The soluble ectodomain of Ret^{C634Y} inhibits both the wild-type and the constitutively active Ret

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Substitution of Cys-634 in the extracellular domain of the Ret tyrosine kinase receptor causes its dimerization and activation of its transforming potential. To gain further insight into the molecular basis leading to Ret activation we purified a mutant protein consisting of the entire ectodomain of the Ret carrying a Cys-634 → Tyr substitution (EC-Ret^{C634Y}). The protein is glycosylated, like the native one, and is biologically active. By using an *in vitro* cell system we show that EC-Ret^{C634Y} inhibits the membrane-bound receptor Ret^{C634Y}, interfering with its dimerization. Furthermore, we demonstrate that EC-Ret^{C634Y} competes with the wild-type Ret receptor for ligand binding. The results presented support the notion of the possible involvment of glial cell line-derived neurotrophic factor (GDNF) with multiple endocrine neoplasia type 2A (MEN2A) tumours, and describe a useful tool for generating molecular mimetics directed towards specific mutations of the *ret* oncogene.

Key words: multiple endocrine neoplasia type 2A (MEN2A), oncogene, receptor, tyrosine kinase.

INTRODUCTION

The *ret* proto-oncogene is expressed in tissues of neural crest origin, including the sympathetic ganglia, adrenal medulla, thyroid C cells and excretory system of the developing kidney. It encodes a transmembrane receptor belonging to the protein tyrosine kinase family. Four distinct ligands for Ret have been identified, all polypeptide growth factors of the glial cell line-derived neurotrophic factor (GDNF) family. Each of these ligands binds specifically to the four members of the glycosylphosphatidylinositol-anchored receptor α1-4 (GFRα1-4) family. Ligand-dependent activation of Ret implicates its recruitment to the GDNF–GFRα complex leading to Ret dimerization and autophosphorylation at specific cytoplasmic Tyr residues [1–4]. The overall organization of the Ret extracellular region was found recently to constitute four cadherin-like domains at the N-terminus and a single cysteine-rich domain close to the boundary of the transmembrane domain [5]. Mutations in the Ret extracellular region have been implicated in multiple endocrine neoplasia type 2A (MEN2A) syndrome, familial medullary thyroid carcinoma and Hirschsprung's disease (HSCR). In the case of familial medullary thyroid carcinoma and MEN2A syndrome, mutations in the cysteine-rich domain of specific cysteine residues engaged in intramolecular disulphide bridges result in the formation of an unpaired cysteine residue, causing the dimerization by intermolecular disulphide bridges of two mutated Ret molecules. These mutations convert Ret into a dominant transforming gene and induce constitutive activation of its intrinsic tyrosine kinase activity, leading to congenital and sporadic cancers in neuroendocrine organs [3,6,7]. On the other hand, deletions and point mutations distributed along the entire *ret* gene have been described in sporadic and familial cases of HSCR [8–10]. HSCR-associated mutations in the extracellular region impair the correct maturation of the receptor, its exposure on the cell surface and its biological activity [3,11].

Here we asked whether disrupting a cysteine residue in the cysteine-rich domain would make a soluble protein for the Ret ectodomain able to compete with the constitutive activity of the *ret* oncogene. With this aim, we purified a protein spanning the entire Ret extracellular domain carrying a Cys-634 → Tyr substitution (EC-Ret^{C634Y}) and show that it acts as an inhibitory competitor for the membrane-bound Ret^{C634Y} receptor. Furthermore the mutated EC-Ret^{C634Y} polypeptide retains the ability to inhibit the GDNF stimulation of the wild-type form of the receptor.

EXPERIMENTAL

Miscellaneous

Protein concentration was determined by the Bradford assay using BSA as the standard. SDS/PAGE analysis, under reducing and non-reducing conditions, was carried out according to Laemmli in the presence and absence of 2-mercaptoethanol, respectively. After SDS/PAGE, the proteins were revealed by Coomassie Brilliant Blue or silver staining, or by immunoblotting analysis (see below). Treatment with peptide N-glycosidase F (New England Biolabs) was performed according to the protocol supplied by the manufacturer. Molar concentrations were calculated on the basis of the following molecular mass values: 120 kDa for the extracellular domains of wild-type Ret (Ret^{wt}) and Ret^{C634Y} (EC-Ret^{wt} and EC-Ret^{C634Y}); 290 kDa for the extracellular domain of Ret^{wt} fused to the Fc region of human IgG1 (Ret-Fc); 30 kDa for GDNF; and 190 kDa for soluble GFRα1.

Immunoblotting analysis

Following SDS/PAGE, proteins were electroblotted on to PVDF membranes (Millipore), and detected with the indicated primary antibodies and peroxidase-conjugated secondary antibodies

Abbreviations used: GDNF, glial cell line-derived neurotrophic factor; GFRα1-4, glycosylphosphatidylinositol-anchored receptor α1-4; HSCR, Hirschsprung's disease; MEN2A, multiple endocrine neoplasia type 2A; Ret^{wt}, wild-type Ret; EC-Ret^{wt}, extracellular domain of wild-type Ret; EC-Ret^{C634Y}, Ret extracellular domain carrying a Cys-634 → Tyr substitution; ERK, extracellular signal-regulated kinase; Ret-Fc, Ec-Ret^{wt} fused to the Fc region of human IgG1; TEV, tobacco etch virus.

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using the enhanced chemiluminescence system (Amersham Biosciences). The primary antibodies used were as follows: anti-Ret (C-19) and anti-(extracellular signal-regulated kinase) (ERK1; C-16; Santa Cruz Biotechnology), anti-(phospho-Ret) (Cell Signalling), anti-(mouse Ret extracellular domain) (rmRet; R&D Systems) and anti-(phospho-44/42 mitogen-activated protein kinase) (E10; Cell Signalling) monoclonal antibodies. When indicated, membranes were stripped in 62.5 mM Tris/HCl, pH 6.7/0.1 M 2-mercaptoethanol/2 % SDS for 30 min at 55 °C. For the immunoblots shown in Figures 4–6 the statistical analysis was performed on at least three independent experiments.

Cell culture

PC12 cells were grown in RPMI 1640 (Gibco) supplemented with 10 % horse serum and 5 % heat-inactivated fetal calf serum. PC12/wt, PC12/MEN2A and PC12-α1/wt cell lines, expressing Ret9^{wt}, Ret9^{C634Y} and Ret9^{wt} + GFRα1 proteins, respectively, were grown in the same culture medium with the appropriate selection pressure, as reported previously [12]. To obtain PC12-α1/wt cells, PC12/wt cells were stably transfected with the full-length cDNA of rat GFRα1 (kindly provided by S. Jing, Amgen, Thousand Oaks, CA, U.S.A.) using Lipofectamine 2000 reagent (Invitrogen), in accordance with the manufacturer's suggestions. The transfected cells were selected in the presence of 400 μg/ml G-418 (Invitrogen) for 4 weeks and individual cell colonies were isolated and expanded. When indicated, 2.5 S nerve growth factor (Upstate Biotechnology), GDNF (Promega) or recombinant rat GFRα1-Fc chimaera (R&D Systems) were added to the culture medium.

HEK-293T cells were grown in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10 % ultra-low IgG fetal bovine serum (Gibco) and 2 mM L-glutamine.

Preparation of cell extracts

Cells were washed twice in ice-cold PBS, then lysed in 50 mM Tris/HCl buffer, pH 8.0, containing 150 mM NaCl, 1 % Nonidet P-40, 2 μg/ml aprotinin, 1 μg/ml pepstatin, 2 μg/ml leupeptin and 1 mM Na_3VO_4 (lysis buffer). The solution was centrifuged at 16000 ***g*** for 30 min at 4 °C and the residue was discarded.

Cloning strategy and expression of EC-Ret^{wt}, EC-Ret^{C634Y} and Ret-Fc

The cDNA encoding EC-Ret^{wt} or EC-Ret^{C634Y} was fused at the C-terminus with a fragment containing two IgG-binding domains of *Staphylococcus aureus* protein A, modified from the TAPTag cassette-based vector kindly provided by Dr B. Seraphin, Centre de Genetique Moleculaire, CNRS, Gif sur Yvette, France [13]. A tobacco etch virus (TEV) protease cleavage site was introduced to allow recovery of untagged EC-Ret during the purification procedure. The chimaeric fragment was thus subcloned into a pcDNA 3.1(+) expression vector (Invitrogen). The expression vector containing the cDNA encoding the extracellular domain of Ret fused with the DNA encoding the Fc region of human IgG1 was from Dr S. Jing, Amgen Inc., Thousand Oaks, CA, U.S.A. [14].

EC-Ret^{wt}, EC-Ret^{C634Y} or Ret-Fc plasmids were transiently transfected into HEK-293T cells at 90 % confluence plated on to five 15 cm plates using the Lipofectamine 2000 reagent. Culture medium was harvested 48 h after the transfection and replaced with fresh medium which was harvested 24 h following the addition. The medium from the two withdrawals (200 ml final volume) was concentrated by Amicon-10 ultrafiltration (15 ml final volume), aliquoted and stored at − 80 °C.

Purification of EC-Ret^{wt} and EC-Ret^{C634Y}

Step 1: affinity chromatography

The concentrated culture medium (6 ml) was dialysed against 10 mM Tris/HCl buffer, pH 8.0, containing 0.15 M NaCl and 0.05 % Tween 20 (buffer A) and loaded on to an IgG–Sepharose 6 Fast Flow column (0.5 cm × 3 cm; Amersham Biosciences) which was equilibrated with the same buffer at room temperature. The sample was incubated with rotation with IgG beads for 2 h at 4 °C, then the column was washed with 30 vol. of buffer A and 10 vol. of buffer A plus 0.5 mM EDTA. The bound proteins were eluted by incubation in 1.5 ml of buffer A plus 0.5 mM EDTA and 1 mM dithiothreitol supplemented with 100 units of recombinant TEV enzyme (Invitrogen) for 2 h at room temperature with gentle mixing. The recovered sample was dialysed against 10 mM Tris/HCl, pH 8.0, and concentrated by a vacuum centrifuge (Savant).

Step 2: gel-filtration chromatography

The sample from the affinity-chromatography column was loaded on to a Superdex 200 High Load column (Amersham Biosciences; 1 cm × 30 cm) connected to an FPLC system, and eluted with 10 mM Tris/HCl, pH 8.0, supplemented with 0.2 M NaCl, at a flow rate of 0.3 ml/min and at room temperature. The fractions containing the Ret proteins (30 μg) were pooled, dialysed against 10 mM Tris/HCl, pH 8.0, concentrated as described above and stored at − 80 °C.

Purification of Ret-Fc

The culture medium (6 ml) was dialysed against 10 mM Tris/HCl, pH 8.0, and loaded on to a Protein A–Sepharose CL-4B column (0.5 cm × 3 cm; Amersham Biosciences) which was equilibrated with the same buffer at 4 °C. The sample was incubated with rotation with the beads overnight at 4 °C, and then the column was washed with 0.1 M Tris/HCl, pH 8.0, at a flow rate of 0.5 ml/min until the absorbance at 280 nm returned to the baseline. The bound proteins were eluted with 0.1 M glycine buffer, pH 3.0. The recovered sample (90 μg) was dialysed against 10 mM Tris/HCl, pH 8.0, concentrated as described above and stored at − 80 °C.

Heat denaturation of EC-Ret^{C634Y}

A 5 ml mixture containing 5 μg of EC-Ret^{C634Y} in RPMI medium was incubated at 80 °C and protein aggregation was followed during heating by measuring the absorbance at 450 nm against a control solution of medium heated as above. The maximal turbidity at 450 nm was taken as 100 % aggregation. After a 30 min incubation the temperature was shifted to 37 °C and the mixture was added to PC12/MEN2A cells. The protein turbidity was monitored during a 20 min incubation at 37 °C in an identical mixture assessed as a control.

RESULTS AND DISCUSSION

Purification and structural characterization of EC-Ret^{wt} and EC-Ret^{C634Y}

Large amounts of the Ret receptor tyrosine kinase extracellular domain were produced by transiently transfecting HEK-293T

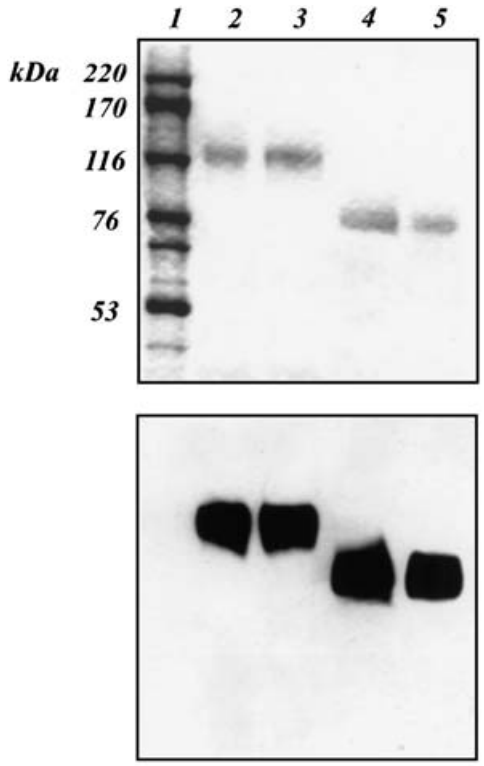

Figure 1 EC-Ret^{wt} and EC-Ret^{C634Y} are glycosylated

SDS/PAGE (10 % acrylamide) of a pure sample of EC-Ret^{wt} or EC-Ret^{C634Y} before (lanes 2 and 3, respectively) and following treatment with peptide N-gycosidase F (lanes 4 and 5, respectively). Molecular-mass standards are shown (lane 1). Proteins were revealed by Coomassie Brilliant Blue staining (upper panel) or by immunoblotting analysis with anti-(Ret extracellular domain) antibodies (lower panel).

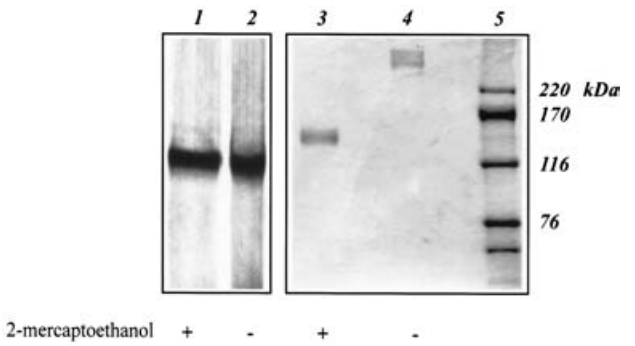

Figure 2 EC-Ret^{C634Y} does not dimerize

SDS/PAGE (7 % acrylamide) of a pure sample of EC-Ret^{C634Y} (lanes 1 and 2) or Ret-Fc (lanes 3 and 4) in the presence or absence of 2-mercaptoethanol as indicated. Molecular-mass standards are shown (lane 5). The apparent molecular mass of Ret-Fc in lane 4 was calculated by extrapolation. Proteins were revealed by silver staining.

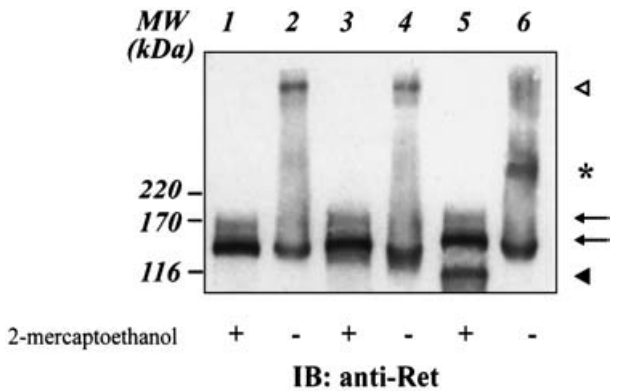

Figure 3 EC-Ret^{C634Y} dimerizes with membrane-bound Ret^{C634Y}

PC12/MEN2A cells (160000 cells/3.5 cm plate) were treated for 10 min with mixtures (2 ml final volume of RPMI medium) containing 20 μg of EC-Ret^{wt} or EC-Ret^{C634Y}. Cells were washed twice in ice-cold PBS, lysed and cell lysates were subjected to SDS/PAGE (6 % acrylamide) in the presence or absence of 2-mercaptoethanol as shown and immunoblotted (IB) with anti-Ret antibodies. Lanes 1 and 2, control cell lysates; lanes 3 and 4, lysates from cells treated with EC-Ret^{wt}; lanes 5 and 6, lysates from cells treated with EC-Ret^{C634Y}. Molecular-mass standards (MW) are shown. Bands are marked as follows: ◁, homodimer Ret^{C634Y} receptor; *, heterodimer of EC-Ret^{C634Y}/membrane-bound Ret^{C634Y} receptor; arrows, 170 and 150 kDa Ret^{C634Y} proteins; ◀, EC-Ret^{C634Y} protein.

cells with recombinant constructs, carrying either the Cys-634 → Tyr substitution or the corresponding wild-type allele. In these constructs the region corresponding to the extracellular domain of the Ret receptor was fused at its C-terminus with the two IgG-binding domains of *S. aureus* Protein A. A TEV protease cleavage site was included at the N-terminus of Protein A to get rid of the tag in the purification procedure. Since the constructs lack the Ret transmembrane domain, the resulting fusion proteins are released in the culture medium. We purified the secreted proteins by using a procedure lacking denaturing steps, which allows one to obtain high yields of the native proteins in a few days. Briefly, the concentrated medium was fractionated by affinity selection on IgG matrix and the Ret-containing samples eluted in the presence of the TEV protease. The eluate was loaded on to a gel-filtration chromatographic column to remove the TEV protease as well as traces of contaminants remaining after the affinity chromatography (for details see the Experimental section).

The gel-filtration chromatography gave rise to almost pure proteins consisting of the untagged EC-Ret^{wt} and EC-Ret^{C634Y}, with apparent molecular masses of 120 kDa as judged by SDS/PAGE analysis (Figure 1, lanes 2 and 3). The yield of purified protein was approx. 350 ng/ml of culture medium. It has been reported [5] that Ret in its mature form is glycosylated and sensitive to deglycosylation by peptide N-glycosidase F. We wondered whether the expression and the purification procedures we used still preserved the glycosylation of the proteins. As shown in Figure 1, both EC-Ret^{wt} and EC-Ret^{C634Y} were largely glycosylated since peptide N-glycosidase F treatment resulted in an electrophoretic mobility increase to approx. 80 kDa (Figure 1, lanes 4 and 5).

Since *in vivo* the Cys-634–Tyr mutation causes dimerization of Ret by formation of interchain disulphide bonds, we asked whether the purified EC-Ret^{C634Y} preparation contained a fraction of homodimeric molecules. SDS/PAGE analysis of EC-Ret^{C634Y} showed that the protein migrates as a monomer under reducing and non-reducing conditions (Figure 2, compare lanes 1 and 2) and its molecular mass under non-reducing conditions was comparable with that of EC-Ret^{wt} (results not shown), thus making unlikely the presence of homodimeric molecules in the preparation. In addition the elution volume of EC-Ret^{wt} and EC-Ret^{C634Y} on Superdex 200 column corresponded to a molecular mass of 120 kDa (results not shown), which was compatible with an homogeneous preparation of the extracellular domain of Ret in a monomeric form. The finding that the EC-Ret^{C634Y} protein does not exist as a disulphide-linked dimer is probably due to the presence of the unpaired cysteine just close to the C-terminus of the protein (the EC-Ret^{C634Y} protein ending at amino acid 636). On the other hand,

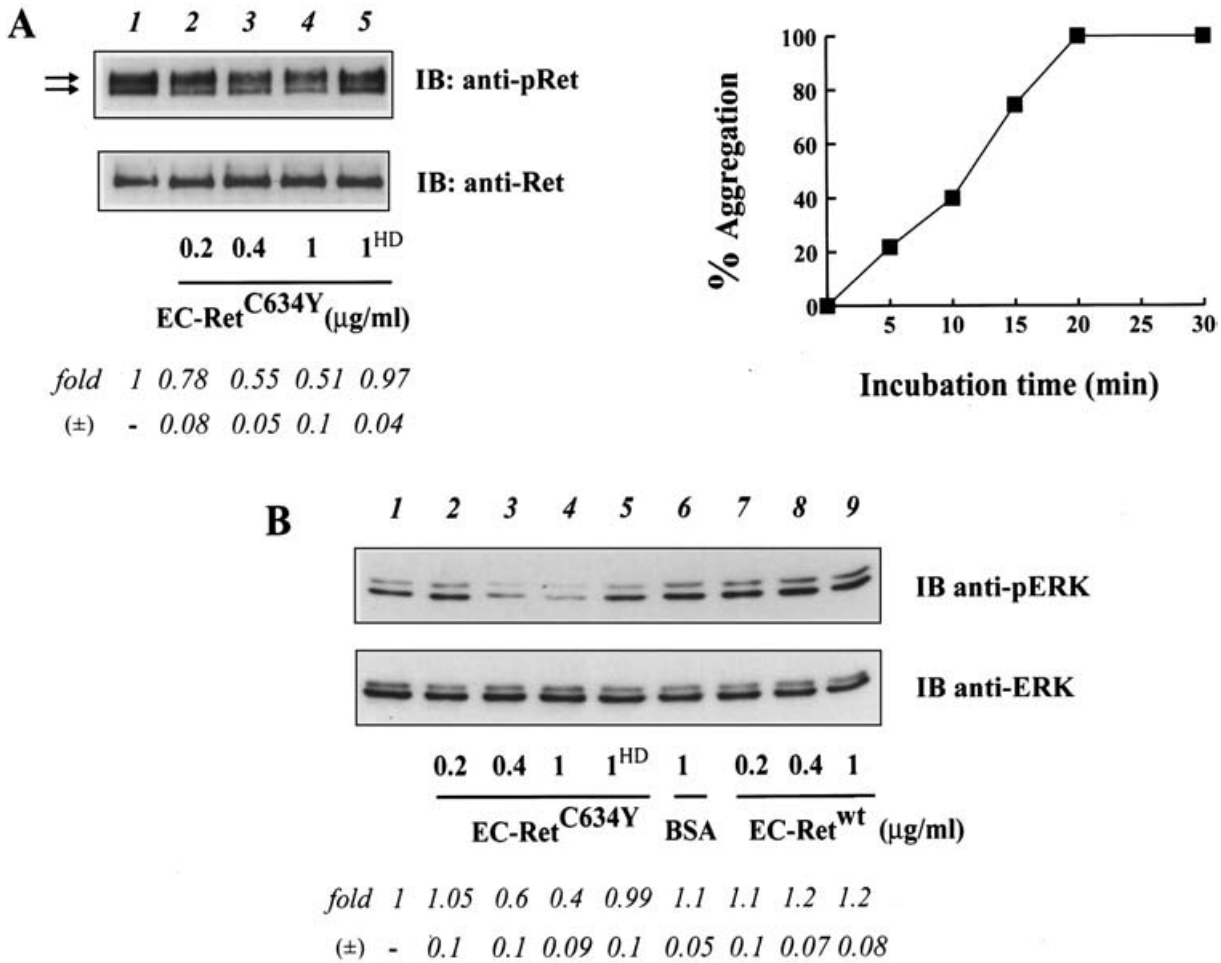

Figure 4 EC-Ret^{C634Y} inhibits Ret activity

(**A**) Left-hand panel: PC12/MEN2A cells were starved for 16 h and then treated for 10 min with mixtures (5 ml final volume of RPMI medium) containing increasing amounts of EC-Ret^{C634Y} or 1 μg/ml heat-denatured EC-Ret^{C634Y} (HD) as described in the Experimental section. Cell lysates were immunoblotted with anti-(phospho-Ret) antibodies (upper panel); the two autophosphorylated 170 and 150 kDa Ret bands are marked by arrows. To confirm equal loading the same filter was stripped and hybridized with anti-Ret antibodies (lower panel). Right-hand panel: aggregation profile of EC-Ret^{C634Y} in the time course of heating at 80 °C. (**B**) PC12/MEN2A cells were starved for 16 h and then treated for 10 min with mixtures (5 ml final volume of RPMI medium) containing increasing amounts of EC-Ret^{C634Y} or EC-Ret^{wt}, 1 μg/ml heat-denatured EC-Ret^{C634Y} (HD) or 1 μg/ml BSA. Cell lysates were immunoblotted with anti-(phospho-ERK) antibodies (upper panel); the same filter was stripped and hybridized with anti-ERK antibodies (lower panel). Relative abundances are shown by the fold values below (**A**), left-hand panel, and (**B**), compared with control values (set to 1; lane 1); these were calculated using the NIH Image program, based on the sum of the two Ret- (**A**) or ERK- (**B**) specific bands, and S.D. values are shown underneath ($n = 3$).

Ret-Fc migrates as a band of 145 kDa under reducing conditions (Figure 2, lane 3) and of approx. 290 kDa under non-reducing conditions (Figure 2, lane 4), compatible with dimer formation, probably due to the presence of the immunoglobulin Fc domain.

EC-Ret^{C634Y} inhibits the Ret^{C634Y} activity and the receptor-dependent downstream signalling

Missense mutations in cysteine residues located close to the transmembrane boundary in the Ret extracellular domain cause homodimer formation and constitutive receptor activation. As a consequence of this event, on the cell surface the mutated Ret receptor exists in a balance between the monomeric and homodimeric forms, the latter being generated by the formation of novel disulphide bridges between two monomeric molecules of the mutated receptor [6]. We thought that if the soluble monomer EC-Ret^{C634Y} dimerizes with membrane-bound monomer Ret^{C634Y}, it might outcompete homodimer formation and ultimately inhibit Ret activity. To verify this hypothesis, we took advantage of a PC12-derived cell line that expresses the human Ret^{C634Y} oncogene (PC12/MEN2A). Lysates from cells treated with EC-Ret^{wt} or EC-Ret^{C634Y} were analysed by SDS/PAGE under reducing or non-reducing conditions followed by immunoblotting with anti-Ret antibodies. As expected, the extract of PC12/MEN2A cells analysed under reducing condition revealed the presence of the two species of 170 and 150 kDa corresponding to different glycosylation states of Ret (Figure 3, lane 1). Under non-reducing conditions, in addition to the Ret monomeric forms we observed a species of approx. 340 kDa, corresponding to the homodimeric form of the receptor (Figure 3, lane 2), confirming the presence on the cell surface of the monomeric and homodimeric forms of mutated Ret. No change in the electrophoretic pattern was observed following treatment of the cells with EC-Ret^{wt} protein (Figure 3, compare lanes 3 and 4 with lanes 1 and 2). Interestingly, the treatment of the cells with EC-Ret^{C634Y} caused the appearance on the reducing gel of a band of approx. 120 kDa, probably corresponding to EC-Ret^{C634Y} protein

that has formed a heterodimer with the membrane-bound receptor (Figure 3, lane 5). Furthermore, the heterodimer EC-Ret^{C634Y}/membrane-bound Ret^{C634Y} is compatible with the species of approx. 290 kDa present on the gel, in the absence of the reducing agent (Figure 3, lane 6). These findings indicate that the soluble extracellular domain of Ret may interact with the full-length Ret receptor and that the heterodimerization depends on the presence of the unpaired cysteine on both proteins.

Further, we decided to verify whether the EC-Ret^{C634Y} protein could inhibit Ret activity by competing for homodimer formation between the mutated Ret molecules. With this aim, PC12/MEN2A cells were treated with increasing amounts of EC-Ret^{C634Y} and then lysed. Lysates were analysed by immunoblotting with antibodies for the Tyr-phosphorylated Ret. As expected, basal levels of Ret phosphorylation were constitutively high in PC12/MEN2A cells (Figure 4A, left-hand panel, lane 1). Treating cells with increasing amounts of EC-Ret^{C634Y} (from 0.2 to 1 μg/ml) inhibited Ret phosphorylation up to 50% (Figure 4A, lanes 2–4). As an appropriate control to ascertain the specificity of the EC-Ret^{C634Y} inhibitory effect we decided to utilize a preparation of heat-denatured EC-Ret^{C634Y} protein. Light-scattering measurements showed that aggregation occurred in a solution of 1 μg/ml EC-Ret^{C634Y} upon heating at 80 °C (Figure 4A, right-hand panel) and no decrease of the turbidity was observed on shifting the temperature to 37 °C (results not shown), indicating that protein denaturation was irreversible. On treating PC12/MEN2A cells with the heat-denatured EC-Ret^{C634Y} sample no change in Ret phosphorylation was observed (Figure 4A, left-hand panel, lane 5), demonstrating that the correct folded structure of the protein is necessary to achieve *ret* oncogene inhibition. These results show that the soluble EC-Ret^{C634Y} monomer may act as functional inhibitor of the Ret^{C634Y} intrinsic tyrosine kinase activity.

Given that EC-Ret^{C634Y} inhibits Ret activity, we asked whether it could interfere with the downstream signalling mediated by Ret^{C634Y}. We therefore measured the phosphorylation levels of the downstream Ret effector, ERK, in PC12/MEN2A cells. As reported previously [15], in these cells the presence of the active allele causes ERK to be phosphorylated constitutively (Figure 4B, lane 1). Consistent with the inhibitory effects on Ret tyrosine phosphorylation, incubating the cells in the presence of EC-Ret^{C634Y} reduced ERK phosphorylation in a dose-dependent manner (Figure 4B, lanes 2–4). The highest inhibition value (60%) was observed at a EC-Ret^{C634Y} final concentration of 1 μg/ml.

The inhibitory effect was specific since no change in ERK phosphorylation was achieved in the presence of heat-denatured EC-Ret^{C634Y} (Figure 4B, lane 5) or BSA (Figure 4B, lane 6). In addition, we verified that EC-Ret^{wt}, lacking unpaired cysteine residues, is not able to dimerize with Ret and cause its inhibition. In fact, treating cells with increasing amounts of EC-Ret^{wt} did not modify the ERK phosphorylation levels (Figure 4B, lanes 7–9).

Taken together these results indicate that EC-Ret^{C634Y} is able to dimerize with membrane-bound Ret^{C634Y} leading to the formation of inactive heterodimeric molecules, and that heterodimerization depends on the presence of the unpaired cysteine on both proteins. The outcome of this event is the inhibition of the intrinsic tyrosine kinase activity of the mutated receptor and, ultimately, the impairment of signal transmission to ERK.

EC-Ret^{wt} competes with Ret^{wt} for binding to GDNF

As described previously, Ret activity may be stimulated by GDNF in the presence of GFRα1 either bound to the cell membrane or added exogenously as a soluble protein [5,14]. Therefore we wondered whether EC-Ret^{wt} would inhibit the GDNF-induced Ret activity. To this aim, we utilized a PC12-derived cell line that expresses both human Ret^{wt} and GFRα1 (PC12-α1/wt). Cells were treated with GDNF in the presence of increasing amounts

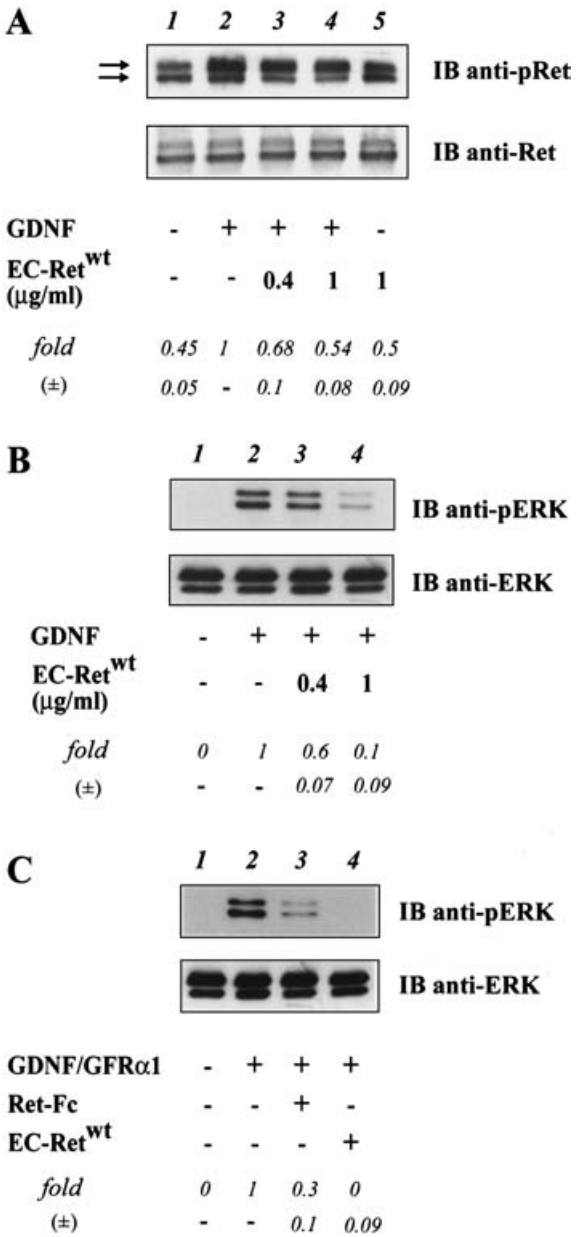

Figure 5 EC-Ret^{wt} competes with the receptor for ligand binding

(**A**, **B**) PC12-α1/wt cells were starved for 16 h and then treated for 10 min with preincubated mixtures (5 ml final volume of RPMI medium) including GDNF (1.6 nM) and increasing amounts of EC-Ret^{wt}. (**A**) Cell lysates were immunoblotted with anti-(phospho-Ret) antibodies (upper panel); the two autophosphorylated 170 and 150 kDa Ret bands are marked with arrows. The same filter was stripped and hybridized with anti-Ret antibodies (lower panel). (**B**) Cell lysates were immunoblotted with anti-(phospho-ERK) antibodies (upper panel); the same filter was stripped and hybridized with anti-ERK antibodies (lower panel). (**C**) PC12/wt cells were starved for 16 h and then treated for 10 min with preincubated mixtures (5 ml final volume of RPMI medium) including GDNF (1.6 nM), soluble GFRα1 (1.6 nM) and Ret-Fc or EC-Ret^{wt} (8.3 nM). Cell lysates were immunoblotted with anti-pERK antibodies (upper panel); the same filter was stripped and hybridized with anti-ERK antibodies (lower panel). In all panels, relative abundances (fold) compared with control values (set to 1; lane 2) and S.D. were calculated as reported in the legend to Figure 4 ($n = 4$).

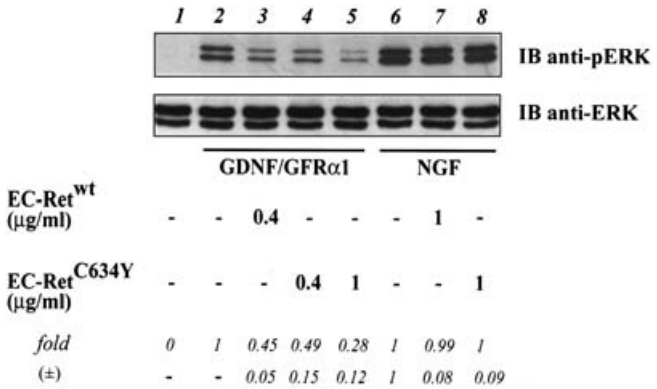

Figure 6 EC-Ret^{C634Y} competes with the receptor for ligand binding

PC12/wt cells were starved for 16 h and then treated for 10 min with preincubated mixtures (5 ml final volume of RPMI medium) including GDNF (1.6 nM), soluble GFRα1 (1.6 nM) and 0.4 μg/ml EC-Ret^{wt} or increasing amounts of EC-Ret^{C634Y}. Alternatively, the cells were treated for 5 min with preincubated mixtures (5 ml final volume) including nerve growth factor (NGF; 100 ng/ml) and 1 μg/ml EC-Ret^{wt} or EC-Ret^{C634Y}. Cell lysates were immunoblotted with anti-(phospho-ERK) antibodies (upper panel); the same filter was stripped and hybridized with anti-ERK antibodies (lower panel). The relative abundances (fold) over the controls (set to 1; lanes 2 and 6) and S.D. were calculated as reported in the Figure 4 legend ($n = 3$).

of EC-Ret^{wt}, then lysed, and lysates analysed by immunoblotting with anti-(phospho-Ret) antibodies. As shown in Figure 5(A), GDNF stimulates Ret tyrosine phosphorylation (Figure 5A, compare lanes 2 and 1), furthermore treating cells with 1 μg/ml EC-Ret^{wt} (corresponding to an ectodomain/GDNF molar ratio of 5:1) kept these levels close to basal (Figure 5A, compare lanes 4 and 1). The presence of EC-Ret^{wt} alone did not affect the Ret basal phosphorylation (Figure 5A, lane 5). This finding indicates that the EC-Ret^{wt} may compete with the Ret receptor for binding to GDNF.

We decided to determine whether the EC-Ret^{wt} could inhibit the Ret-mediated signal transmission to ERK. As shown in Figure 5(B), in PC12-α1/wt cells ERK phosphorylation was undetectable in the absence of ligand (Figure 5B, lane 1) and strongly stimulated by GDNF (Figure 5B, lane 2). EC-Ret^{wt} decreased the GDNF-induced ERK phosphorylation in a dose-dependent manner (Figure 5B, lanes 3 and 4), 1 μg/ml EC-Ret^{wt} being effective in bringing ERK phosphorylation close to the basal level (Figure 5B, compare lanes 4 and 1). No further decrease of ERK phosphorylation was achieved at a 20-fold molar excess of EC-Ret^{wt} (results not shown), possibly due to the formation of non-functional EC-Ret^{wt} multimers.

We wondered whether EC-Ret^{wt} could interfere with GDNF-induced stimulation even in cells treated *in trans* with soluble GFRα1. With this aim, we took advantage of a distinct PC12-derivative cell line expressing human Ret^{wt} (PC12/wt) adding GFRα1 *in trans*. Since it has been reported that GDNF-induced Ret activation in the presence of soluble GFRα1 is inhibited by the dimeric Ret-Fc fusion protein [5,14], we analysed the effects of EC-Ret^{wt} and, as a control, Ret-Fc on the induction of ERK phosphorylation by GDNF. As shown in Figure 5(C), GDNF induced ERK phosphorylation (Figure 5C, compare lanes 2 and 1). Consistent with results obtained in PC12-α1/wt cells, EC-Ret^{wt} inhibited the GDNF-dependent ERK phosphorylation to the basal level (Figure 5C, lane 4); monomeric EC-Ret^{wt} was even more effective than a similar amount of dimeric Ret-Fc (Figure 5C, compare lanes 4 and 3). These results strongly suggest that EC-Ret^{wt}, in spite of its monomeric status, competes with membrane-bound Ret for GDNF binding.

EC-Ret^{C634Y} inhibits the Ret^{wt}-downstream signalling initiated by GDNF

To better elucidate the inibitory effect of EC-Ret^{C634Y} we wondered whether, notwithstanding the Cys-634 mutation, EC-Ret^{C634Y} could also compete with the Ret^{wt} allele for binding to the ligand. We treated the PC12/Ret^{wt} cells with EC-Ret^{C634Y} and analysed the levels of ERK phosphorylation upon stimulation with GDNF (Figure 6). As expected, stimulation with GDNF induced ERK phosphorylation (Figure 6, compare lanes 2 and 1). Adding increasing amounts of EC-Ret^{C634Y} resulted in a drastic reduction of ERK phosphorylation (Figure 6, lanes 4 and 5) at similar extents to EC-Ret^{wt} (Figure 6, lane 3). The effect was maximal when the EC-Ret^{C634Y}/GDNF/GFRα1 molar proportions were 5:1:1. In addition, the ectodomain-induced inhibition of ERK phosphorylation was specific for GDNF-stimulated cells since it was undetectable upon nerve growth factor stimulation (Figure 6, compare lanes 7 and 8 with lane 6).

The fact that EC-Ret^{C634Y}, but not EC-Ret^{wt}, inhibits ERK phosphorylation to similar extents in PC12/Ret^{wt} and in PC12/MEN2A indicates that the EC-Ret^{C634Y} molecule acts as inhibitor of Ret^{C634Y} and Ret^{wt} receptors by two distinct mechanisms. In the case of PC12/MEN2A cells, EC-Ret^{C634Y} forms disulphide bonds with the mutated protein exposed on the cell surface, thus inhibiting the constitutive activation of the receptor. In the case of PC12/Ret^{wt} cells, EC-Ret^{C634Y} probably binds to the GDNF–GFRα1 complex, thus sequestering available ligand in the medium. This might indicate that, in spite of the Cys-634 $\rightarrow$ Tyr mutation, EC-Ret^{C634Y} is also able to bind GDNF and implicates that, in MEN2A-associated tumours, GDNF might interact with the active mutated Ret allele, possibly favouring or stabilizing dimer formation. The possible role of Ret ligands in MEN2A associated tumours, even though controversial, has been suggested by *in vitro* and *in vivo* findings [16–18].

Finally we would like to underline the fact that the functional role of the EC-Ret^{C634Y} molecule as a potential inhibitor of mitogenic effects of the Ret is encouraging for the development of a novel strategy to target the *ret* oncogene. In this respect we are currently investigating the biological effects of a long and continuous exposure of living cells to the inhibitory ectodomain molecule as a tool for suppressing the Ret^{C634Y}-transformed phenotype.

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