated at 3×10^5 cells in a 60-mm diameter tissue culture dish 24–36 hr before transfection. The PC12 cells were transfected using the Lipofectin reagent (GIBCO/BRL), as reported (13). Transient transfections were carried out with 2 μg of reporter plasmid together with 0.5 μg of activated *ret* mutants.

Immunoprecipitation and Immunoblotting. Between 10^6 and 10^7 cells were washed twice in ice-cold TBS (20 mM Tris·HCl, pH

Abbreviations: NGF, nerve growth factor; MEN, multiple endocrine neoplasia; ret-wt, wild-type *ret*; CAT, chloramphenicol acetyltransferase.

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8.0/150 mM NaCl) and lysed in a buffer containing 50 mM *N*-2-hydroxyethylpiperazine-*N'*2-ethane-sulfonic acid (Hepes, pH 7.5), 1% (vol/vol) Triton X-100, 50 mM NaCl, 5 mM EGTA, 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium ortho vanadate, 2 mM phenylmethylsulfonyl fluoride, 0.2 μg each of aprotinin and leupeptin per ml, and 4 mM diisopropylfluorophosphate, as reported (14). Protein lysates were clarified by centrifugation at 10,000 × *g* for 15 min, and the supernatant was processed for immunoblotting or for immunoprecipitation as reported (14). Protein concentrations were estimated by a modified Bradford assay (Bio-Rad). Equal amounts of protein were incubated with antibody, as indicated [rabbit anti-*ret* or Trk C-14 and Trk 763 (Santa Cruz Biotechnology)], for 2 hr at 4°C and subsequently incubated with protein A-Sepharose CL4-B (Pharmacia) for 2 hr at 4°C. Immunoprecipitates were washed three times with the above lysis buffer, rinsed once with double-distilled H_2O, and boiled in Laemmli buffer for 5 min before electrophoresis. Immunoprecipitates were subjected to SDS/7.5% PAGE under reducing conditions and were analyzed by immunoblotting with anti-*ret* polyclonal antibodies, anti-phosphotyrosine monoclonal antibodies (G410; Upstate Biotechnology), or anti-Trk antibodies [Trk C-14 and 763 (Santa Cruz Biotechnology)]. The immunoblot was subsequently stained with the horseradish peroxidase-conjugated secondary antibody as appropriate and with the Amersham enhanced chemiluminescence (ECL) system. The polyclonal antibody (anti-*ret*) was generated against a fusion protein in which the tyrosine kinase domain of human *ret* is fused to the bacterial glutathione *S*-transferase (14).

Chloramphenicol Acetyl Transferase (CAT) Assay. Cell extracts were prepared 72 hr after transfection and CAT activity was determined as described (13, 15). Briefly, CAT activity was analyzed by thin-layer chromatography (TLC) with 95% chloroform/5% methanol. After running, the individual sections from the TLC plate, corresponding to acetylated and nonacetylated chloramphenicol, were counted in a scintillation counter. The conversion of acetylated versus non-acetylated form of chloramphenicol ^{14}C was calculated as the average of at least three experiments, each being made in duplicate.

Expression Plasmids. pNGFI-A-CAT (C4) contains sequences from −1150 to +200 bp relative to the *NGFI-A* promoter transcriptional start site (16) fused to the CAT gene (kindly provided by Moses V. Chao, Cornell University, Ithaca, NY). The pvgf-CAT contains the *vgf* promoter, the 5′ noncoding region, and the first methionine (from −803 to +710) fused inframe with the initiating methionine of the CAT gene (17). The ret-wt, retMEN2A, and retMEN2B mutants were inserted in the LTR-3 vector that contained the resistance marker *Escherichia coli gpt* (9).

Northern Blot Analysis. Total RNA was extracted by a modification of the guanidine thiocyanate method (18). Total RNA (20 μg) was size-fractionated on a denaturing formaldehyde agarose gel and blotted onto nylon filters (Hybond-N; Amersham). To obtain *NGFI-A* and *SCG10* probes, 60-mer oligonucleotides were synthesized according to the published sequence and subsequently ^{32}P-labeled by using the Klenow fragment of the *E. coli* DNA polymerase and a 3′-terminal specific 9-mer. The *vgf* probe used was excised from the pV2–2 plasmid (17). ^{32}P-Labeling of the probes was performed with the random oligonucleotide primer kit (Amersham). Hybridization and washing were carried out under stringent conditions: 1× SSC, 0.1% SDS, and 60°C. Autoradiography was performed using Kodak X-AR films at −70°C for 1–7 days with intensifying screens.

RESULTS

Expression of retMEN2A and retMEN2B Alleles in PC12 Cells. PC12 cells were transfected with expression vectors for retMEN2A (Cys-634 → Tyr), retMEN2B (Met-918 → Thr), or ret-wt, or with the vector alone (LTR-3). Transfected cells were selected for resistance to mycophenolic acid, and individual clones and mass populations, were subsequently isolated and analyzed. All the vector-transfected clones (30 of 30; data not shown) and ret-wt (45 of 45) were morphologically indistinguishable from the parental cells, displaying a small rounded morphology. In contrast, the majority of the PC12/MEN2A (25 of 30) and PC12/MEN2B (31 of 35) clones exhibited a flat phenotype with elongated neuritic processes (Fig. 1).

ret expression was analyzed in clones transfected with each construct. *ret* products were detected as 145-kDa and 160-kDa proteins in all of the retMEN2A and retMEN2B clones exhibiting the flat morphology (an example is shown in Fig. 2*A*). The *ret* products were then analyzed by immunoblot with anti-phosphotyrosine monoclonal antibodies. A predominant phosphoprotein of 160 kDa and a minor component of 145 kDa were detected (Fig. 2*B*), thus indicating that, consistently to what has been observed in other model systems (8, 9), these mutated *ret* alleles are constitutively active in PC12 cells. *ret* products were detected also in ret-wt-transfected PC12 clones, and, as expected, their phosphorylation was undetectable in these cells (data not shown). Finally, the retMEN2A- (5 of 30) and retMEN2B-transfected (4 of 35) clones, which did not exhibit the flat phenotype, were negative for *ret* products (data not shown).

retMEN2A and retMEN2B Induce the Expression of a Neuronal Phenotype in PC12 Cells. To determine whether the flat morphology induced by the *ret* mutants in PC12 cells was associated with the expression of a neuronal phenotype, we examined the expression of *NGFI-A* (also known as *zif/268*, *Egr1*, *Krox24*, *PC1*, *Tis28*, and *d2*), *SCG10*, and *vgf* genes in retMEN2A- and retMEN2B-transfected clones. *NGFI-A* is a ubiquitously expressed gene, rapidly induced by mitogenic signals in several cell types and by NGF in PC12 cells (19). *vgf* and *SCG10* are neuron-specific genes; enhanced expression of these genes in PC12 cells is associated with NGF-induced neuronal differentiation and depends on protein synthesis. (20–24). In Fig. 3, the results obtained with representative clones are shown in comparison to parental PC12, with or without NGF treatment. In cell clones expressing retMEN2A and retMEN2B constructs, *NGFI-A*, *SCG10*, and *vgf* transcripts were present at higher levels than in untreated parental cells and similar to those found in NGF-induced PC12 cells (Fig. 3). In vector-transfected PC12 clones, the levels of these transcripts were comparable to those found in the untreated parental cell line (data not shown). These observations indicate that the retMEN2A and retMEN2B alleles were able to induce a differentiative expression program in PC12 cells.

To further confirm these expression data and to exclude that expression of the differentiated phenotype could be attributed to clonal variations or phenotype selection within the transfected PC12 cell population, we measured the ability of retMEN2A and retMEN2B alleles to induce transcription driven by the *NGFI-A* or *vgf* promoters in transient expression assays. Fig. 4*A* shows that both mutated *ret* alleles induced the expression of the CAT reporter driven either by the *NGFI-A* (compare lanes 2 and 3 to lane 1) or by the *vgf* promoter (compare lanes 5 and 6 to lane 4). Similarly, *ret* mutants were able to induce transcription from the neuron-specific enolase promoter (data not shown). Expression of the ret-wt allele was able to sustain some transcription by the same promoters, albeit at levels ten times lower, as compared with retMEN2A (Fig. 4*B* and data not shown).

Lack of Terminal Differentiation of PC12/MEN2A and PC12/MEN2B Transfectants. Because of the implication of the retMEN2A and retMEN2B alleles in promoting hyperplasia of neuroendocrine cells, it was of interest to evaluate the effects of their expression on PC12 cell proliferation. We determined the duplication rate in PC12/MEN2A, PC12/MEN2B, and vector-transfected clones. The results obtained with two representative clones are shown in Fig. 5; the growth rate of PC12/MEN2A and PC12/MEN2B cells did not differ significantly from that of the parental cells. However, the retMEN2A and the retMEN2B transfectants exhibited a lower saturation density (≈10^6 cells/dish) than the parental PC12

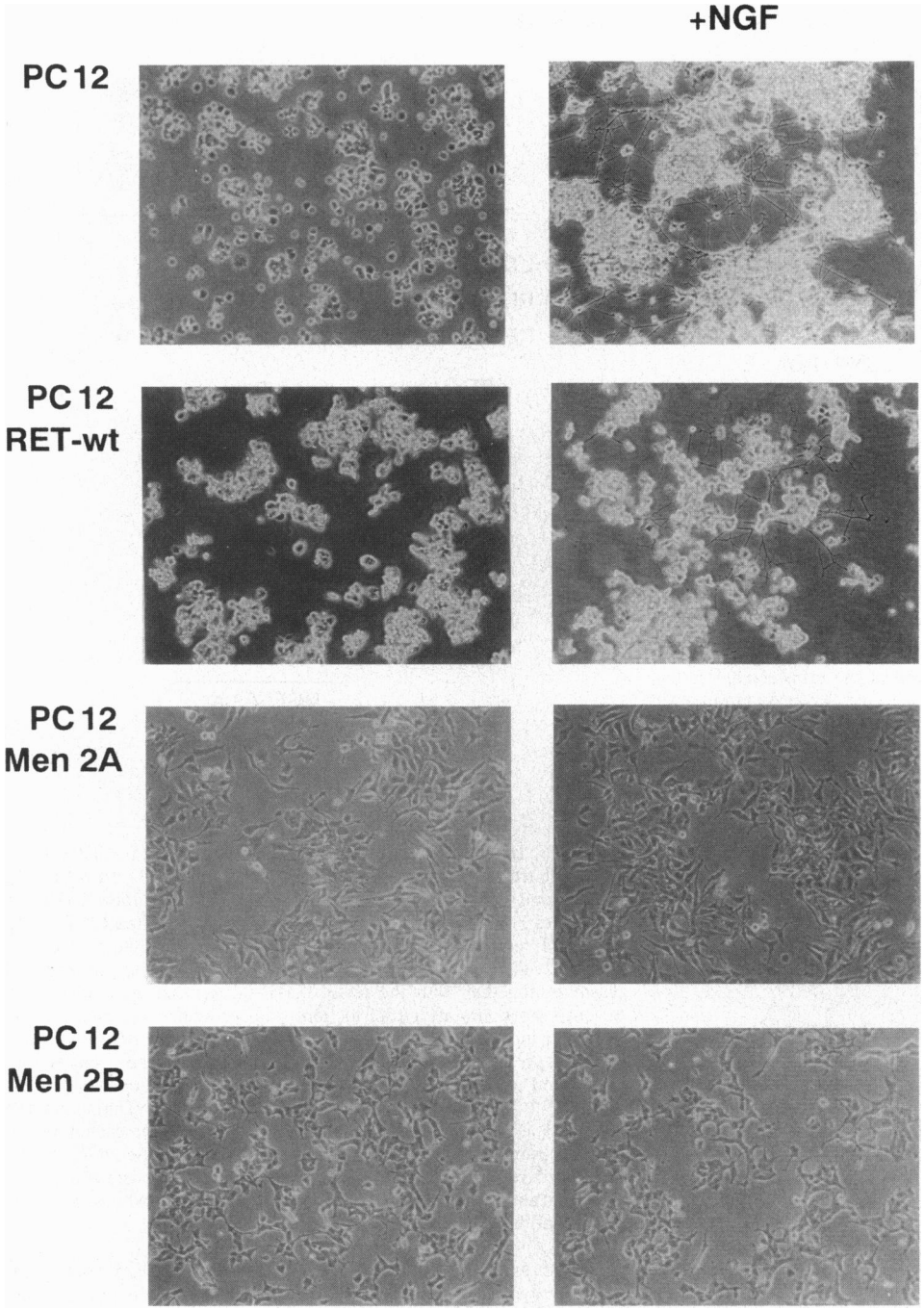

FIG. 1. Effect of retMEN2A and retMEN2B oncogenes on PC12 cell morphology. Cell clones were cultured for 1 week and then treated for 72 hr with NGF (100 ng/ml) as indicated (*Right*), or left untreated (*Left*). Phase contrast micrographs were taken of the following cell clones: PC12 parental cells; cells transfected with the ret-wt (PC12/RET-wt-cl.9); cells transfected with the retMEN2A mutant (PC12/MEN2A-cl.3); and cells transfected with retMEN2B mutant (PC12/MEN2B-cl.7).

cells ($\geq 10^7$ cells/dish). This was not due to reduced proliferation, since these PC12 transfectants could easily be subcultured and kept as continuous cell lines. Thus, the reduction in saturation density probably reflects the larger average size and flat morphology of the retMEN2 transfectants. In addition, when inoculated subcutaneously in nude mice, PC12/MEN2A and PC12/MEN2B cells were still able to generate large tumors in a few weeks, with a latency period similar to that of the parental PC12 cells or vector-transfected cell clones (data not shown). We conclude that the differentiation program induced by constitutively active isoforms of *ret* in PC12 does not include terminal differentiation and growth arrest.

Expression of retMEN2A and retMEN2B Alleles Renders PC12 Cells Insensitive to NGF. The lack of terminal differentiation of PC12/MEN2A and PC12/MEN2B transfectants posed the question as to whether these cells are still sensitive to NGF-induced terminal differentiation (12). Thus we have analyzed the effects of NGF on the growth rate of the PC12 transfectants; results obtained in representative clones are shown in Fig. 5. The growth rate of the retMEN2A- and retMEN2B-expressing cells was not affected by NGF treatment. Conversely, NGF was able to inhibit the duplication rate of the parental PC12 cells as well as of the vector transfected cells (data not shown). We further confirmed these data by determining, in PC12/MEN2A and PC12/MEN2B clones, the rate of thymidine incorporation, which, in contrast to what we observed in the parental PC12 cells, was not depressed by NGF treatment (data not shown).

We also compared the neurite outgrowth in representative clones of PC12/MEN2A and PC12/MEN2B (five for each construct) after exposure to NGF. No evident changes in morphology or neurite extension were observed in NGF-treated PC12/MEN2A and PC12/MEN2B cells (Fig. 1), whereas the parental PC12 and ret-wt-transfected cells responded to NGF, acquiring the typical neurite-bearing sympathetic neurone-like state (Fig. 1). Accordingly, NGF treat-

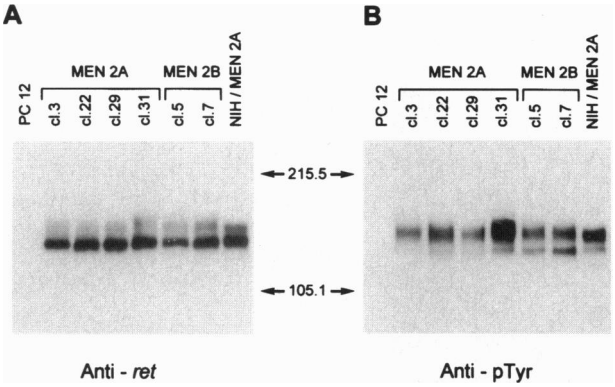

FIG. 2. Expression of mutant *ret* proteins in PC12 cells. Total cellular proteins were extracted from parental PC12, PC12/MEN2A, and PC12/MEN2B cells and from NIH 3T3 cells transfected with the retMEN2A allele (9). Protein from each lysate (2 mg) was immunoprecipitated with anti-*ret* antibodies, then divided in two aliquots, separated on SDS/PAGE, and immunoblotted with either (*A*) anti-*ret* antibodies or (*B*) anti-phosphotyrosine monoclonal antibody (Upstate Biotechnology). PC12 cells express low amounts of *ret* transcripts; however, expression of the endogenous *ret* protein in the parental cells was undetectable in our experimental conditions. These results were typical and representative of three different experiments. By a direct immunoblot analysis, all the retMEN2A and retMEN2B PC12 transfected clones showing a flat morphology, scored positive for the expression of the transfected construct (data not shown). Moreover, we have selected two mass populations, expressing each one of the *ret* mutants (data not shown), which we used to confirm the experiments described below.

ment of PC12/MEN2A and PC12/MEN2B cells had no detectable effects on the expression levels of *NGFI-A* and *vgf* transcripts (data not shown).

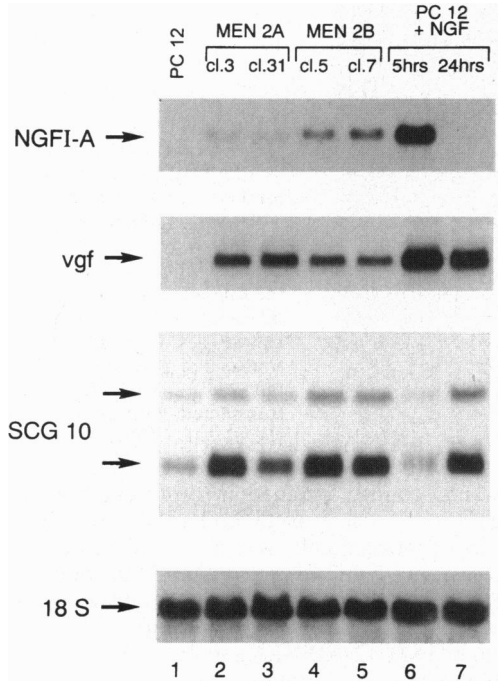

FIG. 3. Gene expression induced by *ret* mutants in PC12. Northern blot hybridization of total cellular RNA (20 μg) from the following cell lines: lane 1, parental PC12 cells; lane 2, PC12/MEN2A-cl.3; lane 3, PC12/MEN2A-cl.31; lane 4, PC12/MEN2B-cl.5; lane 5, PC12/MEN2B-cl.7; lane 6, PC12 incubated with NGF for 5 hr; and lane 7, PC12 incubated with NGF for 24 hr. ^{32}P-Labeled probes were *NGFI-A*, *SCG10*, and *vgf* as indicated. An 18S ribosomal RNA probe was used to normalize the relative RNA amounts present in each lane. These results were typical and representative of three experiments. They were confirmed on four additional retMEN2A and four additional retMEN2B transfectants and on the respective mass populations (data not shown).

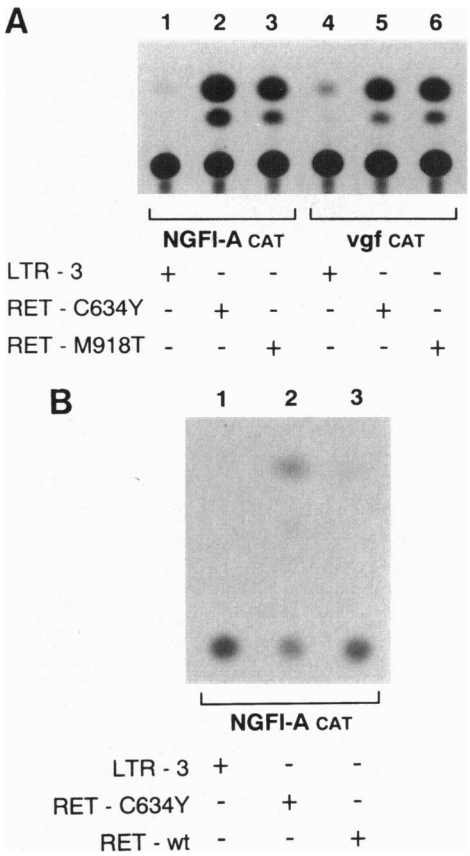

FIG. 4. Induction of early response genes by *ret* mutant alleles. PC12 cells were transfected with the following: (*A*) the LTR-3 vector (0.5 μg) (lanes 1 and 4); retMEN2A (0.5 μg) (lanes 2 and 5); or retMEN2B (0.5 μg) (lanes 3 and 6) plasmids, together with the pNGFI-A-CAT or the pvgf-CAT reporter plasmids (2 μg); and (*B*) the LTR-3 vector (0.5 μg) (lane 1), retMEN2A (0.5 μg) (lane 2), or the ret-wt (0.5 μg) (lane 3) plasmids, together with the pNGFI-A-CAT reporter plasmid (2 μg). Seventy-two hours after transfection, total proteins were isolated and promoter induction determined by CAT assay (see *Materials and Methods*). The results represent an example of three separate transfections performed in duplicate; the results of individual transfection varied by less than 25%. Values of the average relative promoters induction are indicated as fold increases above the basal activity of each reporter plasmid transfected together with the LTR-3 vector: the *NGFI-A* promoter with retMEN2A, 13 ± 2; with retMEN2B, 12 ± 2; and with ret-wt, 1.5 ± 0.5; and the *vgf* promoter with retMEN2A, 6 ± 1; with retMEN2B, 5 ± 1; and with ret-wt, 1 ± 0.5.

However, the extent of NGF-stimulated NGF receptor, *TRKA*, tyrosine phosphorylation was comparable in *ret* transfectants to that found in the parental cell line (Fig. 6), thus suggesting that, upon NGF treatment, *TRKA* kinase activity, in PC12/MEN2A and PC12/MEN2B cells, was active.

DISCUSSION

The results presented here demonstrate that the expression of two constitutively active *ret* alleles (retMEN2A and retMEN2B), in the rat pheochromocytoma cell line PC12, induces a flat-adherent phenotype, together with the expression of neuron-specific transcripts. However, the differentiation program, activated by mutant *ret* alleles appears aberrant, in that no inhibition of cell proliferation has been detected.

Moreover, expression of the *ret* mutants rendered the PC12 cells insensitive to the NGF-induced neurite outgrowth, early gene expression, and inhibition of cell proliferation, even in the presence of functional *TRKA*. Thus, we can speculate that constitutively active *ret* mutants inactivate, or subtract, some critical substrate to the NGF-induced signaling, thus rendering the cells unresponsive to the NGF control on cell proliferation. Alterna-

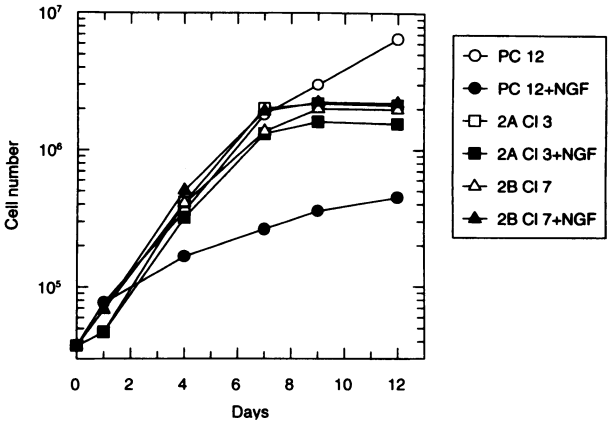

FIG. 5. Analysis of growth properties in the mutant *ret*-transfected PC12 cells. Twenty-four hours after plating PC12, PC12/MEN2A-cl.3, and PC12/MEN2B-cl.7 cells were treated with NGF (100 ng/ml), as indicated. Culture medium was changed every 3 days. Cells were grown in triplicate cultures, and cell number was determined at the indicated time intervals. The initial cell concentration was 2×10^4 cells/dish. The results shown are the average data from two independent experiments, each made in triplicate. The variation among different experiments was <10%. Moreover, these results were confirmed by analyzing five independent clones for each construct and the respective mass populations (data not shown).

tively, *ret* mutants could induce cell differentiation by using an alternative pathway that inhibits *TRKA* signaling. Indeed, NGF-induced differentiation depends on the endogenous *ras*, *raf*, and mitogen-activated protein kinase pathway (24, 25). Conversely, *ret*-induced NIH 3T3 cell transformation is not associated with *raf* or mitogen-activated protein kinase activation, despite activation of *ras*, as shown by the accumulation of GTP-bound *ras* (14). This appears to support the idea that *ret* mutants use alternative signaling pathways, which include *ras* in association with other still unidentified intracellular targets.

A number of oncogenes induce the expression of a differentiated phenotype in PC12 cells, including *TRK*-T1, v-*src* v-*ras*, v-*raf*, and *ErbB2* (26–31). Conversely, expression of *Wnt-1* or of the adenovirus *E1A* oncogene in PC12 cells results in the induction of a flat phenotype coupled to unresponsiveness to NGF induction of neuron-specific genes and morphological differentiation (32, 33). Expression of the *ret* mutants in PC12 cells induces a flat phenotype that strongly resembles that of the *Wnt-1*-expressing cells (32). However, *ret*-transfected cells express high levels of two neuronal markers, *SCG10* and *vgf*, whose expressions are undetectable in the PC12/*Wnt-1* and NGF-stimulated PC12/*Wnt-1* cells.

Thus, mutant *ret* expression induces a unique phenotype in PC12 cells, characterized by biochemical differentiation, absence of terminal differentiation, poorly differentiated morphology, and an inability to respond to NGF stimulation. This phenotype is in good agreement with the notion that the expression of these *ret* mutants is associated with the initial hyperplastic phase of the neuroendocrine tumors characteristic of MEN2A, MEN2B, and familial medullary thyroid carcinoma. Indeed, in these syndromes, germ-line mutations of *ret* are associated with hyperplasia of thyroid C-cells and chromaffin cells of the adrenal medulla (11). These cells, however, still express the differentiated functions typical of the resting normal counterparts (2, 11). We therefore propose that *ret*-induced impairment of the correct differentiative program and of the response to external cell signals causes the hyperplasia of neuroendocrine cells, which constitutes the initial step toward malignancy.

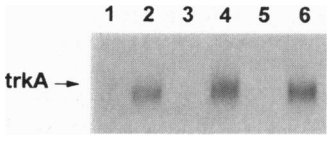

FIG. 6. Phosphorylation of *TRKA* after NGF treatment. Cell lysates were from: parental PC12 cells (lanes 1 and 2); PC12/MEN2A-cl.3 (lanes 3 and 4); PC12/MEN2B-cl.7 (lanes 5 and 6). Cell lysates were either untreated (lanes 1, 3, and 5) or treated with NGF (100 ng/ml) for 5 min (lanes 2, 4, and 6). Equal amounts of proteins from each cell line (4 mg) were immunoprecipitated with anti-TRK (C-14, Santa Cruz Biotechnology) antibodies and then separated and immunoblotted with anti-phosphotyrosine antibody (4G10). Similar results were obtained by using another anti-TRK (763, Santa Cruz Biotechnology) with two other clones tested for each transfectant and with the respective mass populations (data not shown).

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