The EMBO Journal vol.15 no.11 pp.2717–2725, 1996

Molecular heterogeneity of *RET* loss of function in Hirschsprung's disease

Francesca Carlomagno, Gabriella De Vita, Maria Teresa Berlingieri, Vittorio de Franciscis, Rosa Marina Melillo, Vittorio Colantuoni[1], Matthias H.Kraus[2], Pier Paolo Di Fiore[2,3], Alfredo Fusco[4] and Massimo Santoro[5]

Centro di Endocrinologia ed Oncologia Sperimentale del CNR, c/o Dipartimento di Biologia e Patologia Cellulare e Molecolare, Facoltà di Medicina e Chirurgia, Università di Napoli 'Federico II', via S.Pansini 5, 80131 Naples, [1]Dipartimento di Biochimica e Biotecnologie Mediche, CEINGE, Centro di Ingegneria Genetica, Università degli Studi di Napoli, via S.Pansini 5, 80131 Naples, [2]IEO, European Institute of Oncology, via Ripamonti 435, 20141 Milan, [3]Istituto di Microbiologia, University of Bari and [4]Dipartimento di Medicina Sperimentale e Clinica, Facoltà di Medicina e Chirurgia di Catanzaro, Università di Reggio Calabria, via T.Campanella 5, 88100 Catanzaro, Italy

[5]Corresponding author

The *RET* proto-oncogene encodes a receptor with tyrosine kinase activity (RET) that is involved in several neoplastic and non-neoplastic diseases. Oncogenic activation of *RET*, achieved by different mechanisms, is detected in a sizeable fraction of human thyroid tumors, as well as in multiple endocrine neoplasia types 2A and 2B (MEN2A and MEN2B) and familial medullary thyroid carcinoma tumoral syndromes. Germline mutations of *RET* have also been associated with a non-neoplastic disease, the congenital colonic aganglionosis, i.e. Hirschsprung's disease (HSCR). To analyse the impact of HSCR mutations on *RET* function, we have introduced into wild-type *RET* and activated RET^{MEN2A} and RET^{MEN2B} alleles three missense mutations associated with HSCR. Here we show that the three mutations caused a loss of function of *RET* when assayed in two model cell systems, NIH 3T3 and PC12 cells. The effect of different HSCR mutations was due to different molecular mechanisms. The HSCR972 (Arg972→Gly) mutation, mapping in the intracytoplasmic region of *RET*, impaired its tyrosine kinase activity, while two extracellular mutations, HSCR32 (Ser32→Leu) and HSCR393 (Phe393→Leu), inhibited the biological activity of *RET* by impairing the correct maturation of the RET protein and its transport to the cell surface.

Keywords: cell transformation/megacolon/oncoprotein/RET/tyrosine kinase

Introduction

RET encodes a tyrosine kinase (TK) transmembrane receptor (RET) for an as yet unknown ligand (Takahashi *et al.*, 1985, 1988). Somatic rearrangements activating the transforming potential of *RET* have been described in human papillary thyroid carcinomas (Grieco *et al.*, 1990). Several observations suggest that *RET* serves a role in the migration and differentiation of neural crest-derived cell lineages. *RET* expression is found in migrating neural crest cells and in the developing peripheral and central nervous systems (Pachnis *et al.*, 1993). Moreover, activated *RET* isoforms are able to induce the differentiation of rat pheochromocytoma (Califano *et al.*, 1995) as well as of human neuroblastoma cells (D'Alessio *et al.*, 1995). Finally, RET accumulates during axon regeneration of the rat hypoglossal nerve (V.de Franciscis *et al.*, manuscript submitted).

Consistent with its predicted role in neurogenesis, *RET* plays a role in the pathogenesis of disorders of the neural crest ectoderm. Point mutations of *RET* are responsible for the autosomal dominant cancer syndromes, multiple endocrine neoplasia types 2A and 2B (MEN2A and MEN2B) and familial medullary thyroid carcinoma (FMTC) (Donis-Keller *et al.*, 1993; Mulligan *et al.*, 1993; Carlson *et al.*, 1994; Eng *et al.*, 1994; Hofstra *et al.*, 1994). 'Gain of function' of *RET* caused by these mutations is believed to be the initiating event of MEN2 syndromes, promoting multiple foci of hyperplasia in the target neuroendocrine organs (for a review see Smith *et al.*, 1994). Indeed, RET^{MEN2A} and RET^{MEN2B} alleles behave as dominant oncogenes in NIH 3T3 cells (Asai *et al.*, 1995; Santoro *et al.*, 1995).

RET is also involved in the pathogenesis of Hirschsprung's disease (HSCR), which is a developmental disorder of the autonomic innervation of the gut. It is regarded as the consequence of premature arrest of the craniocaudal migration of neural crest-derived enteric neurons towards the anal end of the rectum, which occurs between weeks 5 and 12 of gestation. This causes the absence of autonomic ganglion cells within intestinal parasympathetic Meissner's and Auerbach's plexuses and, as a consequence, a functional obstruction resulting in megacolon (Okamoto and Ueda, 1967). A linkage analysis has demonstrated that one HSCR susceptibility locus is located on chromosome 10q11.2, where the *RET* gene was mapped (Angrist *et al.*, 1993; Lyonnet *et al.*, 1993). Indeed, partial deletions of chromosome 10, which encompass the *RET* locus, were detected in some HSCR patients (Martucciello *et al.*, 1992; Luo *et al.*, 1993), and point mutations scattered along the *RET* sequence were found in several familial and sporadic HSCR cases (Attie *et al.*, 1994, 1995; Edery *et al.*, 1994; Pelet *et al.*, 1994; Romeo *et al.*, 1994; Angrist *et al.*, 1995).

There is evidence that 'loss of function' of *RET* is associated with HSCR. The targeted disruption of *RET* causes a lack of enteric ganglion cells of the myenteric and submucosal plexuses in homozygous mice (Schuchardt *et al.*, 1994). Moreover, although mutations of *RET* described in HSCR patients are heterogeneous, some

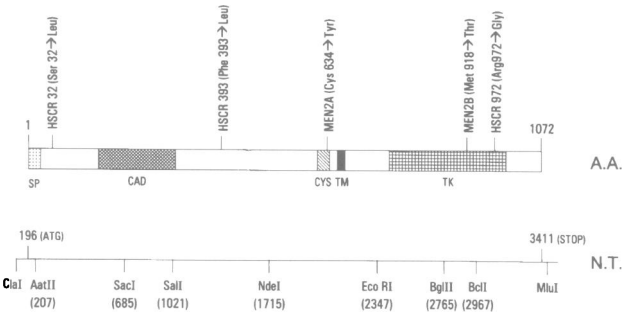

Fig. 1. Schematic representation of *RET* mutants. Top: a scheme of the RET protein (short isoform; Takahashi *et al.*, 1988). The mutations associated with HSCR, MEN2A and MEN2B are shown. SP, signal peptide; CAD, cadherin homologous domain; CYS, cysteine-rich region; TM, transmembrane region. Bottom: RET cDNA. The restriction sites used in this study are represented here.

HSCR mutations are obviously endowed with an inactivating effect; for example, apart from the HSCR cases characterized by the deletion of one allele of *RET* (Martucciello *et al.*, 1992; Luo *et al.*, 1993), in other HSCR cases non-sense or frame-shift mutations predict the synthesis of probably non-functional truncated RET proteins (Edery *et al.*, 1994; Romeo *et al.*, 1994). However, in most cases HSCR mutations are of the missense type and, in some instances, these mutations affect the intracellular domain of *RET*; this suggests that they might act on its intrinsic kinase activity. Indeed, some intracellular HSCR mutations have been shown to abolish the kinase and biological activities of *RET* (Pasini *et al.*, 1995). Mutations of the extracellular domain have also been described; in these HSCR cases no immediate structural linkage to RET kinase activity can be envisaged.

Here we demonstrate that both intracellular and extracellular mutations of *RET* associated with HSCR invariably lead to loss of function, albeit with different molecular mechanisms. Therefore our results strongly support the concept that 'loss of function' of *RET* is involved in the pathogenesis of HSCR.

Results

'Loss of function' effect of HSCR mutations in NIH 3T3 cells

Three HSCR mutations, found respectively in exons 2 (HSCR32; Ser32→Leu), 6 (HSCR393; Phe393→Leu; Edery *et al.*, 1994) and 17 (HSCR972; Arg972→Gly; Romeo *et al.*, 1994) of the *RET* proto-oncogene, were generated by recombinant PCR and introduced into two activated *RET* constructs: RET^{MEN2A} and RET^{MEN2B}. RET^{MEN2A} carries an activating mutation (Cys634→Tyr) in the extracellular domain, and RET^{MEN2B} is activated by a Met918→Thr substitution in the TK domain. Specifically, the HSCR972 mutation, affecting the TK domain of *RET*, was introduced into the RET^{MEN2A} construct. The HSCR32 and HSCR393 mutations, located in the extracellular domain, were individually introduced into the RET^{MEN2B} construct. In addition, the three HSCR mutations were also introduced into a vector encoding the wild-type *RET* (RET^{wt}). All the constructs were cloned in the long terminal repeat (LTR) vector. The structure of *RET* and the position of the introduced mutations are shown schematically in Figure 1.

Table I. Transforming efficiency of different *RET* constructs in NIH 3T3 fibroblasts

Transfected DNA[a]	FFU/pmol of DNA[b]
LTR vector	$<10^0$
RET^{wt}	$<10^0$
RET^{MEN2A}	5×10^3
RET^{MEN2B}	3×10^3
RET^{wt}/HSCR32	$<10^0$
RET^{wt}/HSCR393	$<10^0$
RET^{wt}/HSCR972	$<10^0$
RET^{MEN2B}/HSCR32	$<10^1$
RET^{MEN2B}/HSCR393	2×10^2
RET^{MEN2A}/HSCR972	$<10^1$

[a]All the constructs were cloned in the LTR vector.
[b]Transforming activity was corrected for the efficiency of transfection calculated in parallel plates subjected to marker selection. Results are the means of three experiments performed in duplicate.

Because oncogenic *RET* mutants are endowed with transforming activity in NIH 3T3 cells, we chose to analyse the biological effects of HSCR-associated mutations in this system. As shown in Table I, RET^{MEN2A} and RET^{MEN2B} exhibited readily detectable transforming activity. In contrast, the RET^{MEN2A}/HSCR972 and RET^{MEN2B}/HSCR32 double mutants lacked transforming ability, and the RET^{MEN2B}/HSCR393 construct showed a transforming efficiency at least 10-fold lower than RET^{MEN2B}. RET^{wt} was not transforming, as reported previously (Takahashi *et al.*, 1988; Santoro *et al.*, 1995). In addition, consistent with the 'loss of function' effect associated with HSCR mutations, the three HSCR mutations were not transforming when inserted in the RET^{wt} construct (RET^{wt}/HSCR32, RET^{wt}/HSCR393 and RET^{wt}/HSCR972).

'Loss of function' effect of HSCR mutations in PC12 cells

PC12 is a rat pheochromocytoma cell line characterized by the ability to differentiate to a sympathetic neuron-like state in response to nerve growth factor (NGF; Greene and Tishler, 1976; D'Arcangelo and Halegoua, 1993). In these cells, active forms of *RET* (*RET*/PTC oncogenes) are able to induce the transcription of genes involved in neuronal differentiation (Califano *et al.*, 1995). Thus, we investigated the effect of HSCR mutations in the PC12 cell line, which shares embryological derivation with the cellular targets of HSCR. First, the different *RET* constructs were tested for their ability to activate the expression of the pNGFI-A–chloramphenicol acetyl transferase (CAT) plasmid in a transient assay. In pNGFI-A–CAT, the CAT gene is under the transcriptional control of the NGFI-A promoter. NGFI-A (also known as zif/268, Egr1, Krox24, PC1, TIS8 and d2) is an immediate-early response gene whose expression is rapidly induced upon the NGF treatment of PC12 cells and also after growth factor stimulation of fibroblasts (Milbrandt, 1987; Lemaire *et al.*, 1988; Janssen-Timmen *et al.*, 1989).

A dose–response curve obtained by cotransfecting increasing amounts of the RET^{wt}, RET^{wt}/HSCR972, RET^{MEN2A} and RET^{MEN2A}/HSCR972 constructs with a constant amount of reporter plasmid, is shown in Figure 2A. A single-dose experiment using the other plasmids is reported in Figure 2B. The average results of three independent transfections, each performed in duplicate,

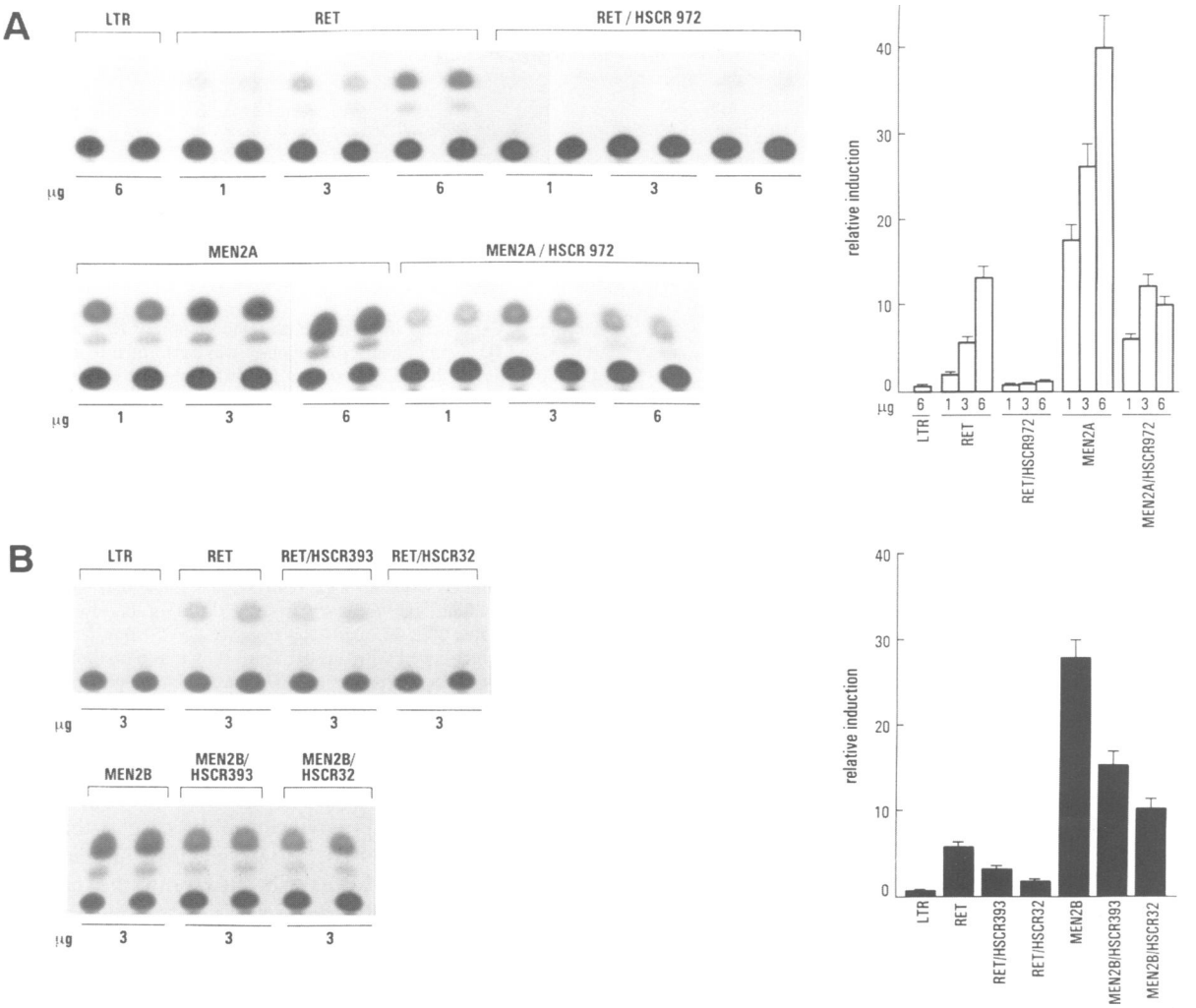

Fig. 2. Induction of the NGFI-A promoter by wild-type and mutant *RET* versions. PC12 cells were transfected with 2 μg pNGFI-A–CAT and different amounts of *RET* constructs. At 60 h after transfection, total proteins were isolated and promoter induction was determined by a CAT assay (see Materials and methods). Representative CAT assays and the bar charts of the relative induction are reported on the left and right, respectively. CAT activities are shown as fold increases above the basal activity of pNGFI-A–CAT reporter gene transfected alone. The results represent the average of three separate experiments performed in duplicate. Variation between the experiments was <25% of the mean. (**A**) NGFI-A promoter induction, obtained by transfecting 2 μg reporter with 1, 3 and 6 μg of the different *RET* constructs, as indicated. (**B**) Transfection of 2 μg reporter with 3 μg of the indicated *RET* constructs.

are reported in the bar charts on the right-hand side of the figure. The LTR vector alone did not induce any activation, whereas RET^{wt} was able to induce a detectable activation of the reporter gene. These effects were specific, because no stimulation was observed when either pBLCAT2 or RSVCAT was used as the reporter (data not shown). The transfection of RET^{MEN2A} (Figure 2A) or RET^{MEN2B} (Figure 2B) plasmids resulted in a marked induction of CAT activity (>4-fold above RET^{wt} and >30-fold above the LTR control). The activity of RET^{wt} in the PC12 system, as opposed to its inability to transform NIH 3T3 cells, might reflect either a higher sensitivity of the employed technique to the low level of intrinsic kinase activity of RET^{wt}, or an autocrine production of a *RET* ligand by PC12 cells; alternatively, it might be the result of a high level of *RET* expression with consequent ligand-independent dimerization. Whatever the case, this phenomenon allowed us to study the effect of the HSCR mutations both in RET^{wt} and activated *RET* constructs.

The HSCR972 mutation consistently inhibited the *RET*-induced activation of NGFI-A promoter. In particular, HSCR972 abolished the low activating ability of RET^{wt} and strongly reduced the function of RET^{MEN2A} (Figure 2A). Similar results were obtained when the HSCR32 and HSCR393 mutations were inserted into the RET^{wt} and RET^{MEN2B} constructs. The HSCR32 mutation nearly abolished the activity of the RET^{wt} construct and caused a 3-fold reduction of the activity of RET^{MEN2B}. The HSCR393 mutation reduced to ~50% the activity of both RET^{wt} and RET^{MEN2B} (Figure 2B). Thus, as in the case of the focus assay in NIH 3T3 cells, the HSCR393 mutation reduced *RET* activity to a lesser extent compared with the other two HSCR mutations.

We have also investigated the ability of the different *RET* constructs to induce the expression of the promoter of the *vgf* gene, which is specifically active in neuroendocrine cells (Van den Pol *et al.*, 1989) and has been demonstrated previously to be induced efficiently by only activated *RET* constructs or NGF, among several extracellular signals, in PC12 but not other cell systems (Possenti *et al.*, 1992;

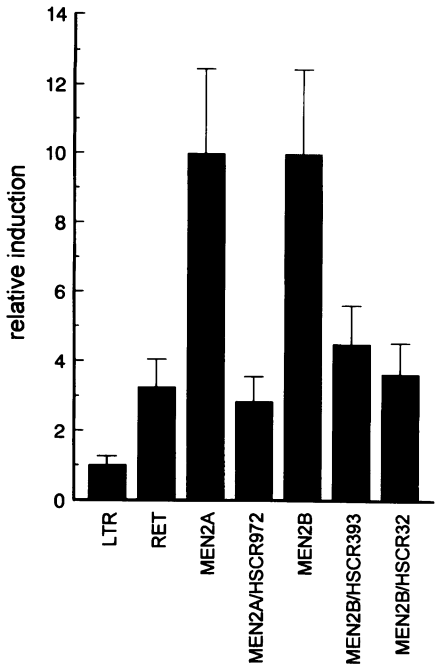

Fig. 3. Induction of the *vgf* promoter by wild-type and mutant *RET* versions. PC12 cells were transfected with 2 μg p*vgf*–CAT and 1 μg of the different *RET* constructs, as indicated. At 60 h after transfection, total proteins were isolated and promoter induction was determined by a CAT assay (see Materials and methods). The bar charts of relative induction are reported: CAT activities are shown as fold increases above the basal activity of the p*vgf*–CAT reporter gene transfected alone. The results represent the average of three separate experiments. Variation between the experiments was <25% of the mean.

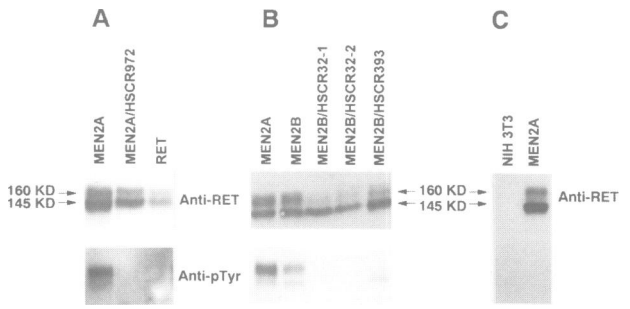

Fig. 4. Expression and tyrosine phosphorylation of *RET* mutants. (**A** and **B**) A preliminary immunoblot was performed to evaluate the expression of the RET protein in the different transfectants. RET^{MEN2A} was expressed at higher levels (3-fold) than RET^{MEN2B}, as reported elsewhere (Santoro *et al.*, 1995); RET^{MEN2B}/HSCR32 and RET^{MEN2B}/HSCR393 were expressed at 2-fold lower levels than RET^{MEN2B}; finally, RET^{MEN2A}/HSCR972 was expressed at 4- to 5-fold lower levels than RET^{MEN2A} (data not shown). A total of 500 μg total lysate from cells expressing RET^{MEN2A} and comparable amounts of RET proteins from the other transfectants were immunoprecipitated with a polyclonal antibody to RET. One half of the immunoprecipitate was immunoblotted with anti-RET (anti-RET) and one half with a monoclonal antibody to pTyr (anti-pTyr). 500 μg total lysate from untransfected NIH 3T3 cells and from RET^{MEN2A} transfectants immunoprecipitated with anti-RET are shown as a control of the specificity of the antibodies (**C**). The molecular weights of the ~160 and ~145 kDa RET proteins are indicated. The results are typical and representative of three experiments.

Califano *et al.*, 1995; D.Califano *et al.*, manuscript submitted). When the different constructs were transiently transfected in PC12, the three HSCR mutations consistently inhibited the *RET*-induced activation of the *vgf* promoter cloned in the p*vgf*–CAT plasmid. In Figure 3, we report the results obtained with the three HSCR mutations cloned in the active *RET* constructs. A similar inhibitory effect was exerted by the same HSCR mutations on the function of RET^{wt} (data not shown).

Molecular heterogeneity of HSCR 'loss of function'

Marker-selected mass populations of NIH 3T3 cells transfected with the different *RET* constructs were obtained. RET protein expression was evaluated by immunoprecipitation with specific anti-RET antibodies followed by immunoblotting with the same antibodies. RET products have been detected as ~145 and ~160 kDa isoforms (Takahashi *et al.*, 1991). The ~160 kDa species represents a mature glycosylated protein present on the cell surface, whereas the ~145 kDa form is an immature glycosylated protein, carrying high mannose oligosaccharides, that is present in the endoplasmic reticulum and is sensitive to endoglycosidase H treatment (Takahashi *et al.*, 1993). The immunoblot reported in Figure 4 shows that both species were present in RET^{wt}, RET^{MEN2A} and RET^{MEN2B} transfectants. The HSCR972 mutation introduced into the RET^{MEN2A} allele did not alter this migration pattern (Figure 4A, lane MEN2A/HSCR972). Conversely, the two extracellular HSCR mutations, introduced into the RET^{MEN2B} construct, reduced the abundance of the higher molecular weight form of the *RET* protein, this effect being much more pronounced in the case of the HSCR32 mutation (Figure 4B). However, low amounts of the ~160 kDa band were also visible in the case of the RET^{MEN2B}/HSCR32 transfectants. A second independent mass population transfected with RET^{MEN2B}/HSCR32 was obtained and confirmed these observations (Figure 4B, compare lanes MEN2B/HSCR32-1 and -2). Moreover, the strong reduction in the ~160 kDa band was also observed in two mass populations of RET^{wt}/HSCR32 transfectants (data not shown). Untransfected NIH 3T3 cells were negative when analysed with the same antibodies (Figure 4C).

To test the kinase activity of the various HSCR mutants, we initially measured their phosphotyrosine (pTyr) content. As shown in Figure 4, in agreement with an earlier report (Santoro *et al.*, 1995), no tyrosine phosphorylation of the RET^{wt} product was detectable, whereas RET^{MEN2A} and RET^{MEN2B} products showed high amounts of pTyr. In both RET^{MEN2A} (Figure 4A, lane MEN2A) and RET^{MEN2B} (Figure 4B, lane MEN2B) the pTyr:protein ratio was higher for the ~160 than for the ~145 kDa band (compare anti-RET with anti-pTyr), suggesting that the ~160 kDa form is more active. The level of tyrosine phosphorylation of the HSCR mutants paralleled their biological activity. Phosphorylation was reduced greatly in the case of the partially active RET^{MEN2B}/HSCR393 mutant (Figure 4B). The RET^{MEN2A}/HSCR972 and RET^{MEN2B}/HSCR32 products were not phosphorylated (Figure 4A and B). No tyrosine phosphorylation of the RET^{wt}/HSCR mutants was observed, as expected (data not shown).

Next we measured the ability of the HSCR mutants to autophosphorylate in an immunocomplex kinase assay because the intrinsic kinase activity of normal and activated RET products was shown previously to correlate with their tyrosine phosphorylation *in vivo*. As shown previously

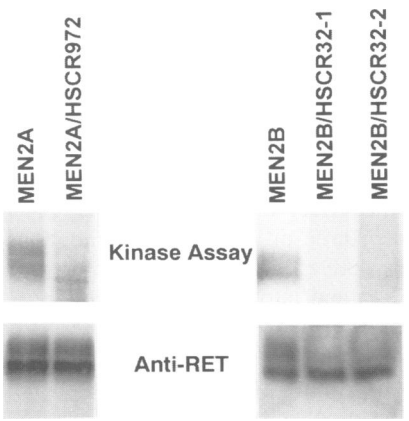

Fig. 5. Immunocomplex kinase assay. A total of 500 μg protein lysate from RET^{MEN2A} transfectants and comparable amounts of RET proteins from RET^{MEN2A}/HSCR972, RET^{MEN2B} and two independent RET^{MEN2B}/HSCR32 (-1 and -2) transfectants were immunoprecipitated with a polyclonal antibody to RET. One half of the immunoprecipitate was subjected to an *in vitro* immunocomplex kinase assay (kinase assay) and one half was immunoblotted with an anti-RET antibody (anti-RET). The results are typical and representative of three experiments.

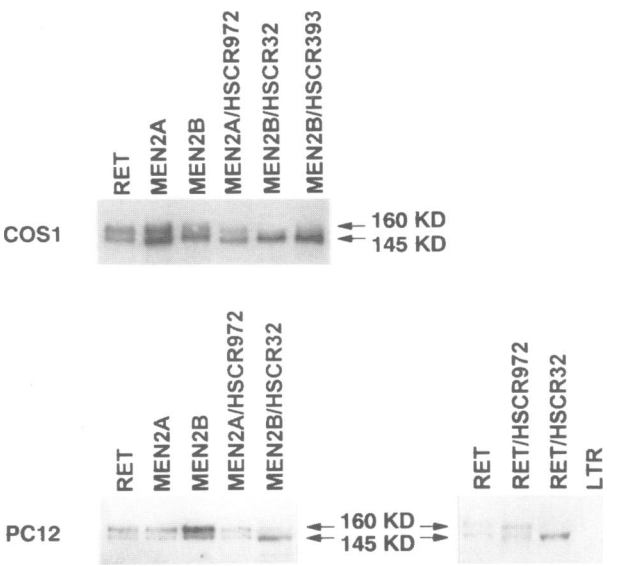

Fig. 6. Expression of *RET* mutants in COS1 and PC12 cells. COS1 cells were transfected with 20 μg of the different *RET* constructs, and 2 days after transfection protein lysates were obtained and 50 μg total lysate were separated on a 7.5% SDS–polyacrylamide gel. PC12 cells were transfected with the different *RET* constructs. Transfected cells were selected for their ability to grow in the presence of mycophenolic acid. 100 μg total protein lysate were separated on a 7.5% SDS–polyacrylamide gel. The gels were blotted and reacted with anti-RET antibodies. The molecular weights of the ~160 and ~145 kDa RET proteins are indicated.

(Santoro *et al.*, 1995), RET^{wt} products displayed little, if any, *in vitro* kinase activity (data not shown), whereas RET^{MEN2A} and RET^{MEN2B} became readily autophosphorylated *in vitro* (Figure 5). RET^{MEN2A}/HSCR972 and RET^{MEN2B}/HSCR32 proteins did not display a detectable *in vitro* kinase activity (Figure 5, lanes MEN2A/HSCR972 and MEN2B/HSCR32-1 and -2). The lower molecular weight bands present in the MEN2A/HSCR972 lane probably represent non-specifically immunoprecipitated proteins, because they were not detected when similar immunoprecipitates were blotted with two different anti-RET antibodies (data not shown). Conversely, the double mutant RET^{MEN2B}/HSCR393 showed a reduction in the kinase activity of only two-thirds, consistent with its biological activity and *in vivo* pTyr content (data not shown).

To validate these observations in another cell system and to exclude the possible consequences of phenotype selection within the transfected mass populations, we analysed the expression of the same constructs when transiently transfected in COS1 cells. RET products were readily detected when 20 μg of the different plasmid DNAs were transfected. Figure 6 shows that, as in the case of the stable NIH 3T3 transfectants, the COS1 cells transfected with *RET* constructs harboring the HSCR32 (MEN2B/HSCR32) or HSCR393 (MEN2B/HSCR393) mutation showed a strong reduction of the ~160 kDa protein that was in contrast unaltered in RET^{MEN2A}, RET^{MEN2B} and more importantly RET^{MEN2A}/HSCR972 transfectants.

When RET^{MEN2A} and RET^{MEN2B} alleles were stably expressed in PC12 cells, they were able to induce differentiated characteristics with the expression of neuronal markers, a conversion from a round to a flat morphology and an insensitivity to NGF (D.Califano *et al.*, manuscript submitted). Because, as shown above, PC12 cells were found to be a useful system to discriminate between active *RET* and inactive *RET*/HSCR alleles, *RET* constructs harboring the three HSCR mutations were stably transfected in these cells and marker-selected mass populations obtained. A loss of the differentiative effect exerted by active *RET* was caused by the HSCR mutations (G.De Vita, F.Carlomagno and M.Santoro, unpublished results). Protein lysates from these cells were analysed for the expression of RET protein. As in the case of NIH 3T3 and COS1 transfectants, PC12 transfected with *RET* constructs harboring the HSCR32 (RET/HSCR32 and MEN2B/HSCR32) mutation showed a reduction of the ~160 kDa protein that was in contrast unaltered in the case of the HSCR972 mutation (RET/HSCR972 and MEN2A/HSCR972; Figure 6). As in the other cell systems, the HSCR393 mutation caused a less pronounced reduction of the ~160 kDa protein (data not shown).

To define more clearly the alteration caused by extracellular HSCR mutations, we focused on the HSCR32 mutation which affected *RET* function most dramatically. The strong reduction in intensity of the ~160 kDa *RET* band in HSCR32 transfectants suggested that this mutation might affect the correct maturation and/or transport to the cell surface of RET. In fact, it has been demonstrated that the ~160 kDa isoform is that present on the cell surface and is probably the one endowed with kinase activity *in vivo* (see above).

A preliminary experiment with tunicamycin indicated that the RET product encoded by the RET^{MEN2B}/HSCR32 construct was correctly synthesized as a ~120 kDa core polypeptide, as in the case of the other *RET* constructs (data not shown). We performed a subcellular fractionation of NIH 3T3 transfectants expressing RET^{MEN2A}, RET^{MEN2B} and the double mutants RET^{MEN2B}/HSCR32 and RET^{MEN2B}/HSCR393; all the RET isoforms were found in the membrane compartment. The purity of individual fractions was assayed by using as specific markers the platelet-derived growth factor (PDGF) receptor and the

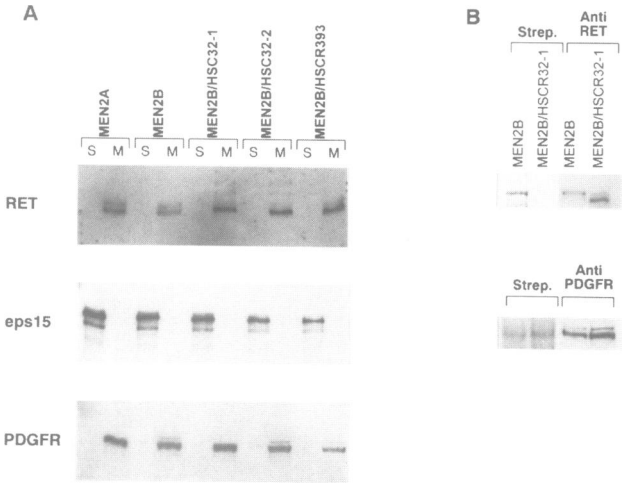

Fig. 7. Cellular localization of RET^{MEN2B}/HSCR32 products. (**A**) Subcellular localization of products of RET^{MEN2A}, RET^{MEN2B}, RET^{MEN2B}/HSCR32 (mass populations -1 and -2) and RET^{MEN2B}/HSCR393. Subcellular fractions were obtained as described in Materials and methods. The percentage of the soluble (S) with respect to the membrane fraction (M) was calculated after separation from the nuclear fraction (which was negative for RET expression; data not shown). For the S and M fractions, protein amounts deriving from comparable numbers of cells were immunoprecipitated with anti-RET antibodies and analysed by a Western blot with the same antibodies. Aliquots of each fraction representative of the same number of cells (5×10^5) were analysed with anti-PDGF receptor and with anti-eps15 antibodies as a control of their purity. (**B**) Biotinylation of the 160 kDa RET protein present on the cell surface. Cells expressing the RET^{MEN2B} and RET^{MEN2B}/HSCR32 mutants (mass population -1) were labeled with biotin and immunoprecipitated with anti-RET antibody. Samples were fractionated on a 7.5% SDS–polyacrylamide gel; one half of the specific immunoprecipitate was immunoblotted with anti-RET antibodies (right) and one half with the streptavidin–peroxidase complex (left). 1 mg of each lysate was immunoprecipitated with anti-PDGF receptor antibodies and blotted with streptavidin–peroxidase or anti-PDGF receptor antibodies as a control of efficient biotinylation. The results are representative of two experiments.

TK substrate *eps15*, which has been demonstrated to be exclusively cytosolic (Fazioli *et al.*, 1993), for the membrane and the cytosolic compartment, respectively (Figure 7A).

Then we performed *in vivo* biotin-labeling of the cell surface proteins of the *RET* transfectants. Cellular lysates were immunoprecipitated with anti-RET antibodies and subsequently stained with streptavidin-peroxidase or anti-RET antibodies. The results of these experiments are shown in Figure 7B. The ~160 kDa protein was the only *RET* product biotinylated in the case of the RET^{MEN2B} transfectant (lane MEN2B), indicating that the ~160 but not the ~145 kDa protein is a cell surface protein, as demonstrated previously (Asai *et al.*, 1995). No *RET* biotinylated product was observed in the case of RET^{MEN2B}/HSCR32 (lane MEN2B/HSCR32-1), although the labeling of this cell line was successful, as demonstrated by the efficient biotinylation of the PDGF receptor (Figure 7B). Thus, we conclude that the RET products affected by the HSCR32 mutation are located in the membrane compartment but are not correctly transported to the cell surface.

Discussion

In this study we investigated the effects on *RET* function of three different missense mutations associated with Hirschsprung's disease. Pasini *et al.* (1995) have recently demonstrated that HSCR mutations affecting the intracellular domain of *RET* cause a loss of function of an oncogenic rearranged form of *RET* lacking the extracellular domain (*RET*/PTC2 oncogene). To circumvent the absence of a known ligand for *RET* and to investigate the effect of extracellular HSCR mutations, we introduced three different HSCR mutations into two constitutively active full-length *RET* isoforms (RET^{MEN2A} and RET^{MEN2B}) and into RET^{wt}. Two biological detection systems, represented by NIH 3T3 and PC12 cells, were used. The advantage the PC12 system afforded was 2-fold. First, it allowed us to study the HSCR phenotypes in a 'closer to physiological setting'. In fact, the ability of *RET* to influence the differentiation of neural crest-derived cells is probably the function that is lost in HSCR. Moreover, the unexpected finding that RET^{wt} was able to induce transcription from the NGFI-A and *vgf* promoters in PC12 cells (albeit at low levels) allowed us to investigate the effect of HSCR mutations also in the RET^{wt} gene background, thus closely reproducing the events of HSCR.

As reported by Pasini *et al.* (1995), the Arg972→Gly substitution caused a marked inhibition of RET kinase activity and of its biological effects. However, HSCR972 mutants were correctly synthesized and maturated. Thus, direct impairment of the kinase activity is likely to be the primary lesion caused by this mutation. The molecular mechanisms underlying this inhibition are still unknown. The Arg972 residue is located in the conserved subdomain 10 of kinases; moreover, it is conserved in some cytoplasmic TKs such as *abl* and *lyn*, and is followed immediately by a proline residue that is also highly conserved in TKs (Hanks *et al.*, 1988). Therefore, it is conceivable that this amino acid sequence, which can be disrupted by the HSCR972 mutation, is involved in the correct folding of the catalytic core.

The alterations caused by the extracellular HSCR mutations are more complex, being characterized by impairment of the kinase activity and aberrant intracellular transport. RET products normally consist of two molecular species of ~145 and ~160 kDa (Takahashi *et al.*, 1991). The ~160 kDa protein represents the mature fully glycosylated form, and is expressed on the cell surface; the ~145 kDa protein is probably a partially glycosylated immature product which accumulates intracellularly (Takahashi *et al.*, 1991; Asai *et al.*, 1995). The ~160 kDa form is probably the one endowed with kinase activity *in vivo*, as witnessed by its at least 20-fold higher pTyr content compared with the ~145 kDa isoform.

In the HSCR32 and HSCR393 mutants, the degree of reduction of kinase and of biological activities correlated with the decrease in the amount of cell surface ~160 kDa RET isoform. Thus, it appears that the impaired kinase activity of these two mutants is a consequence of their altered maturation and lack of exposure on the cell surface. Alternatively, as a second possibility, one can postulate that HSCR extracellular mutations alter the folding of RET in such a way as to inhibit, at the same time, both kinase activity and intracellular transport. The first possibility is supported by the low level of tyrosine phosphorylation of the ~145 kDa product encoded by active RET alleles, although one cannot exclude the fact

that the intracellular ~145 kDa RET is exquisitely sensitive to phosphatase action.

It is not known how the extracellular HSCR mutations affect RET maturation and transport. The extracellular domain of RET contains a cadherin-related domain of ~110 amino acids which encompasses putative Ca^{2+} binding sites including the D–X–D motif (Schneider, 1992). Phylogenetic conservation of this structure suggests relevance for RET function (Iwamoto *et al.*, 1993; Schuchardt *et al.*, 1995). By mutagenizing the D–X–D motif (Asp300→Lys) in a RET^{MEN2A} construct, Asai *et al.* (1995) showed a reduction of its transforming ability. Moreover, this Asp300→Lys substitution impaired the intracellular transport of RET and caused a dramatic decrease of the cell surface 160 kDa isoform without affecting the abundance of the lower molecular weight form present in the endoplasmic reticulum (Asai *et al.*, 1995). These results are similar to our findings concerning the HSCR32 and HSCR393 mutations.

Residues Ser32 and Phe393 are outside the cadherin domain. They are, however, also conserved in mouse and chicken RET (Iwamoto *et al.*, 1993; Schuchardt *et al.*, 1995). Thus, they may be necessary for the correct folding of the RET protein and for its transport through the Golgi complex, or for the process of N-linked glycosylation. The fact that the HSCR32 mutant, the most severely affected one, is entirely present in the membrane fraction, although not on the plasma membrane, suggests that these processes are altered as a consequence of this mutation.

The HSCR phenotype might represent the final outcome of genetically different diseases, as demonstrated by the existence of other susceptibility loci such as the endothelin-B receptor gene (Puffenberger *et al.*, 1994). In the HSCR cases in which *RET* is involved, the 'loss of function' phenotype of the mutated HSCR alleles of *RET* poses the question as to how these mutations contribute to the pathogenesis of the disease. The inheritance of these HSCR cases associated with *RET* mutations appears to be dominant with variable penetrance (for a review see Smith *et al.*, 1994). A simple possibility is that the kinase-impaired HSCR products exert a dominant negative effect on the function of the wild-type *RET* allele. Pasini *et al.* (1995) demonstrated that the kinase function of an oncogenically activated *RET* construct (RET/PTC2 oncogene) is inhibited by coexpression with a construct carrying intracytoplasmic HSCR mutations. We tried extensively, but without success, to demonstrate such a dominant negative effect by cotransfecting HSCR mutants with activated *RET* isoforms in both NIH 3T3 and PC12 cells (data not shown). Our failure to demonstrate any negative dominance could be an artefact of the model systems, i.e. high levels of expression of the activated isoforms might have overwhelmed the inhibitory action of the HSCR mutants. However, given the high sensitivity of the detection systems used, one would have expected to see at least some effect. In addition, in the case of the extracellular transport-impaired HSCR mutants, a possible mechanism of negative dominance is not immediately obvious. Thus, in addition to negative dominance, other mechanisms such as reduced gene dosage (see for example the HSCR cases associated with the deletion of one *RET* allele or with the synthesis of truncated proteins) might play a role in the pathogenesis of Hirschsprung's disease associated with *RET* alterations.

Materials and methods

Engineering of expression vectors

RET nucleotide positions are according to the published sequence (Takahashi *et al.*, 1988; Itoh *et al.*, 1992), and the numbering of the *RET* exons is according to Kwok *et al.* (1993). The LTR–RET^{wt} eukaryotic expression vector encoding the short isoform (1072 amino acids) of the RET protein is described elsewhere (Santoro *et al.*, 1995). The LTR–RET^{MEN2A} and LTR–RET^{MEN2B} expression vectors encode for *RET* mutants carrying a Cys634→Tyr and a Met918→Thr mutation, respectively (Santoro *et al.*, 1995). To obtain the HSCR mutations, PCR fragments containing the required mutation were generated by recombinant PCR using LTR–RET^{wt} as a template. PCRs were performed according to Higuchi (1990). Briefly, two primary PCRs (a 'left' and a 'right' reaction) were performed using standard PCR conditions (AmpliTaq; Perkin Elmer-Cetus Co.). This yielded two products overlapping in the sequence corresponding to the reverse primer of the 'left' PCR and the forward primer of the 'right' PCR; the mutations were introduced as part of these overlapping PCR primers. The oligonucleotide primer sequences used are shown in Table II. The products of the primary PCRs were then purified by agarose gel electrophoresis. In all, 10 ng of the purified PCR products of the 'left' and the 'right' primary PCRs were annealed and elongated with five PCR cycles (95°C for 1 min, 37°C for 2 min, 72°C for 1 min), followed by 15 cycles of a secondary PCR, using the 5′- and 3′-most oligonucleotides as primers. The recombinant PCR products were cloned in the pT7Blue T vector (Novagen) and completely sequenced using the Sequenase Kit (USB). Finally, the fragments containing the mutations were excised by digestion with appropriate restriction enzymes (*Aat*II and *Sac*I for HSCR32, *Sal*I and *Nde*I for HSCR393, *Bcl*I and *Mlu*I for HSCR92) and cloned in the LTR–RET^{wt}, LTR–RET^{MEN2A} or LTR–RET^{MEN2B} plasmids. The resulting expression vectors were RET^{wt}/HSCR32, RET^{wt}/HSCR393, RET^{wt}/HSCR972, RET^{MEN2B}/HSCR32, RET^{MEN2B}/HSCR393 and RET^{MEN2A}/HSCR972. They were sequenced in both strands of the regions that underwent genetic manipulations to verify that the predicted structures were achieved after the recombination procedures and that no additional mutations were introduced during the cloning steps.

Other expression vectors

The pNGFI-A–CAT vector was kindly provided by M.V.Chao. It contains sequences from position −1150 to +200 of the NGFI-A promoter fused to the CAT gene (Janssen-Timmen *et al.*, 1989). p*vgf*–CAT contains the *vgf* promoter, the 5′ non-coding region and the first methionine (from −803 to +710) fused in-frame with the initiating methionine of the CAT gene (Possenti *et al.*, 1992). The pBLCAT2 and RSV–CAT vectors contain the CAT gene under the control of the HSV thymidine kinase promoter (position −105 to + 51) and of the Rous sarcoma virus promoter, respectively (Lukow and Schutz, 1987).

Cell culture and transfection experiments

NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS) and were transfected using the calcium phosphate precipitation method, as described elsewhere (Di Fiore *et al.*, 1987). Transformed foci were scored at 3 weeks. The transforming efficiency was calculated in focus-forming units (FFU) per pmol of added DNA after normalization for the efficiency of colony formation in parallel dishes subjected to marker selection in mycophenolic acid. Marker-selected mass populations of transfected NIH 3T3 cells were obtained by taking advantage of the *Escherichia coli gpt* gene of the LTR vector, which confers resistance to mycophenolic acid. PC12 cells were grown in RPMI 1640 medium (Gibco, BRL) supplemented with 10% horse serum and 5% FCS. For transient transfection assays, cells were plated at 3×10^5 cells in 60 mm diameter tissue culture dishes 24–36 h prior to transfection. Transfection was performed using the lipofectin reagent following the manufacturer's instructions (Gibco, BRL). First, the pNGFI-A–CAT plasmid was chosen as the target because of its very low basal level of activity in PC12 cells (<0.5% of chloramphenicol conversion; Califano *et al.*, 1995). All transfections were carried out with 2 μg reporter plasmid (pNGFI-A–CAT, pBLCAT2 or RSVCAT), together with various amounts of the *RET* constructs, as indicated in each figure. The same DNA concentration was reached by

Table II. Oligonucleotide primer sequences used for the PCRs

	Mutated nucleotide	PCR	Primer
HSCR32	TCG→TTG	left	5′-ATGGCGAAGGCGACGTCCGGT-3′ (forward) 5′-TAAGCATCCCTC(A)AGAAGTAGAGGC-3′ (reverse)
		right	5′-GCCTCTACTTCT(T)GAGGGATGCTTA-3′ (forward) 5′-TCGGTTCTCCCGAATGCGGAAGGA-3′ (reverse)
HSCR393	TTC→TTA	left	5′-TGACCGTGTACGACGAGGACGAC-3′ (forward) 5′-CACCGACACGTT(T)AAGTGGAGCA-3′ (reverse)
		right	5′-TGCTCCACTT(A)AACGTGTCGGTG-3′ (forward) 5′-CAGCCGTCTCTTGCTGACTGCAC-3′(reverse)
HSCR972	AGG→GGG	left	5′-AAGCTCGTTCATCGGGACTTG-3′ (forward) 5′-CAGTT GTCTGGCC(C)CTCCATCCGGTG-3′ (reverse)
		right	5′-CACCGGATGGAG(G)GGC CAGACAACTG-3′ (forward) 5′-ACGCGTACAGCGGTGCTAGAATC-3′ (reverse)

adding various amounts of the LTR control vector. To generate PC12 cells stably transfected with the different constructs, 10 μg plasmid DNA were transfected onto 30% confluent PC12 cells in 100 mm dishes by using the lipofectin reagent (Gibco, BRL); mass populations were selected in HAT medium. COS1 cells were transfected using LipofectAMINE (Gibco, BRL). Approximately 4.5×10^6 cells were seeded in 150 mm dishes and transfected with 20 μg DNA using 100 μl LipofectAMINE in a 16 ml volume. Transfections were stopped after 5 h by adding medium containing 20% heat-inactivated fetal bovine serum. Cells were then incubated with OptiMEM I medium supplemented with GlutaMAX I (Gibco, BRL) for an additional 2 days, and finally protein extracts were prepared.

CAT assays

Cell extracts were prepared 60 h after transfection, and CAT activity was analysed by thin-layer chromatography (TLC) with 95% chloroform–5% methanol, as described previously (Califano *et al.*, 1995). Each experimental point was cut from the TLC plate and counted. For each experiment, the percentage of conversion to the acetylated form of chloramphenicol ^{14}C was then calculated. The results of at least three experiments, made in duplicate, were plotted on an arbitrary scale as relative promoter induction.

Protein studies

Immunoprecipitation and immunoblotting experiments were performed as in Santoro *et al.* (1994). Briefly, cells were lysed in a buffer containing 50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5), 1% (v/v) Triton X-100, 50 mM NaCl, 5 mM EGTA, 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride and 0.2 μg each of aprotinin and leupeptin per ml. Lysates were clarified by centrifugation at 10 000 *g* for 15 min, and the supernatant was processed for immunoblotting or immunoprecipitation as described previously (Santoro *et al.*, 1994). Protein concentration was estimated by a modified Bradford assay (Bio-Rad). To analyse the tyrosine phosphorylation of the various mutants, comparable amounts of RET were immunoprecipitated and assayed for their pTyr content. Antibodies against RET included a polyclonal antibody to the RET TK domain (Santoro *et al.*, 1994) and, as a control in some experiments, another polyclonal antibody directed against a COOH-ter peptide of the cytoplasmic domain of human RET (residues 1011–1027). Other antibodies were a polyclonal antibody specific for the *eps15* gene product (Fazioli *et al.*, 1993), the PDGF (R)-B (958) antibody raised against type B PDGF receptor (Santa Cruz Biotechnology), and the 4G10 anti-pTyr monoclonal antibody (Upstate Biotechnology, Lake Placid, NY). Immunoblots were subsequently stained with appropriate secondary antibodies and revealed with the Amersham ECL system. The immunocomplex kinase assay was performed as published previously (Santoro *et al.*, 1995). Tunicamycin treatment was performed by culturing cells in the presence of the drug (5 μg/ml) for 16 h, as described previously (Takahashi *et al.*, 1991). Biotin labeling of the intact cells (5×10^6) was performed essentially as described previously (Asai *et al.*, 1995). Briefly, cells were washed three times with biotinylation buffer (10 mM HEPES, pH 8.8, 150 mM NaCl). Biotin *N*-hydroxysuccinimide ester (Pierce) was dissolved in dimethyl sulfoxide at a concentration of 20 mg/ml and added to cells at a final concentration of 500 μg/ml. After 20 min at 20°C, the reaction was terminated by the addition of Dulbecco's modified Eagle's medium. Cells were washed with 10 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, and lysed in immunoprecipitation buffer. The resulting immunoblot was developed with streptavidin–biotin peroxidase complex (Amersham) and the Amersham ECL system. Subcellular fractionation of *RET*-expressing NIH 3T3 cells was performed as described previously (Lanzi *et al.*, 1992). Cells were lysed in hypotonic buffer (20 mM HEPES, pH 7.5, 5 mM EGTA, proteases and phosphatase inhibitors). Nuclei were recovered by centrifugation at 1000 *g*. The supernatant was centrifuged at 100 000 *g* for 45 min to separate the soluble and membrane fractions. Aliquots from each fraction were subjected to immunoprecipitation and immunoblotting.

Acknowledgements

We are grateful to Prof. Giancarlo Vecchio for his support during the course of this work. We thank Dr Moses V.Chao for the pNGFI-A and Dr Roberta Possenti for the p*vgf*–CAT plasmid. This study was supported in part by the Associazione Italiana per la Ricerca sul Cancro (AIRC), by the Progetto Finalizzato ACRO, Sottoprogetto 2, Biologia Molecolare and by the Progetto Finalizzato Biotecnologia e Biostrumentazione.

References

Angrist,M. *et al.* (1993) A gene for Hirschsprung disease (megacolon) in the pericentromeric region of human chromosome 10. *Nature Genet.*, **4**, 351–356.

Angrist,M., Bolk,S., Thiel,B., Puffenberger,E.G., Hofstra,R.M., Buys,C.H., Cass,D.T. and Chakravarti,A. (1995) Mutation analysis of the RET receptor tyrosine kinase in Hirschsprung disease. *Hum. Mol. Genet.*, **4**, 821–830.

Asai,N., Iwashita,T., Matsuyama,M. and Takahashi,M. (1995) Mechanism of activation of the *ret* proto-oncogene by multiple endocrine neoplasia 2A mutations. *Mol. Cell. Biol.*, **15**, 1613–1619.

Attie,T., Pelet,A., Sarda,P., Eng,C., Edery,P., Mulligan,L.M., Ponder, B.A.J., Munnich,A. and Lyonnet,S. (1994) A 7 bp deletion of the RET proto-oncogene in familial Hirschsprung's disease. *Hum. Mol. Genet.*, **3**, 1439–1440.

Attie,T. *et al.* (1995) Diversity of RET proto-oncogene mutations in familial and sporadic Hirschsprung disease. *Hum. Mol. Genet.*, **4**, 1381–1386.

Califano,D. *et al.* (1995) Activated RET/PTC oncogene elicits immediate early and delayed response genes in PC12 cells. *Oncogene*, **11**, 107–112.

Carlson,K.M., Dou,S., Chi,D., Scavarda,N., Toshima,K., Jackson,C.E., Wells,S.A., Goodfellow,P. and Donis-Keller,H. (1994) Single missense mutation in the tyrosine-kinase catalytic domain of the RET proto-oncogene is associated with multiple endocrine neoplasia type 2B. *Proc. Natl Acad. Sci. USA*, **91**, 1579–1583.

D'Alessio,A., De Vita,G., Calì,G., Nitsch,L., Fusco,A., Vecchio,G., Santelli,G., Santoro,M. and De Franciscis,V. (1995) Expression of the RET oncogene induces the SK-N-BE neuroblastoma cells to differentiate. *Cell Growth Diff.*, **6**, 1387–1394.

D'Arcangelo,G. and Halegoua,S. (1993) A branched signaling pathway for nerve growth factor is revealed by Src-, Ras-, and Raf-mediated gene inductions. *Mol. Cell. Biol.*, **13**, 3146–3155.

Di Fiore,P.P., Pierce,J.H., Kraus,M.H., Segatto,O., King,C.R., Schlessinger,J. and Aaronson,S.A. (1987) erbB-2 is a potent oncogene when overexpressed in NIH-3T3 cells. *Science*, **237**, 178–181.

Donis-Keller,H. *et al.* (1993) Mutations in the RET proto-oncogene are associated with MEN2A and FMTC. *Hum. Mol. Genet.*, **2**, 851–856.

Edery,P. *et al.* (1994) Mutations of the RET proto-oncogene in Hirschsprung's disease. *Nature*, **367**, 378–380.

Eng,C. *et al.* (1994) Point mutation within the tyrosine kinase domain of the RET proto-oncogene in multiple endocrine neoplasia type 2B and related sporadic tumors. *Hum. Mol. Genet.*, **3**, 237–241.

Fazioli,F., Minichiello,L., Matoskova,B., Wong,W.T. and Di Fiore,P.P. (1993) Eps15, a novel tyrosine kinase substrate, exhibits transforming activity. *Mol. Cell. Biol.*, **13**, 5814–5827.

Greene,L.A. and Tishler,A.S. (1976) Establishment of a noradrenergic clonal cell line of rat pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl Acad. Sci. USA*, **73**, 2424–2428.

Grieco,M. *et al.* (1990) PTC is a novel rearranged form of the ret proto-oncogene and is frequently detected *in vivo* in human thyroid papillary carcinomas. *Cell*, **60**, 557–563.

Hanks,S.K., Quinn,A.M. and Hunter,T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science*, **241**, 42–52.

Higuchi,R. (1990) Recombinant PCR. In Innis,M.A., Gelfand,D.H., Sninski,J.J. and White,T.J. (eds), *PCR Protocols: A Guide to Methods and Applications*. Academic Press Inc., San Diego, CA, pp. 177–183.

Hofstra,R.M.W. *et al.* (1994) A mutation in the RET proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. *Nature*, **367**, 375–376.

Itoh,F. *et al.* (1992) Identification and analysis of the ret proto-oncogene promoter region in neuroblastoma cell lines and medullary thyroid carcinomas from MEN2A patients. *Oncogene*, **7**, 1201–1206.

Iwamoto,T., Taniguchi,M., Asai,N., Ohkusu,K., Nakashima,I. and Takahashi,M. (1993) cDNA cloning of mouse ret proto-oncogene and its sequence similarity to the cadherin superfamily. *Oncogene*, **8**, 1067–1091.

Janssen-Timmen,U., Lemaire,P., Mattéi,M.G., Revelant,O. and Charnay,P. (1989) Structure, chromosome mapping and regulation of the mouse zinc-finger gene Krox-24; evidence for a common regulatory pathway for immediate-early serum-response genes. *Gene*, **80**, 325–336.

Kwok,J.B.J., Gardner,E., Warner,J.P., Ponder,B.A.J. and Mulligan,L.M. (1993) Structural analysis of the human Ret proto-oncogene using exon trapping. *Oncogene*, **8**, 2575–2582.

Lanzi,C. *et al.* (1992) Identification of the product of two oncogenic rearranged forms of the RET proto-oncogene in papillary thyroid carcinomas. *Oncogene*, **7**, 2189–2194.

Lemaire,P., Relevant,O., Bravo,R. and Charnay,P. (1988) Two mouse genes encoding potential transcription factors with identical DNA-binding domains are activated by growth factors in cultured cells. *Proc. Natl Acad. Sci. USA*, **85**, 4691–4695.

Lukow,B. and Schutz,G. (1987) CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. *Nucleic Acids Res.*, **15**, 5490.

Luo,Y. *et al.* (1993) Close linkage with the RET proto-oncogene and boundaries of deletion mutants in autosomal dominant Hirschsprung disease. *Hum. Mol. Genet.*, **11**, 1803–1808.

Lyonnet,S. *et al.* (1993) A gene for Hirschsprung disease (megacolon) maps to the proximal long arm of chromosome 10. *Nature Genet.*, **4**, 346–350.

Martucciello,G., Bicocchi,M.P., Dodero,P., Lerone,M., Cirillo,M.S., Puliti,A., Gimelli,G., Romeo,G. and Jasonni,V. (1992) Total colonic aganglionosis associated with interstitial deletion of the long arm of chromosome 10. *Pediatr. Surg. Int.*, **7**, 308–310.

Milbrandt,J. (1987) A nerve growth factor-induced gene encodes a possible transcriptional regulatory factor. *Science*, **238**, 797–799.

Mulligan,L.M. *et al.* (1993) Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. *Nature*, **363**, 458–460.

Okamoto,E. and Ueda,T. (1967) Embryogenesis of intramural ganglia of the gut and its relation to Hirschsprung disease. *J. Pediatr. Surg.*, **10**, 437–443.

Pachnis,V., Mankoo,B. and Costantini,F. (1993) Expression of the c-RET proto-oncogene during mouse embryogenesis. *Development*, **119**, 1005–1017.

Pasini,B. *et al.* (1995) Loss of function effect of RET mutations causing Hirschsprung disease. *Nature Genet.*, **10**, 35–40.

Pelet,A., Attie,T., Goulet,O., Eng,C., Ponder,B.A.J., Munnich,A. and Lyonnet,S. (1994) De-novo mutations of the RET proto-oncogene in Hirschsprung's disease. *Lancet*, **344**, 1769–1770.

Possenti,R., Di Rocco,G., Nasi,S. and Levi,A. (1992) Regulatory elements in the promoter region of *vgf*, a nerve growth factor-inducible gene. *Proc. Natl Acad. Sci. USA*, **89**, 3815–3819.

Puffenberger,E.G., Hosoda,K., Washington,S.S., Nakao,K., deWit,D., Yanagisawa,M. and Chakravarti,A. (1994) A missense mutation of the endothelin-B receptor gene in multigenic Hirschsprung's disease. *Cell*, **79**, 1257–1266.

Romeo,G. *et al.* (1994) Point mutations affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease. *Nature*, **367**, 377–378.

Santoro,M., Wong,T.W., Aroca,P., Santos,E., Matoskova,B., Grieco,M., Fusco,A. and Di Fiore,P.P. (1994) An epidermal growth factor receptor/*ret* chimera generates mitogenic and transforming signals: evidence for a *ret*-specific signaling pathway. *Mol. Cell. Biol.*, **14**, 663–675.

Santoro,M. *et al.* (1995) Germ-line mutations of MEN2A and MEN2B activate RET as a dominant transforming gene by different molecular mechanisms. *Science*, **267**, 381–383.

Schneider,R. (1992) The human proto-oncogene ret: a communicative cadherin? *Trends Biochem. Sci.*, **17**, 468–469.

Schuchardt,A., D'Agati,V., Larsson-Blomberg,L., Costantini,F. and Pachnis,V. (1994) Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature*, **367**, 380–383.

Schuchardt,A., Srinivas,S., Pachnis,V. and Costantini,F. (1995) Isolation and characterization of a chicken homolog of the c-ret proto-oncogene. *Oncogene*, **10**, 641–649.

Smith,D.P., Eng,C. and Ponder,B.A.J. (1994) Mutations of the RET proto-oncogene in the multiple endocrine neoplasia type 2 syndromes and Hirschsprung disease. *J. Cell Sci.*, **18**, 43–49.

Takahashi,M., Ritz,J. and Cooper,G.M. (1985) Activation of a novel human transforming gene, ret, by DNA rearrangement. *Cell*, **42**, 581–588.

Takahashi,M., Buma,Y., Iwamoto,T., Inaguma,Y., Ikeda,H. and Hiai,H. (1988) Cloning and expression of the ret protooncogene encoding a tyrosine kinase with two potential transmembrane domains. *Oncogene*, **3**, 571–578.

Takahashi,M., Buma,Y. and Taniguchi,M. (1991) Identification of the ret proto-oncogene products in neuroblastoma and leukemia cells. *Oncogene*, **6**, 297–301.

Takahashi,M., Asai,N., Iwashita,T., Isomura,T., Miyazaki,K. and Matsuvama,M. (1993) Characterization of the RET proto-oncogene products expressed in mouse L cells. *Oncogene*, **8**, 2925–2929.

Van den Pol,A.N., Decavel,C., Levi,A. and Paterson,B. (1989) Hypothalamic expression of a novel gene product, VGF: immunocytochemical analysis. *J. Neurosci.*, **9**, 4122–4137.

Received on November 8, 1995; revised on February 9, 1996