RET-Familial Medullary Thyroid Carcinoma Mutants Y791F and S891A Activate a Src/JAK/STAT3 Pathway, Independent of Glial Cell Line–Derived Neurotrophic Factor

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Abstract
The RET proto-oncogene encodes a receptor tyrosine kinase whose dysfunction plays a crucial role in the development of several neural crest disorders. Distinct activating RET mutations cause multiple endocrine neoplasia type 2A (MEN2A), type 2B (MEN2B), and familial medullary thyroid carcinoma (FMTC). Despite clear correlations between the mutations found in these cancer syndromes and their phenotypes, the molecular mechanisms connecting the mutated receptor to the different disease phenotypes are far from completely understood. Luciferase reporter assays in combination with immunoprecipitations, and Western and immunohistochemistry analyses, were done in order to characterize the signaling properties of two FMTC-associated RET mutations, Y791F and S891A, respectively, both affecting the tyrosine kinase domain of the receptor. We show that these RET-FMTC mutants are monomeric receptors which are autophosphorylated and activated independently of glial cell line–derived neurotrophic factor. Moreover, we show that the dysfunctional signaling properties of these mutants, when compared with wild-type RET, involve constitutive activation of signal transducer and activator of transcription 3 (STAT3). Furthermore, we show that STAT3 activation is mediated by a signaling pathway involving Src, JAK1, and JAK2, differing from STAT3 activation promoted by RETC634R which was previously found to be independent of Src and JAKs. Three-dimensional modeling of the RET catalytic domain suggested that the structural changes promoted by the respective amino acids substitutions lead to a more accessible substrate and ATP-binding monomeric conformation. Finally, immunohistochemical analysis of FMTC tumor samples support the in vitro data, because nuclear localized, Y705-phosphorylated STAT3 as well as a high degree of RET expression at the plasma membrane was observed. (Cancer Res 2005; 65(5): 1729-37)

Introduction
The RET proto-oncogene encodes a receptor tyrosine kinase which is expressed in neural crest–derived cells, including enteric and sympathetic neurons, adrenal chromaffin cells, and parafollicular C cells of the thyroid gland (1). Ligands of the RET receptor are members of the glial cell line–derived neurotrophic factor (GDNF) family: GDNF, neurturin, persephin, and artemin. They are able to activate RET in the presence of GFR-α1 glycosyl phosphatidylinositol anchored coreceptors (2–7).

Activation of RET by its ligand results in transphosphorylation of multiple tyrosine residues that, in turn, act as docking sites and interact with specific adaptor proteins to trigger downstream signaling pathways crucial for the survival and differentiation of neural crest–derived lineages, as well as kidney organogenesis (8–10). Phosphotyrosine residues 905, 981, 1,015, 1,062, and 1,096 serve as docking sites for Grb7/10, c-Src, phospholipase C-γ, Shc/Enigma/Frs2/IRIS1-2/Dok4-5, and Gbr2, respectively (11–15). In general, the signaling pathways activated by RET include Ras-mitogen-activated protein kinases, PI3K, c-Jun NH2-terminal kinase, p38, ERK-5, phospholipase C-γ, and signal transducer and activator of transcription 3 (STAT3) (16–18). Additionally, RET can activate members of the Rho family of GTPases, including Rho, Rac, and Cdc42, that are involved in the reorganization of the cytoskeleton and are responsible for cell motility and morphology (19, 20). Tyrosine residues located at the catalytic domains such as Y687, Y806, Y809, Y900, Y826, and Y1029 have also been shown to be phosphorylated in response to RET activation but their downstream signaling pathways have not yet been elucidated (21, 22).

Specific germ line missense mutations in the RET proto-oncogene leading to a constitutive activation of the receptor cause a dominant inherited cancer syndrome called multiple endocrine neoplasia type 2 (MEN2) and a familial form of medullary thyroid cancer (FMTC). Depending on the affected tissues, two different clinical subtypes of MEN2 can be recognized. MEN2A is characterized by MTC, pheochromocytoma, and hyperplasia of the parathyroid. MEN2B is characterized by MTC, pheochromocytoma, but instead of hyperplasia of the parathyroid, patients develop neuromas in tongue, lips, and eyelids, as well as intestinal ganglionneuromas. In FMTC, only the C cells of the thyroid become malignant (23).

Mutations located in the cysteine-rich domain of RET (codons 609, 611, 618, 620, 630, and 634) give rise to MEN2A and FMTC. Distinct mutations found in the cysteine kinase domain of the receptor can give rise to FMTC (codons 768, 790, 791, 804, and 891) or to MEN2B (codons 883 and 918; ref. 24). Interestingly, mutations in the cysteine-rich domain (codons 609, 611, 618, and 620) are not only found in families with MEN2A/FMTC but also in patients with Hirschsprung’s disease, a congenital malformation characterized by an absence of enteric ganglia cells in the distal part of the colon, or patients having a combination of MEN2 and Hirschsprung’s disease (25, 26).

As mentioned, FMTC mutations can be localized both in the cysteine-rich motif as well as in the catalytic domain of the protein. In
this report, we investigate the activation and downstream signaling routes triggered by two RET-FMTC mutants, Y791F (27), and S891A (28), both mutations are localized in the tyrosine kinase domain of the receptor.

Materials and Methods

Cell Lines and Cell Culture Reagents. SK-N-SH, COS-7, HepG2, HEK293, and MZ-CRC-1 (29) cells were grown in DMEM supplemented with 10% FCS (Life Technologies, Rockville, MD), 100 µg/mL penicillin, 100 µg/mL streptomycin and 2 mmol/L-glutamine. MTC-TT cells (30) were grown in RPMI supplemented with 15% FCS (Life Technologies), 100 µg/mL penicillin, 100 µg/mL streptomycin, and 2 mmol L-glutamine.

Cells were stimulated with 50 or 100 ng/mL GDNF where indicated (Prepotech, Rocky Hill, NJ). Different inhibitors were employed: U0126 (Promega, Madison, WI), AG1296, AG490, and PP2 were purchased from Calbiochem (San Diego, CA).

Expression and Reporter Plasmids. The pRC-cytomegalovirus-RETwt (RETWT) plasmid encoding the short form of the human RET proto-oncogene cDNA was used to create the RET S891A (RETS891A) and RETY791F (RETY791F) mutants by site-directed mutagenesis according to the manufacturer’s instructions (Stratagene, La Jolla, USA) using the following forward and reverse primers: RETS891A 5′-GGAAGATGAAAGATTGGCGATTTCGGCTTGC-3′ and RETS891A 3′-GGGACAAGCCGAAATCCGAAATCTTCATCTTCC-5′, RETY791F 5′-CCACATGTCAATCAAATTGTGTGGGGCTTGAGCCACGATGCCCC-3′, and RETY791F 3′-GGGCCATGCTGGCTGCAGGCCACACAATTTGATGACATGTGG-5′, respectively. Following mutagenesis, all constructs were fully sequenced.

The Mercury Pathway Profiling Luciferase System #K2049-1 (Clontech, Palo Alto, CA) containing several pathway-specific reporter constructs was used. We also tested the pIRE-ti-Luc (containing two copies of the interleukin-6 response element of the intercellular adhesion molecule-1 promoter) and pIREmut-ti-Luc reporters (17). The dominant-negative (DN) JAK1 and JAK2 constructs were kindly provided by Dr. Lu-Hai Wang (Department of Microbiology, Mount Sinai School of Medicine, New York, NY) and were described previously (31). The DN-Src plasmid was a gift from Dr. Yung H. Wong (Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China) and was described previously (32).

Luciferase Reporter Assays. Cells were transfected using the calcium phosphate precipitation method as described previously (17, 33).

Western Blotting and Immunoprecipitation. The following antibodies (1:1,000) were used in protein analyses: phosphotyrosine 4G10 (Upstate, New York, NY), RET (H-300), phospho-Y1062RET, STAT3 (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA), phospho-Y981RET (15), phospho-ERK1/2, ERK1/2, and phospho-STAT3 (Tyr705). Cell Signaling Technology, New England Biolabs, United Kingdom). Western blotting and immunoprecipitations were done as described previously (17).

Blue Native SDS-PAGE. Cells were lysed on ice with lysis buffer [10 mmol Tris-Cl (pH 7.4), 150 mmol NaCl, 2 mmol EDTA, 1% Nonidet P40, 1 mmol sodium vanadate, 10 µg/mL aprotinin, 2 µg/mL leupeptin, 0.2 mmol phenylmethylsulfonyl fluoride, 10% glycerol] and blue native-PAGE was done as described previously (34).

Immunohistochemistry. From the tumor cell lines, MTC-TT and MZ-CRC-1, 10 x 10^6 cells were fixed with buffered formalin (pH 7.4) for

Figure 1. Signaling properties of RETWT, RETY791F, and RETS891A (A, B and D). SK-N-SH cells were transfected with either RETWT, RETY791F, or RETS891A in combination with different reporter plasmids and stimulated with GDNF as indicated. Columns, mean fold induction of normalized reporter activity; bars, ± SD of triplicates. C, COS-7 cells were transfected with RETWT, RETY791F, and RETS891A as indicated. Cell lysates were subjected to RET immunoprecipitation (IP) and immunoblotting (IB) with antiphosphotyrosine and RET antibodies, respectively. COS-7 cell were transfected with RETWT, RETS891A, and RETS891A in combination with different reporter plasmids and stimulated with GDNF as indicated. Columns, mean fold induction of normalized reporter activity; bars, ± SD of triplicates. C, COS-7 cells were transfected with RETWT, RETY791F, and RETS891A as indicated. Cell lysates were subjected to RET immunoprecipitation (IP) and immunoblotting (IB) with antiphosphotyrosine and RET antibodies, respectively. COS-7 cell were transfected with RETWT, RETY791F, and RETS891A, respectively. Cell lysates were analyzed using blue native PAGE followed by Western analysis using an anti-RET antibody.
1 hour at 4°C. After washing with PBS, 150 μL plasma (Sigma coagulation control level 1; C7916) was added, followed by 50 μL thromboplastine/Ca²⁺ (Sigma, St. Louis, MO; T7280). This was incubated for 10 minutes at 37°C in a water bath. The gel was embedded in paraffin. Immunohistochemistry was done on five tumor samples from independent patients all carrying the Y791F-RET mutation, and it was done as described previously (35).

Structural Modeling of RET Cytoplasmic Tyrosine Kinase Domain. The crystal structure of the human fibroblast growth factor receptor 1 (PDB accession code 1FGK) determined at 2.0 Å resolution (36) was selected as the template to construct a model of the RET tyrosine kinase domain. The tyrosine kinase domains of fibroblast growth factor receptor 1 and RET (RETK) share 50% amino acid sequence identity. The homology models of the wild-type and mutant RETKs were constructed using MODELLER (37) version 7, as implemented in DS Modeling 1.1 (Accelrys, Inc., San Diego, CA) using standard settings. The quality of the models was evaluated using WHAT IF (38) and Procheck (39). Quality parameters of the models, such as structure Z scores and G factors, were comparable to those of the template structure. A short energy minimization using constraints on the backbone atoms was done using the CHARMM (40) module of DS Modeling 1.1.

Results

Glioma Cell Line–Derived Neurotropic Factor–Dependence of RET\(^{WT}\), RET\(^{Y791F}\), and RET\(^{S891A}\) Signaling. In order to determine the signaling properties of the wild-type RET receptor in response to GDNF, SK-N-SH cells, which are GFRα-1 positive (data not shown), were transfected with an RET\(^{WT}\) expression plasmid in combination with different luciferase reporter constructs. GDNF treatment (100 ng/mL) resulted in activation of the SRE, NF-κB, and, to a lesser extent, the IRE reporters (Fig. 1A), where the HSE, activator protein and GRE reporters were not activated. When SK-N-SH cells were transiently transfected with either RET\(^{Y791F}\) or RET\(^{S891A}\) expression plasmids in combination with RET\(^{WT}\), RET\(^{Y791F}\), and RET\(^{S891A}\), and treated or untreated with GDNF, respectively. Cell lysates were Western analyzed using antibodies against phospho-Y705-STAT3, STAT3, and RET, respectively.

Figure 2. RET\(^{Y791F}\) and RET\(^{S891A}\) induce constitutive STAT3 transactivation. SK-N-SH (A), HepG2, and COS-7 cells (B and D) were transfected with IRE and IRE-mut reporter constructs in combination with STAT3\(^{α}\), STAT3\(^{β}\), RET\(^{Y791F}\), and RET\(^{S891A}\) expression plasmids as indicated. C, HEK293 cells transfected with RET\(^{WT}\), RET\(^{Y791F}\), and RET\(^{S891A}\), and treated or untreated with GDNF, respectively. Cell lysates were Western analyzed using antibodies against phospho-Y705-STAT3, STAT3, and RET, respectively.

To determine whether the observed GDNF-independent signaling of these FMTC mutants is reflected by constitutive receptor autophosphorylation, COS-7 cells were transfected with RET\(^{WT}\), RET\(^{Y791F}\), and RET\(^{S891A}\), respectively. Cell lysates were subjected to anti-RET immunoprecipitations followed by Western analysis using anti-phosphotyrosine and anti-RET antibodies. Whereas tyrosine phosphorylation was not observed in the wild-type RET receptor; strong GDNF-independent phosphorylation was observed in both mutant receptors. Tyrosine phosphorylation of both mutant receptors was not further increased by GDNF stimulation (data not shown).

These results supported the findings obtained in the reporter assays where these mutants activated reporter activity in the absence of GDNF.
Monomeric and Dimeric RET Proteins. Iwashita et al. (41) showed that RET$^{s891a}$ functions as a monomeric oncoprotein. Whether the Y791F mutant signals as a monomer or as a dimer is at present unknown (24). In order to determine the monomeric/dimeric state of RET$^{Y791F}$, COS-7 cells were transfected with wild-type and mutant RET expression plasmids, after which cell lysates were analyzed using blue native gel electrophoresis and Western blotting. RET$^{c634r}$, a known dimer, RET$^{s891a}$ (monomeric) and RET$^{wt}$ (monomeric) were included as controls. From these studies, we concluded that, like the S891A mutant, the Y791A mutant functions as a monomeric receptor (Fig. 1C).

STAT3 Activation by RET$^{Y791F}$ and RET$^{S891A}$. GDNF-independent activation of the IRE reporter by RET$^{Y791F}$ and RET$^{S891A}$ was observed. As the IRE reporter contains specific binding sites for members of the STAT family of transcription factors (42) and because it has previously been shown that the RET receptor contains two STAT3-specific docking sites (Y752 and Y928; ref. 17), we determined whether the observed IRE transactivation by RET$^{Y791F}$ and RET$^{S891A}$ was mediated by STAT3. For this purpose, SK-N-SH cells were transiently transfected with an IRE-Luc reporter in combination with STAT3$^a$ and RET$^{S891A}$. In case the IRE reporter was cotransfected with RET$^{S891A}$, a 5-fold increase in IRE reporter activity was observed. When STAT3$^a$ was also included, IRE reporter activation increased to 20-fold. Overexpression of STAT3$^a$ alone had no effect on reporter activity (Fig. 2A). In case STAT3$^b$, a splice variant of STAT3$^a$ which lacks the COOH-terminal tail of the molecule, was cotransfected, RET$^{S891A}$-induced activation of the IRE luciferase reporter progressively decreased when increasing amounts of STAT3$^b$ were used (Fig. 2A). These results indicate that activation of the IRE reporter by RET$^{S891A}$ is mediated by STAT3. Furthermore, when an IRE reporter was used in which the STAT3 binding sites are mutated (IRE-mut-Luc), no reporter activation was observed on cotransfection with RET$^{S891A}$. To confirm that these results are not cell type–dependent, we did similar experiments using HepG2 and COS-7 cells. In both cell lines, potentiation of IRE activity in response to RET$^{S891A}$ by STAT3$^a$ and inhibition of IRE-Luc activation by STAT3$^b$ was again observed (Fig. 2B). When RET$^{Y791F}$ was used, similar results as observed with RET$^{S891A}$ were obtained (Fig. 2D).

To further substantiate that FMTC-RET mutants can activate STAT3, the effect of these mutants on STAT3$^Y$-705 phosphorylation was determined. HEK293 cells were transiently transfected with RET$^{wt}$, RET$^{Y791F}$, and RET$^{S891A}$ expression plasmids, treated in the

![Figure 3](image-url)
presence (50 ng/mL) and in the absence of GDNF, and cell lysates were Western analyzed. Both FMTC mutants (RET<sup>Y791F</sup> and RET<sup>S891A</sup>) induced an increase in STAT3-Y705 phosphorylation levels where overexpression of RET<sup>WT</sup> did not result in STAT3 phosphorylation. STAT3 tyrosine phosphorylation induced by both FMTC mutants was not further increased by GDNF stimulation (Fig. 2C).

**RETY791F and RETS891A Activate STAT3 through a Src-, JAK1-, and JAK2-Dependent Pathway.** To determine whether the activation of STAT3 by RET<sup>Y791F</sup> and RET<sup>S891A</sup> was mediated by the tyrosine kinase domain of RET exclusively (17) or whether other cytoplasmic kinases contribute to this process (43, 44), different inhibitors of known STAT3 activators were used in SK-N-SH cells transiently expressing RET<sup>S891A</sup> in combination with the IRE-Luc reporter (Fig. 3A). First, we showed that the tyrosine kinase inhibitor AG1296 (20 and 40 μmol/L) reduced IRE reporter activity in a dose-dependent manner suggesting that the kinase domain of RET is indeed involved in STAT3 activation. To confirm that AG1296 was indeed inhibiting RET activity, cells expressing RET<sup>S891A</sup> were treated with increasing amounts of AG1296 (1, 5, 10, 20, and 40 μmol/L, respectively) and they were Western analyzed using a phospho-RETY981 antibody. A clear reduction of RET tyrosine 981 phosphorylation, at concentrations of 20 and 40 μmol/L, was observed, demonstrating that indeed AG1296 was inhibiting RET activity/phosphorylation. This effect, reduction of phosphorylation, was also observed in downstream RET-dependent signaling protein pathways such as STAT3 and ERK1/2 (Fig. 3A).

When cells were treated with JAK inhibitor AG490 (20 and 40 μmol/L), a dose-dependent decrease in IRE reporter activity was observed, suggesting that besides RET, JAK is also involved in the activation of STAT3. Moreover, the treatment of cells with Src inhibitor PP2 (30 nmol/L) led to a reduction of IRE reporter activity as well. Furthermore, the mitogen-activated protein/extracellular signal-regulated kinase inhibitor U0126 (10 μmol/L) partially inhibited IRE reporter activity, suggesting the involvement of a mitogen-activated protein/extracellular signal-regulated kinase/mitogen-activated protein kinase pathway in regulating the transcriptional activity of STAT3 through phosphorylation of serine 727 (45). The same experiment was done with STAT3α overexpression, and the same results were obtained (data not shown).

However, none of the inhibitors completely abolished IRE reporter activation by FMTC-RET, suggesting that there is interplay between these molecules during signaling. To further confirm the involvement of Src and JAKs, we determined the effect of dominant-negative forms of Src (32), JAK1 (containing a three-amino acid change in the conserved region VIII of the kinase domain) and JAK2 (containing a lysine to alanine mutation in the ATP binding site; ref. 31) on IRE reporter activation by RET<sup>Y791F</sup> and RET<sup>S891A</sup>. Overexpression of DN-Src resulted in decreased IRE reporter activity by both FMTC mutations in a dose-dependent manner (Fig. 3B). IRE reporter activity was also markedly decreased by overexpression of either DN-JAK1 or DN-JAK2 (Fig. 3D). These results suggest that RET<sup>Y791F</sup> and RET<sup>S891A</sup> activate STAT3 through a pathway involving Src, JAK1, and JAK2.

Similar experiments were done with RET<sup>C634R</sup>. In contrast to the results obtained for the FMTC mutant, the Src inhibitor PP2 does not have any effect on IRE reporter activation by RET<sup>C634R</sup> (Fig. 3C).

The same results were obtained using a dominant-negative Src...
We determined the phosphorylation status of tyrosine 981, the tyrosine residue involved in Src activation. We show that Tyr-981 is more strongly phosphorylated in both FMTC mutants than in the case of RET C634R, when compared with the level of tyrosine 1062 phosphorylation, which proved to be more phosphorylated in the case of the RET C634R mutant compared with both FMTC mutants. It has to be noticed that the higher levels of phospho-Y981 displayed by RET S891A when compared with the RET Y791F are supported by the dominant-negative data, where the S891A mutant showed a higher sensitivity for DN-Src. These findings suggest that tyrosine 981 in the FMTC mutants plays a key role in Src activation.

The JAK inhibitor AG490 shows a slight inhibition of the IRE reporter activation by C634R (Fig. 3C), but to a lesser extent than FMTC-mediated activation, which was almost completely inhibited. Using DN-JAK1 and DN-JAK2 (data not shown), we were able to observe that this slight inhibition could be JAK1-dependent as no effect on the IRE reporter was observed for DN-JAK2, whereas DN-JAK1 was slightly inhibited. From these experiments, we concluded that indeed, RET C634R activates STAT3 through a pathway independent of Src and JAK2.

Immunohistochemical Analysis of RET in Familial Medullary Thyroid Carcinoma Tumor Samples and Tumor Cell Lines. Analysis of tumor sections from five patients carrying germ line RET Y791F mutation with an anti-RET antibody displayed a high degree of RET staining at the plasma membrane (Fig. 4A-C). Paraffin-embedded tumor cell lines MTC-TT (MEN2A) and MZ-CRC-1 (MEN2B) were also analyzed for RET expression as positive controls, showing a high degree of the RET receptor at the plasma membrane as well (Fig. 4D).

Immunohistochemical Analysis of Phospho-Y705-STAT3 and STAT3 in Familial Medullary Thyroid Carcinoma Tumor Samples and Tumor Cell Lines. As mentioned in the previous paragraph, staining of the same tumor samples and cell lines with anti-STAT3 and anti–phospho-Y705-STAT3 showed high levels of phosphorylated STAT3 in the nucleus in all samples (Fig. 5A-C), whereas STAT3 was detected both in the cytoplasm and the nucleus (Fig. 5D). These results indicate strong activation of STAT3 in MTCs.

Structural Modeling of RET Cytoplasmic Tyrosine Kinase Domain. Structural models of wild-type and mutant RETKs were obtained by comparative modeling using the tyrosine kinase domain of fibroblast growth factor receptor 1 as template. The models show the characteristic bilobate structure of tyrosine kinase catalytic domains. Like the inactivated tyrosine kinase domains of fibroblast growth factor receptors 1 and 2, the model of wild-type RETK shows a relatively open ATP binding site (Fig. 6A). Residue Ser901 is located at the beginning of the activation loop in the cleft between the
NH$_2$- and COOH-terminal lobes and just proximal to the conserved DFG motif (residues 892-894). Comparison between the inactivated (46) and activated (47) structures of the insulin receptor kinase domain shows that at the position before the DFG motif, the activation loop conformations begin to diverge. The activation loop in the inactive conformation blocks access of peptide substrate to the active site and aspartic acid 892 in RET is not properly positioned for interaction with the $\beta$-phosphate group of ATP through coordination with Mg$^{2+}$. Mutation of Ser$^{891}$ in RET to the smaller hydrophobic alanine possibly permits the activation loop to change to a conformation compatible with peptide substrate binding, as well as a proper interaction with the ATP, in the monomeric state (Fig. 6B). The other FMTC mutation, Y791F, is located in the $\beta$4 strand of the NH$_2$-terminal lobe and is spatially located near the conserved WE motif and is partly solvent exposed. Phosphorylation of this residue has not been shown (21). The Y791F mutation probably disrupts interactions with surrounding Glu$^{805}$ and Lys$^{789}$ residues, resulting in a conformational change of the loop connecting the $\alpha$C helix and the $\beta$4 strand. Through the subsequent spatial rearrangement of the $\alpha$C helix, the conformation of the activation loop changes into the active state by interaction of $\alpha$C helix residues with phenylalanine 893 of the DFG motif (Fig. 6C).

**Discussion**

Many groups have investigated how mutations in the RET proto-oncogene can result in constitutive active receptors and which substrates and signaling routes are being activated by these aberrant proteins. Finding mutation-specific alterations in RET signaling properties might help us to understand how different RET-associated cancer syndromes develop. Nevertheless, the oncogenic activation and downstream signaling pathways activated by FMTC mutations located within the catalytic domain of RET have been just partially uncovered (41, 48).

We now show that RET$^{Y791F}$ is active as a monomeric protein and that both RET$^{Y791F}$ and RET$^{S891A}$ are phosphorylated and activated independently of GDNF. Results similar to the observations reported for RET$^{S891A}$ (41). Signaling by the monomeric RET$^{Y791F}$ and RET$^{S891A}$ proteins is GDNF-independent and strongly targets the STAT3 signaling pathway. Although there is evidence that RET-induced signaling is cell type–specific (24), our results indicate that the activation of STAT3 by both RET$^{Y791F}$ and RET$^{S891A}$ mutants was not cell type–dependent as four different cell lines (HepG2, HEK293, COS-7, and SK-N-SH) gave similar results. Previously, it was reported that the oncogenic transactivation of STAT3 by RET$^{C634R}$ (a mutation found in MEN2A patients) was required for cellular transformation, and this process was mediated by the intrinsic

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**Figure 6.** Structural modeling of the RET tyrosine kinase domain affected by mutations Y791F and S891A. Schematic representation of modeled RET-TK showing the NH$_2$-terminal lobe on the left-hand side and the COOH-terminal lobe on the right-hand side. The activation loop is depicted in dark blue and the loop connecting the $\alpha$C helix and the $\beta$4 strand in yellow (A). Activation loop conformation of inactive RET-TK (dark blue) and activated RET-TK (dark gray) diverge at residue Ser$^{891}$. Activation loop in the inactive conformation blocks access of peptide substrate to the active site and Asp$^{892}$ is not properly positioned for interaction with the $\beta$-phosphate group of ATP through coordination with Mg$^{2+}$. Backbone of modeled RET-TK in light gray, activation loop in blue, and $\alpha$C helix in red. The superimposed activation loop of RET-TK in active conformation was modeled using activated insulin tyrosine receptor kinase as template structure. ATP analogue AMP-PNP and tyrosine peptide substrate (purple) coordinates were also taken from this structure (B; ref. 47). Local environment around tyrosine 791: NH$_2$-terminal lobe backbone residues in light gray and backbone residues of the DFG motif in dark gray, $\alpha$C helix in red, and loop connecting the $\alpha$C helix and the $\beta$4-strand in yellow (C).

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tyrosine kinase domain of RET, independently of JAKs and Src (17), data we largely confirm (see Results). Here, we show, from the use of either specific inhibitors or dominant-negative forms of Src, JAK1, and JAK2, that Src and JAKs are implicated in STAT3 activation by RETY791F and RETS891A.

Our results suggest that different disease phenotype–associated RET mutations activate STAT3 through different signaling routes. Nevertheless, it is still unclear if the interaction between c-Src, JAKs and both RETY791F and RETS891A is promoted by direct phosphorylation through the catalytic domain of the receptor itself, or by another parallel mechanism of signaling. Overexpression of JAK1, JAK2, and c-Src in combination with both RETY791F and RETS891A in the absence of STAT3 did not increase IRE reporter activity significantly, but expression of v-Src did enhance it (data not shown), suggesting that aberrant signaling through STAT3 also requires the catalytic domain of the receptor ‘per se’, as it is mediated by a Src/JAK-dependent mechanism that could not be directly linked to the RET receptor itself.

The oncogenic mechanism of activation promoted by the investigated monomeric FMTC-RET mutations located within the catalytic domain differs from the MEN2A/FMTC mutations located at the cysteine-rich motif. The extracellular mutations lead to the formation of covalent dimers due to intermolecular disulfide bounds between RET monomers. Hence, the receptor is activated constitutively and independently of GDNF (49). FMTC mutations affecting the catalytic domain of RET, however, seem to have an effect similar to the MEN2B mutations. These mutations result in monomeric oncoproteins, altering both the catalytic activity and substrate specificity of the receptor due to structural changes of the binding pocket of the tyrosine kinase domain. They lead to an aberrant phosphorylation of substrates preferred by cytoplasmic tyrosine kinases such as c-Src and c-abl (50). We have seen such an effect in RETY791F and RETS891A mutant proteins. However, RETM918T (MEN2B) has been also shown to be GDNF-responsive, suggesting that differences in the mechanism of receptor activation combined with differences in GDNF responsiveness of these receptors, as well as tissue-specific GDNF expression (or related ligands), could give rise to different disease phenotypes (24).

STATs are a family of latent transcription factors that become activated in response to cytokines and growth factors (43). IFN, granulocyte colony-stimulating factor, and cytokines activate STAT3 through their receptors in a JAK-dependent manner, whereas growth factor receptors can phosphorylate STAT3 through their intrinsic tyrosine kinase domains (43, 44). STAT3 has been implicated in the oncogenesis of many types of tumors, such as leukemia, lymphomas, multiple myeloma, breast cancer, and head and neck cancers. In all of these cases, constitutive, ligand-independent activation of STAT3 has been detected (51). In this study, we show that STAT3 plays an important role in the development of MTC, not only based on in vitro data, but also based on the immunohistochemistry analysis of MTCs. Tumors from patients carrying the RETY791F germ line mutation showed a clear nuclear localization of activated STAT3 (phospho-Tyr705) in contrast to normal cells where STAT3 is not phosphorylated and mainly cytoplasmic localized. Therefore, we hypothesize that aberrant STAT3 activation by both FMTC mutants could promote the dysregulation of the transcriptional control of genes playing an important role in tumorigenesis, contributing to the malignant transformation of the C cells resulting in MTC. Immunohistochemical analysis of tumor tissues also revealed high levels of RET protein at the plasma membrane, similar to those observed in MTC-derived cell lines, MTC-TT and MZ-CRC-1. Interestingly in FMTC tumor tissues, cytoplasmic localized RET was also observed. We assume, therefore, that the antibodies also recognize the immature form of the receptor located in the cytoplasm. It remains unclear if the immature form of RET could also be active inside the endoplasmic reticulum while it is being processed and transported to the plasma membrane. How RET, which is normally activated upon dimerization of two proteins, can be active as a monomer is yet unclear. We therefore modeled the RET catalytic domain using the tyrosine kinase domain of the fibroblast growth factor receptor 1 as a template. Our modeling studies suggest that the mutations result in a modification of the tertiary structure of the catalytic domain giving rise to a protein with a more accessible substrate and ATP conformation.

In summary, these results show that FMTC-RET mutants Y791F and S891A constitutively activate STAT3 in vitro, a finding supported by observations in tumor material from FMTC patients. Constitutive ligand-independent signaling of these mutant receptors through STAT3 might contribute to the development of medullary thyroid carcinomas.

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References


Activation of STAT3 by RET in FMTC


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