Inherited activating mutations of Ret, a receptor tyrosine kinase, predispose to multiple endocrine neoplasia (MEN) types 2A and 2B and familial medullary thyroid carcinoma. To investigate the effects induced by acute stimulation of Ret, we transfected both PC12 and NIH 3T3 cells with a molecular construct in which the ligand-binding domain of the epidermal growth factor receptor was fused to the catalytic domain of Ret. Acute stimulation of the chimeric receptor induced PC12 cells to express a neuronal-like phenotype. Moreover, we introduced the dominant mutation, responsible for the multiple endocrine neoplasia type 2B, in the catalytic domain of the Ret chimera. Expression of the mutant chimera, in the absence of ligand stimulation, induces the PC12 cells to acquire a flat morphology with short neuritic processes and transforms the NIH 3T3 cells. Stimulation of the mutant chimera with epidermal growth factor causes a drastic overgrowth of long neuritic processes, with the induction of the sucl-associated protein tyrosine phosphorylation in PC12 cells and higher transforming efficiency in NIH 3T3 cells. These data indicate that the gain-of-function MEN2B mutation does not abrogate ligand responsiveness of Ret and suggest that the presence of Ret ligand could play a role in the pathogenesis of the MEN2B syndrome.

Specific mutations of the ret gene, a receptor tyrosine kinase (1), are responsible for the inheritance of multiple endocrine neoplasia (MEN) type 2A and 2B and familial medullary thyroid carcinoma syndromes (2). MEN2A and MEN2B are distinct hereditary neoplastic syndromes both characterized by an earlier age of tumor onset.(3) Mutations in menin, a transcription factor of the retinoic acid receptor locus, are responsible for the inheritance of multiple endocrine neoplasia (MEN)1 type 2A and 2B and familial medullary thyroid carcinoma syndromes (4, 5). A single point mutation, which results in a Thr for Met substitution at codon 918 within the Ret catalytic domain, is responsible for the MEN2B syndrome (6, 7). These mutations convert Ret into a dominant transforming gene (retMEN2A and retMEN2B alleles) and cause constitutive activation of its intrinsic tyrosine kinase activity (8, 9).

retMEN2A and retMEN2B differ in their mechanisms of activation. In the case of retMEN2A, activation likely results from constitutive receptor dimerization, whereas retMEN2B proteins do not constitutively dimerize and display altered substrate specificity (2, 9, 10). It is presently unknown whether the Ret harboring the MEN2B mutation is fully activated by an intramolecular mechanism. Indeed, if retMEN2B is still sensitive to ligand stimulation, the contribution of active Ret to the resulting phenotype, in the affected tissues, may be in part attributed to the presence of available Ret ligand in the extracellular environment.

We thus investigated the biological effects induced by Ret stimulation in the rat pheochromocytoma cell line, PC12, because of the sensitivity of this system, which retains the ability to differentiate in vitro and also allows discrimination among stimuli from different extracellular signals (11, 12). Indeed, this cell line has been particularly suitable for studying the molecular mechanisms by which ret alleles contribute to the development of neuroendocrine cancer syndromes (13–16).

We have recently shown that chronic expression of active Ret oncoproteins induces the PC12 cells to differentiate toward a neuronal-like phenotype. Yet, we have shown that Ret-induced differentiation is not complete, because the expression of neuronal genes is dissociated from the inhibition of cell proliferation (16).

Because one of the biological mechanisms underlying the choice between differentiation and proliferation in PC12 cells is determined by the extent and duration of the signaling (12), we decided to investigate whether acute stimulation of Ret causes differentiation of the PC12 cells. In addition, we addressed the question of whether constitutive activation, induced by the MEN2B mutation, fully activates the Ret biochemical activity, thus abrogating responsiveness to ligand stimulation.

A potential physiological ligand for Ret has recently been
Ligand Stimulation of EGFR/ret

Ligand Stimulation of EGFR/ret

with the random oligonucleotide primer kit (Amersham). Hybridization and washing were carried out under stringent conditions: 0.1 × SSC, 0.1% SDS, 60 °C. Autoradiography was performed using Kodak X-AR films at ~70 °C for 1–7 days with intensifying screens.

RESULTS

Acute Stimulation of Ret Induces Neurite Outgrowth in PC12 Cells—PC12 cells, stably transfected with the chimera (EGFR/ret) or with the vector alone (LTR-3) (Fig. 1A), were selected for resistance to mycophenolic acid. A mass population and individual clones were then isolated and analyzed. All of the EGFR/ret- and vector-transfected populations appeared morphologically undifferentiated, displaying a small size and round shaped morphology (Fig. 1B and data not shown).

Stimulation of the EGFR/ret chimera with epidermal growth factor (EGF) (100 ng/ml) induced the PC12 cells to change, within 24 h, from a round shaped to a neuron-like morphology with long neurite processes that strikingly resembled that induced by the nerve growth factor (NGF) on the parental cells (Fig. 1B). On the other hand, EGF stimulation (up to 300 ng/ml) of the parental cells and of the vector-transfected cells had little or no effect on cell morphology even after 72 h of continuous treatment (Fig. 1B and data not shown).

The expression and the functional integrity of the EGFR/ret chimeric receptor were tested by immunoprecipitation of Ret products, followed by blotting either with anti-Ret or with anti-phosphotyrosine antibodies (Fig. 1C). A single protein, of 140–150-kDa apparent molecular mass, corresponding to the EGFR/ret product (21), was observed in the PC12-EGF/ret cells but not in the parental cells. In the absence of ligand stimulation, the EGFR/ret receptor displayed some constitutive levels of phosphorylation; however, stimulation with EGF (100 ng/ml) caused a dramatic increase (more than 20-fold) in tyrosine phosphorylation of the receptor (Fig. 1C).

Ret Induces Differentiation in PC12 Cells—NGF-induced differentiation of the PC12 cells is characterized by the expression of a complex pattern of genes, including immediate early genes (fos, krox24) or delayed and late genes (vgf, SCG10, peripherin), the expression of the latter genes being, at least partially, dependent on protein synthesis (26). We decided to investigate whether the Ret-induced neurite outgrowth was associated with the expression of a similar pattern of genes. In Fig. 2, we show that stimulation of the chimera induced the expression of krox24 and vgf (27, 28) at levels similar to those induced by NGF. On the other hand, EGF stimulation was unable to induce any significant vgf expression, and it induced only low levels of krox24 mRNA.

Because EGF is able, on its own, to induce an early gene response, which partially superimposes that induced by NGF, even if to a lower extent, we determined whether stimulation of the chimera could induce tyrosine phosphorylation of a specific target of neurotrophic factor activity in the neuronal cells, Snt (Fig. 3). Tyrosine phosphorylation of Snt has been reported as a qualitative event that discriminates between proliferation signals, induced by serum or EGF, and differentiation signals, such as that induced by NGF, even if its biological function is still poorly understood (23, 29). EGF stimulation of the EGFR/ret chimera resulted in tyrosine phosphorylation of Snt at levels similar to those observed following stimulation with NGF (Fig. 3, compare lane 5 to lanes 3 and 6). On the other hand, EGF had no effect on the parental PC12 cells (Fig. 3, lane 2).

Ligand Stimulation Increases the retMEN2B Activity in PC12 and NIH 3T3 Cells—A single point mutation in the catalytic domain of Ret, which is associated with the MEN2B syndrome, causes constitutive tyrosine kinase activation. This mutation enables Ret to transform the NIH 3T3 cell line and to cause incomplete differentiation of the PC12 cells (9, 16).
address the question of whether the ret/MEN2B is further inducible, we introduced the Met-918 to Thr substitution in the EGFR/ret construct (thus obtaining the EGFR/ret Thr-918). We first evaluated its effects in NIH 3T3 cells in a focus formation assay. Consistent with the "gain of function" effects of the MEN2B mutation (9), EGFR/ret Thr-918 transformed also in the absence of EGF (102 focus-forming units/pmol). EGF stimulation further increased the transforming activity of the EGFR/ret Thr-918 construct (Table I), indicating that also in the presence of a MEN2B mutation, Ret retained responsiveness to ligand triggering.

PC12 cells were then transfected with the EGFR/ret Thr-918 construct, and both a mass population and several independent clones were marker-selected. The morphology of PC12 cells expressing a ret/MEN2B allele (16). Indeed, PC12-EGFR/ret Thr-918 cells were flat and showed the growth of short neurites (Fig. 4). However, they were still responsive to ligand triggering. Twenty-four hours of EGF treatment induced the PC12-EGFR/ret Thr-918 cells to shift toward a more differentiated neuronal phenotype that was, however, clearly different from that induced by NGF on parental cells. As shown in Fig. 4, although EGF induced a pronounced neuritic outgrowth, PC12-EGFR/ret Thr-918 cells still retained a flat shaped cell body that contrasted with the round shape and the high refractility characterizing PC12 cells treated with NGF.

These biological effects were explained by the retained responsiveness of the tyrosine kinase activity of the EGFR/ret Thr-918 construct to ligand stimulation. Consistent with the reported constitutive activation of the tyrosine kinase function of Ret caused by the MEN2B mutation (9), the EGFR/ret Thr-918 protein product showed constitutive levels of tyrosine phosphorylation, both in NIH 3T3 and PC12 cells, which were higher than those of the wild type EGFR/ret chimera (Fig. 5A and data not shown). However, EGF stimulation caused a sharp increase in the phosphorylation of the receptor in both cell lines. On the other hand, phosphorylation of Snr was barely phosphorylated in cells transfected with EGFR/
Cell lysates were incubated with p13 ng/ml NGF (indicated. Supported by the observation that the EGFR/ret chimera was able to induce the tyrosine phosphorylation of Snt, a molecule that is regarded as a specific target of neurotrophic factors (23, 29).

FIG. 2. Gene expression induced by EGFR/ret in PC12 cells. Northern blot analysis is shown of total cellular RNA (20 μg) extracted either from PC12 cells (lanes 1–6) or from PC12 cells transfected with EGFR/ret (lanes 6–8), grown in the presence of NGF (100 ng/ml) or EGF (100 ng/ml) as indicated. The filters were hybridized with either a krox24-specific, or a vgf-specific probe as indicated. Equal gel loading was confirmed by the hybridization with an 18 S-specific ribosomal RNA probe. These results are representative of three independent experiments.

FIG. 3. EGFR/ret stimulation of tyrosine phosphorylation of Snt in PC12. Cells were untreated (lanes 1 and 4) or treated with 100 ng/ml NGF (lanes 3 and 6) or EGF (lanes 2 and 5) for 5 min at 37°C. Cell lysates were incubated with p13'-agarose, eluted, and analyzed by immunoblot with anti-Tyr(P) (αpTyr) antibody. The position of Snt is indicated.

retThr-918, whereas stimulation of the mutant chimera caused its marked tyrosine phosphorylation (Fig. 5B) and overinduction of krox24 gene expression (not shown).

DISCUSSION

Here we report data showing that the ret gene is able to differentiate the PC12 cells and that Ret carrying the MEN2B activating mutation is further inducible by ligand stimulation. To perform this study, we took advantage of an inducible system represented by a chimeric receptor (EGFR/ret) in which the tyrosine kinase activity of Ret was triggerable by EGF. When stimulated with EGF, PC12 cells transfected with EGFR/ret acquired a neuronal phenotype, characterized by long neuritic processes and the expression of immediate early genes such as krox24 and vgf genes. Such phenotype is undistinguishable from that induced by NGF (“NGF phenotype”). This was further supported by the observation that the EGFR/ret chimera was able to induce the tyrosine phosphorylation of Snt, a molecule that is regarded as a specific target of neurotrophic factors (23, 29).

The pattern of neuronal gene induction in PC12 cells, expressing the chronically active retMEN2A and retMEN2B alleles, is similar to that elicited by the acute stimulation of the EGFR/ret chimera. However, PC12-retMEN2A and PC12-retMEN2B cells displayed a less differentiated morphology with respect to EGF-stimulated PC12-EGFR/ret cells, since the former were characterized by a flat cell body and short neuritic processes (“MEN2 phenotype”) (29) and the latter displayed a NGF phenotype. Whether or not these differences resulted from the kinetics of activation of the forms used, namely acute stimulation of EGFR/ret versus chronic activation of retMEN2A and retMEN2B, remains to be determined.

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 (via a MEN2A or via a MEN2B mutation) (2, 3). One possibility is that retMEN2B activity could still be influenced by cell- or tissue-specific biological constraints, such as, for example, the density of the available ligand.

We thus investigated this possibility by using a mutated version of the chimera (EGFR/retThr-918), harboring the MEN2B mutation. Consistent with the notion that MEN2B causes a gain of function of Ret, the EGFR/retThr-918 construct was able to transform NIH 3T3 cells and induce differentiation in PC12 cells, even in the absence of EGF. PC12 cells transfected with EGFR/retThr-918 showed a phenotype indistinguishable from PC12 transfected with retMEN2B, thus confirming
that the expression in PC12 cells of a constitutive active Ret version results in a MEN2 phenotype. It is noteworthy that the MEN2B mutation was less effective in activating Ret function when cloned in the EGFR/ret construct with respect to the full-length ret. Since the difference between EGFR/ret and \textit{ret} resides in their extracellular and transmembrane domains, it is likely that some specific characteristics of such domains confer to Ret this particular susceptibility to the activating effect of the MEN2B mutation.

However, despite the fact that the Met-918 to Thr mutation constitutively activates the chimera, the biological effects of the EGFR/ret\textsuperscript{Thr-918} construct were markedly sensitive to EGF triggering. EGF stimulation caused a marked increase of the transforming ability of the EGFR/ret\textsuperscript{Thr-918} construct and modified the phenotype of PC12-EGFR/ret\textsuperscript{Thr-918} cells, determining the overgrowth of long neuritic processes and a dramatic phosphorylation of Snt. These effects were consistent with the stimulation of tyrosine phosphorylation of EGFR/ret\textsuperscript{Thr-918} caused by EGF.

These results show that the MEN2B mutation does not abrogate ligand responsiveness of Ret. However, we cannot discern whether the stimulation of the mutated Ret enhances the activity of the receptor, without changing the substrate specificity or, more likely, uncovers docking sites for new substrates. This ligand responsiveness may have important implications in the human diseases associated with ret MEN2B mutations. It is likely that some of the differences in the disease phenotype between MEN2A and MEN2B syndromes could depend on the tissue distribution of the Ret ligand and on the different susceptibility of ret MEN2A and ret MEN2B alleles to the action of such a ligand.