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Plaza-Menacho, Ivan

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Chapter 3

Oncogenic RET induces STAT3Ser727 phosphorylation via a RAS/RAF/MEK1-2/ERK1-2 pathway

Submitted

Ivan Plaza-Menacho¹, Tineke van der Sluis², Harry Hollema², Oliver Gimm³, Anthony I. Magee⁴, Charles H. C. M. Buys¹, Robert M. W. Hofstra¹ and Bart J. L. Eggen⁵

¹Department of Medical Genetics ²Department of Pathology, University Medical Center Groningen, The Netherlands. ³Department of Surgery, Martin Luther University, Germany. ⁴Division of Biomedical Sciences, Imperial College, London, UK. ⁵Department of Developmental Genetics, University of Groningen, The Netherlands.
ABSTRACT

Aberrant activation of STAT3 by mutant RET is observed in Multiple Endocrine Neoplasia type 2 (MEN2) associated tumors. This activation is mediated by phosphorylation of Tyr705 which causes STAT3 dimerization and nuclear translocation. In the nucleus, STAT3 transcriptional activity can be enhanced by phosphorylation of Ser727. In this study we show that oncogenic RET, but not wild type RET, induced STAT3-Tyr705 phosphorylation. In addition, oncogenic RET induced constitutive STAT3-Ser727 phosphorylation, whereas RET wild type was only able to induce phosphorylation of Ser727 in a ligand-dependent manner. Using chemical inhibitors, dominant negative constructs and westerns analyses we determined that RET induced STAT3-Ser727 phosphorylation via a pathway involving RAS-RAF-MEK1/2-ERK1/2. We could further show that this STAT3-Ser727 phosphorylation enhanced transcriptional activation of the STAT3 target genes CYCLIND1, ICAM1 AND BCL-XL. Moreover, inhibition of the ERK1/2 pathway resulted in decreased phosphorylation of STAT3-Ser727 and inhibited proliferation of a MEN2-derived tumor cell line. Immunohistochemical analyses of MEN2 tumor samples showed strong nuclear staining of phosphorylated ERK1/2 and Ser727 phosphorylated STAT3. These data show that dysregulation of both the STAT3 and RAS/MAPK pathways by oncogenic RET converges on STAT3-Ser727 which could play an important role in the development of MEN2 tumors.
INTRODUCTION

Signalling by the receptor tyrosine kinase RET is crucial for the development of neural crest-derived cells lineages and kidney organogenesis (1). In the presence of GFR-α(1-4) coreceptors, RET is activated by members of the Glial Derived Neurotrophic Factors (GDNF) family (1). Different activating missense mutations in the RET proto-oncogene cause Multiple Endocrine Neoplasia type 2 (MEN2), a dominant inherited cancer syndrome affecting several neuroendocrine tissues (1). Three different clinical subtypes can be recognized depending on the affected tissues and mutations found: MEN2A, MEN2B and FMTC (1). The molecular mechanisms connecting the mutated receptors with the different clinical subtypes are largely unknown despite clear phenotype-genotype correlations (2).

In MEN2, aberrant STAT3 activation through Tyr705 phosphorylation by mutated RET receptors has been reported (3-5). STAT3 is a latent transcription factor implicated in several types of cancer when aberrantly activated (6-7). Activation of STAT3 is triggered by phosphorylation of Tyr705 in its SH2 domain, resulting in dimerization and nuclear translocation. STAT3 Tyr705 phosphorylation can be mediated by Janus kinases or by growth factor receptors directly (6, 8-9). Moreover, phosphorylation of STAT3 on Ser727, situated in the C-terminal transactivation domain (TAD), results in enhanced transcriptional activation and DNA binding capacity (9). Various kinases have been shown to phosphorylate STAT3 on Ser727, depending on the cytokines and growth factors involved and the cellular context. Examples are: ERK1/2 (10), MSK1 (11), c-Jun N-terminal kinase (12), p38 (9) and TAK1-Nemo-like kinase (13).

In this study we delineated the signalling pathway connecting the RET receptor to STAT3 Ser727 phosphorylation, its biological consequences in RET-mediated tumor cell proliferation and extended our findings to tumor samples from patients carrying germ line activating RET mutations.
MATERIALS AND METHODS

Cell lines and cell culture reagents

HEK293 (human embryonic kidney) and MTC-TT (human thyroid medulla carcinoma) cells were grown as described previously (5), TGW (human neuroblastoma) cells (14) were cultured in RPMI medium containing 10% FCS (Gibco), further supplemented with 100 IU/ml penicillin, 1 mg/ml streptomycin and 2 mM L-glutamine (Invitrogen). To generate stably transfected HEK293 cell lines, \(10^6\) HEK293 cells were plated in 10 cm dishes and transfected with 1µg of RET wild type, RETY791F or RETS891A expression plasmid. 24 hr after transfection, cells were washed and cultured in medium containing 500 µg/ml of G418 (Sigma). Clones were picked after two weeks and screened for RET expression by western analysis. Cells were serum-starved for 1 hour prior to GDNF stimulation (15 min, 50 ng/ml, Prepotech, Rocky Hill, NJ, USA). AG1296, LY and U0126 (Promega, Madison, USA) were used as indicated in the text.

MTT proliferation assays

MTC-TT cells were plated in quadruplicate in 96-well plates (25,000/well) and treated with U0126 (2, 10 and 40 µM). MTC-TT cell proliferation was followed for five days using an MTT assay according to the manufacturer’s (Roche) instructions.

RNA extraction, cDNA synthesis and RT-PCR

The RNAeasy protect mini kit (Qiagen) was used to extract total RNA from HEK293 cell lines. For cDNA synthesis, 200 ng of total RNA was used with ready-to-go-your-prime first strand beads (Amersham Biosciences) using oligo-dT primers as indicated by the manufacturer. For RT-PCR, \(FOS\) (F 5'-TGCCAACTTCATTCCCAGGGT-3', R 5'-TAGTTGGTCTGTCTCGCTTG-3'), EGR-1 (F 5'-TTTGCCAGGAGCGATGAAC-3', R 5'-CCGAAGAGGCCACAACACTT-3') and ACTIN (F 5'-GCTCGTCGTCGACAACGGCT-3', R 5'-CAAAACATGATCTGGGTATCTTCTTCT-3') primers were used and PCR reactions were performed under standard conditions.

Expression and reporter plasmids

The expression plasmids pRC-CMV-RETwt (RETWT), RETS891A, RETY791F, pGL5-STAT3α, STAT3S727A and STAT3β were described previously (5). The dominant negative
(dn) RAS, dominant negative RAF, dominant negative SEK1, pUAS-luc, pGAL4-ELK-1, and the full length promoter-luciferase construct of CYCLIND1, ICAM1 were kindly provided by members of the Department of Hematology (UMCG, Groningen, The Netherlands). The reporter plasmids pTAL-SRE-luc, pIRE-ti-Luc and pDM2-LacZ were described previously (5,15) and the BCL-XL-promoter-luciferase construct was kindly provided by the Department of Pulmonary diseases AZU, Utrecht, The Netherlands.

**Luciferase reporter assays**

HEK293 cells were transfected using a calcium phosphate method as described previously (3, 15). Briefly, 24 hr after transfection, cells were washed and treated with inhibitors (overnight) as indicated, the next day; cells were harvested in lysis buffer (Promega). Luciferase activity was determined using the SteadyLite HTS kit (Perkin Elmer). In all transfections, a β-galactosidase expression plasmid (pDM2LacZ) was included to normalize luciferase activities. β-Galactosidase activity was determined in 100 mM Na2HPO4/NaH2PO4, 1 mM MgCl2, 100 mM 2-mercaptoethanol and 0.67 mg/ml O-nitrophenylgalactopyranoside.

**Western blotting**

Cells were lysed with 10 mM Tris-Cl pH 7.4, 144 mM NaCl, 2 mM EDTA, 1% Nonidet P40, 2 mM DTT, 1 mM Na-vanadate, 87% glycerol, 10 μg/ml aprotinin, 2 μg/ml leupeptin, 0.2 mM PMSF. Lysates were resolved on SDS-PAGE and analyzed using western blotting and ECL (Roche). The following antibodies (1:1000) were used: RET (H-300), phospho-Tyr1062RET, STAT3 (C-20) (Santa Cruz), phospho-Tyr981RET (5), phospho-ERK1/2, ERK1/2 and phospho-STAT3 (Tyr705 and Ser727, Cell Signaling Technology, New England Biolabs, UK). For immunoprecipitation, protein A sepharose beads were coated with ERK1/2 antibodies by 1 hour rotation at 4°C. Beads were washed with lysis buffer, and added to whole cell protein extracts and rotated overnight at 4°C.

**Immunohistochemistry**

Immunohistochemistry was performed on tumor samples of independent patients all carrying germ line FMTC-RET mutations, as described previously (16).
RESULTS

Oncogenic RET induces constitutive STAT3 Tyr705 and Ser727 phosphorylation

The mutants RETY791F and RETS891A (two mutations targeting the tyrosine kinase domain of RET and associated with the FMTC phenotype) signal independent of GDNF and induce STAT3 Tyr705 phosphorylation through a pathway involving Src and JAK (5). To analyze if RETY791F and RETS891A contribute to aberrant transcriptional activity of STAT3, we investigated whether these mutants also induce STAT3 Ser727 phosphorylation. Western analyses of HEK293 cells, transfected with RETWT, RETY791F and RETS891A, indicated that both mutant receptors were activated in the absence of GDNF, as indicated by RET Tyr1062 phosphorylation. These mutants induced STAT3 phosphorylation on Tyr705 and Ser727 (Fig. 1A, left panel). Stimulation of these mutant RET receptors by GDNF did not further enhance STAT3 phosphorylation, neither on Tyr705 (5), nor on Ser727 (data not shown). To further corroborate these findings we analyzed the degree of STAT3 Tyr705 and Ser727 phosphorylation in GDNF-treated human neuroblastoma TGW cells, that express wild type RET and the GFRα1 co-receptor, as well as in a metastatic MTC-derived tumor cell line MTC-TT, that expresses oncogenic RET in which Cys 634 is mutated into an Trp. In TGW cells, stimulation with GDNF (50 ng/ml, 15 min) resulted in activation of RETWT as indicated by high levels of Tyr1062 phosphorylated RET (Fig. 1A central panel). Notably, GDNF stimulation of TGW cells did not result in STAT3 Tyr705 phosphorylation whereas increased levels of STAT3 Ser727 phosphorylation were observed (Fig.1A, central panel). In MTC-TT cells, stimulation with GDNF did not result in increased RETC634R Tyr1062 phosphorylation and also did not further enhance phosphorylation of STAT3 on Tyr705 and Ser727 (Fig. 1A, right panel). These results indicated that oncogenic RET receptors aberrantly activate STAT3 by phosphorylation of Tyr705 and Ser727.

Next, we investigated the effect of oncogenic RET-induced STAT3 Ser727 phosphorylation on the promoter activity of STAT3 target genes. A STAT3 mutant, STAT3S727A in which Ser 727 is mutated into an Ala, was expressed in HEK293 cells in combination with oncogenic RET constructs and various luciferase reporters. Activation of an IRE-reporter by RETS891A was increased by co-expression of STAT3α whereas expression of STAT3S727A reduced reporter activation (Fig. 1B).
Integration of STAT3 and ERK1/2 pathways by oncogenic RET

Figure 1

Oncogenic RET induces constitutive activation of the STAT3 pathway

A) HEK293 cells were transfected with RETWT, RETY791F and RETS891A. TGW and MTC-TT cells were stimulated with GDNF (50 ng/ml; 15 min.) as indicated. Cell lysates were western analyzed with phospho-Tyr1062 RET, RET, phospho-Tyr705 STAT3, phospho-Ser727 STAT3 and STAT3 antibodies, as indicated. B) HEK293 cells were transfected with expression plasmids and luciferase reporters as indicated. The average fold activation of normalized luciferase activity with their SD is depicted.

In order to analyze the role of Ser727 phosphorylation in RET-mediated transcriptional activation of STAT3 target genes, reporter assays were performed using the promoter regions of the CYCLIND1, ICAM1 AND BCL-XL genes fused to a luciferase reporter. In all promoters used, STAT3α potentiates reporter activation by RETS891A whereas co-expression of STAT3S727A resulted in reduced transcriptional activation compared to STAT3α (Fig.1B). From these data we concluded that STAT3 Ser727 phosphorylation contributes to
RET-induced transcriptional activation of STAT3 target genes *CYCLIND1, ICAM1* and *BCL-XL*.

**Deregulation of ERK1/2 by oncogenic RET**

STAT3 Ser727 can be phosphorylated by ERK1/2 (17) and ERK1/2 can be activated by wild type RET (18). To investigate if FMTC-RET mutants aberrantly activate the ERK1/2 pathway, we transfected HEK293 cells with RETWT, RETY791F and RETS891A, in combination with an SRE-luciferase reporter (serum response element), known to be activated by ELK-1, an established ERK1/2 target. Activation of the SRE reporter by both RET mutants was observed when compared to the negative control and RETWT, a 3- and 5-fold induction by RETY791F and RETS891A, respectively (Fig. 2A, left panel). Next, we used a GAL4/ELK-1 reporter system, in which ELK-1 is fused to the DNA binding domain of GAL4, in combination with an UAS-Luc reporter. Robust activation of the UAS-reporter by both mutants was observed (200- and 300-fold for RETY791F and RETS891A, respectively) when compared to an empty expression plasmid and RETWT-induced reporter activity (70-fold). These results indicate that the RET receptor is able to trigger activation of an ERK1/2 pathway that results in ELK-1 activation (Fig. 2A, right panel). Western analyses of HEK293 cells expressing RETWT, RETY791F and RETS891A showed increased levels of ERK1/2 phosphorylation by both mutants (Fig. 2B, left panel). When HEK293 cells that stably express RETWT were stimulated with GDNF (50 ng/ml, 15 min.), phosphorylation of both RET (Tyr1062) and ERK1/2 was observed (Fig. 2B, central panel). To further investigate activation of ERK1/2 by mutant RET receptors; we analyzed the mRNA levels of *FOS* and *EGR-1* genes in HEK293 stably expressing RETY791F and RETS891A using RT-PCR. Up-regulation of both these genes was observed in the mutant cell lines, when compared to control HEK293 and HEK293RETWT cells (Fig. 2B, right panel and data not shown). To complement these experiments, we determined the levels of ERK1/2 phosphorylation in response to GDNF in cell lines expressing either wild type RET (TGW) or MEN2A-RET (MTC-TT). In TGW cells, stimulation with GDNF (50 ng/ml, 15 min.), resulted in RET phosphorylation and activation of ERK1/2 (Fig. 2C, left panel). In MTC-TT cells, RET and ERK1/2 phosphorylation was observed in untreated cells and stimulation with GDNF had no effect (Fig. 2C, right panel). Taking these results together, aberrant activation of ERK1/2 by oncogenic RET is observed in MTCs.
Oncogenic RET activates ERK1/2, in the absence of GDNF

A) HEK293 cells were transfected with expression plasmids and reporters as indicated. The average fold activation of normalized luciferase activity with their SD is depicted. B) HEK293 cells were transfected as indicated and cell lysates were western analyzed using antibodies against: phospho-Tyr1062 RET, RET, phospho-ERK1/2, ERK1/2 and as indicated. HEK293 cells, stably transfected with RETWT, and transiently transfected with GFRα-1, were stimulated with GDNF (50 ng/ml, 10 min) as indicated. RT-PCR on cDNA from HEK293-RETY791F, HEK293-RETS891A, and HEK293 control cells, showing up-regulation of c-FOS and EGR-1 mRNA levels in mutant cell lines. C) Tumor cell lines TGW and MTC-TT were stimulated with GDNF (50 ng/ml, 15 min) and protein cell extracts were western analyzed with phospho-Tyr1062 RET, RET, phospho-ERK1/2 and ERK1/2 antibodies.
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RET induces STAT3 Ser727 phosphorylation via a RAS-RAF-MEK1/2-ERK1/2 pathway

To determine if RET-induced STAT3 Ser727 phosphorylation was mediated through a RAS/ERK1/2 pathway, HEK293 cells expressing RETS891A were treated with the tyrosine kinase receptor inhibitor AG1296. A reduction of RET Tyr981 and Tyr1062 phosphorylation was observed at concentrations of 20 µM AG1296, indicating reduced RET kinase activity (Fig. 3A). A reduction in phosphorylation levels of signaling molecules downstream of RET such as STAT3 on Tyr705 (data not shown and (5)), STAT3 Ser727 and ERK1/2 was observed (Fig. 3A). These results suggest that RET signalling is required for STAT3 Tyr705, STAT3Ser727 and ERK1/2 phosphorylation. Next, GAL4-ELK-1/UAS-Luc reporter assays were used to delineate the pathway from the RET receptor to ERK1/2 activation. Co-expression of either dominant negative (dn)-RAS or dn-RAF resulted in a complete loss of UAS-reporter activation, indicating that RAS and RAF are required for ELK-1 activation by RETY791F and RETS891A (Fig. 3B). In case the IRE-Luc reporter was used, dn-RAS and dn-RAF partially reduced reporter activation by RETS891A where expression of a dn-SEK1 construct (16) had no effect on RETS891A-induced IRE-Luc activity, indicating that RAS and RAF, but not SEK1, are involved in ERK1/2-mediated STAT3 activation. To further confirm the reporter data, we determined the requirement of RAS for ERK1/2 and STAT3 Ser727 phosphorylation in HEK293 cells expressing RETS891A. Expression of dn-RAS in these cells resulted in decreased levels of ERK1/2 and STAT3 Ser727 phosphorylation, respectively (Fig. 3B and C).

To test whether MEK1/2 (a MAPKK upstream of ERK1/2) was activating ERK1/2 in response to RETS891A, we tested the effect of the MEK1/2 inhibitor U0126 on RETS891A-induced UAS-Luc and IRE-Luc activation. U0126 (10-40 µM) reduced both UAS-Luc and IRE-Luc activation by RETS891A by approximately 70% and 50%, respectively (Fig. 3D and E). Western analysis indicated that U0126 was able to completely inhibit RETS891A-induced ERK1/2 and STAT3 Ser727 phosphorylation (Fig. 3F).
Figure 3

STAT3 Ser727 phosphorylation by a RET/RAS/RAF/MEK1-2/ERK1-2 pathway

A) HEK293 cells were transfected with RETS891A and treated with AG1296 as indicated. Western analyses were performed using antibodies against: phospho-Tyr 1062 RET, phospho-Tyr981 RET, phospho-Ser727 STAT3, STAT3, phospho-ERK1/2 and ERK1/2. Samples (+, 20 µM AG1296) were resolved on the same gel but not in adjacent lanes. B-E) Luciferase reporter assays were performed in HEK293 cells transfected with the indicated expression and reporter plasmids and treated with U0126. The average fold activation of normalized luciferase activity with their SD is depicted. B,C) Western analysis were performed with HEK293 cells transiently expressing RETS891A and dn-RAS as indicated, using phospho-Ser727 STAT3, STAT3, phospho-ERK1/2 and ERK1/2 antibodies, respectively. Samples were resolved on the same gel but not in adjacent lanes. F) HEK293 cells were transfected with RETS891A and treated with U0126 as indicated. Cell extracts were western analyzed with antibodies against phospho-Ser727 STAT3, STAT3, phospho-ERK1/2 and ERK1/2 as indicated. Total cell lysates from TGW, stimulated with GDNF (50 ng/ml, 15 min.), and MTC-TT cells were subjected to immunoprecipitation using ERK1/2 antibodies, and western analyzed with STAT3 antibody (IP: ERK1/1, IB: STAT3). Uncoated beads were used as a control and total cell lysates were probed against ERK1/2 (Totals).
To demonstrate that ERK1/2 and STAT3 form a complex during RET signalling, immunoprecipitation was performed in TGW (-/+ GDNF) and MTC-TT cells. Whole cell lysates were immunoprecipitated using ERK1/2 antibody and western analyzed for STAT3. Complex formation between STAT3 and ERK1/2 was seen in GDNF-stimulated TGW cells and in untreated MTC-TT cells (Fig. 4G). From these results we concluded that RET-induced STAT3 Ser727 phosphorylation was mediated by a RAS/RAF/MEK1-2/ERK1-2 pathway.

**Immunohistochemical analysis of tumors samples**

Analysis of tumor samples from five patients carrying a RET S891A germline mutation was performed. All tumor samples displayed high levels of RET expression at the plasma membrane and in the cytoplasm (Fig. 4A and B). Due to space limitations, representative staining of only one biopsy are shown. A biopsy section containing both normal and tumor tissue was taken as a control. In the area containing normal tissue, highly organized tissue with low levels of RET expression was observed (Fig. 3C). In the area containing the carcinoma, disorganized tissue with high levels of RET expression were detected (Fig. 3C, see arrows). Strong nuclear staining for Ser727 phosphorylated STAT3 was seen in all tumor tissues (Fig. 3D, E). Weak staining was observed for Ser727 phosphorylated STAT3 in areas lacking tumor tissue where strong staining was detected in the area containing tumor tissue (Fig. 3F, see arrows). Finally, strong nuclear and cytoplasmic phospho-ERK1/2 staining was observed in MTC tumor samples (Fig. 3H, I and J, see arrows). In areas where no tumor tissue was present, low levels of phosphorylated ERK were detected (Fig. 3J).

**ERK1/2 activity is required for STAT3 Ser727 phosphorylation and MTC-TT cell proliferation**

To investigate the biological significance of the interaction between STAT3 and ERK1/2 for RET mediated tumorigenesis, we determined the effect of the MEK1/2 inhibitor U0126 on the proliferation rate of MTC-TT cells (that express RETC634W). Treatment of MTC-TT cells with U0126 (2, 10 and 40 µM) resulted in a dose dependent inhibition of proliferation (Fig. 5A). In parallel, STAT3 Ser727 and ERK1/2 phosphorylation levels were determined in MTC-TT cells treated with U0126 (10 µM, 1 hr) or the PI3K inhibitor LY (20 µM, 1 hr).
Integration of STAT3 and ERK1/2 pathways by oncogenic RET

Figure 4

Immunohistochemical analysis of MTC- tumor samples
A,B,D,E,G,H) Medullary Thyroid Carcinoma (MTC) biopsies (100x, left panels), (400x, right panels) from patients carrying a germ line RETS891A mutation stained with anti RET, phospho-Ser727 STAT3 and P-ERK1/2 antibodies as indicated. The frame in the left panels indicates the position of the enlargement shown in the right panels; bar is 100 µm. C, F and I). As controls, sections of MTC biopsies containing both normal and tumor tissue are shown. Higher levels of RET, phospho-Ser727 STAT3 and phospho-ERK1/2, as well as disorganized tissue, was observed in the areas containing the carcinoma (see arrows).

Inhibition of the ERK1/2 pathway by U0126 resulted in a loss of STAT3 Ser 727 phosphorylation, whereas the PI3K inhibitor LY had no effect on ERK1/2 and STAT3 S727 phosphorylation (Fig. 5B).

These data indicate that ERK1/2 is required for STAT3 Ser727 phosphorylation and for proliferation of MTC-TT tumor cells.
ERK1/2 activity is required for STAT3 Ser727 phosphorylation and proliferation of MTC-TT cells

A) MTC-TT cells were treated with U0126 (2, 10 and 40 µM) to determine the requirement of ERK1/2 activity for MTC-TT cell proliferation. A representative experiment is depicted; the average of quadruplicates with the SD is given.

B) Western analysis of MTC-TT cells treated with LY (a PI3K inhibitor) and U0126 (a MEK1/2 inhibitor) with phospho-Ser727 STAT3, STAT3 and ERK1/2 antibodies as indicated.
DISCUSSION
Aberrant activation of STAT3 in MEN2 was shown for MEN2A- (3), MEN2B- (4) and FMTC-associated (5) mutations in the RET proto-oncogene. Activation of STAT3 is triggered by phosphorylation of Tyr705 localized in an SH2 domain (8), which causes STAT3 dimerization and translocation to the nucleus. Once in the nucleus, transcriptional activation of STAT3 can be enhanced by phosphorylation of Ser727, located in the carboxy-terminal transcriptional activation domain (10-12). Whether STAT3 Ser727 phosphorylation was induced by FMTC-RET mutants and if STAT Ser727 phosphorylation contributes to aberrant STAT3 transactivity in MEN2 was unknown and has been addressed in this study. First, we show that oncogenic RET is able to induce STAT3 phosphorylation at both Tyr705 and Ser727, independent of GDNF. However, STAT3 Tyr705 phosphorylation was not induced by GDNF-activated wt RET receptors, whereas phosphorylation of STAT3 on Ser727 was observed. These results suggest that STAT3 Tyr705 phosphorylation is specifically associated with oncogenic RET. Moreover, assays using the IRE-luciferase reporter and the promoter regions of STAT3 target genes CYCLIND1, BCL-XL and ICAM-1 as STAT3 responsive reporters demonstrated that mutation of STAT3 Ser727 to Ala reduced the levels of RET-mediated STAT3 transcriptional activity of these targets genes (Fig. 1B). Secondly, we demonstrated that oncogenic RET is also able to constitutively activate the ERK1/2-MAPK pathway as SRE- and Elk1/UAS-luciferase reporter activation was induced by RETY791F and RETS891A, in the absence of GDNF. Western blotting and RT-PCR analysis corroborated the reporter data, showing increased levels of phosphorylated ERK1/2 as well as induction of the ELK1 targets genes FOS and EGR1. Levels of ERK1/2 phosphorylation and SRE and ELK1 reporter activation displayed by RETS891A were higher than RETY791F. These results correlated with the levels of receptor activation and the degrees of STAT3 activation observed (Fig. 1A). These data suggest that the S891A mutant has a higher capacity to trigger proliferative signals. Transformation assays performed in 3T3-NIH cells indeed showed higher transforming capacity of RETS891A then of RETY791, and confirmed the potential oncogenic properties of RETY791F (data not shown). We were able to delineate a RET-mediated signalling pathway promoting STAT3 Ser727 phosphorylation, involving RAS, RAF, MEK1/2 and ERK1/2. These finding were obtained using dominant negative (dn) intermediates of the RAS/MAPK signalling pathway such as dn-RAS and dn-RAF, as well as with the MEK1/2 inhibitor U0126 in luciferase reporter
experiments, immunoprecipitation and western analyses. Expression of dn-RAS (RasN17) and dn-RAF abolished RETY791F- and RETS891A-induced activation of the UAS-luciferase reporter by GAL4-ELK-1 and decreased the phosphorylation levels of ERK1/2 (Fig. 3B). Activation of the IRE reporter by RETS891A and the STAT3 Ser727 phosphorylation levels were reduced by dn-RAS (Fig. 3B), dn-RAF and U0126, indicating that the ERK1/2 pathway is playing a role in the regulation of the RET-STAT3 signaling pathway. The fact that the IRE reporter was not completely blocked by U0126 at concentrations where the SRE and ELK1 reporters were almost completely inhibited is most likely explained by the fact that STAT3-Ser727 phosphorylation potentiates transcriptional activation by STAT3 but is not absolutely required for STAT3 activity. The same results are obtained by expression of a STAT3S727A mutant, which is less transactive than wild type STAT3, but still acts as a transcriptional activator (Fig. 1B). A STAT3 splice variant, STAT3β, that lacks the C-terminal transactivation domain, behaves more like a dn-STAT3 in our studies, as overexpression of this construct could reduce levels of IRE reporter activation by RETS891A (5). Immunoprecipitation and western analyses confirmed our reporter data as we were able to demonstrate GDNF-induced complex formation between ERK1/2 and STAT3 (Fig. 3F) as well as functional association between ERK1/2 and STAT3, as U0126 completely abrogated RET-induced ERK1/2 phosphorylation and reduced the levels of RET-induced STAT3 Ser727 phosphorylation (Fig. 3F). However, a complete inhibition of RET-induced ERK1/2 phosphorylation was obtained at 10 µM U0126 where at this concentration; a partial inhibition of STAT3 Ser727 phosphorylation was observed (Fig. 3F). This result suggests that other, non-MEK1/2-ERK1/2 pathways might be involved in mediating oncogenic RET induced STAT3 Ser727 phosphorylation.

We have tried to investigate such alternatives pathways using the dn-SEK1 (19) in our luciferase reporter experiments (Fig. 3C) and the PI3K inhibitor (LY) in western analysis (Fig. 5B), which indicated that these pathways are not involved in RET-induced STAT3 Ser727 phosphorylation.

Immunohistochemical analyses of tumor samples from patients carrying a germ line RET S891A mutation supported our in vitro data since high levels of RET expression at the plasma membrane was observed in combination with strong nuclear staining of both phospho-Ser727 STAT3 and phospho-ERK1/2. These data, in combination with previous
Integration of STAT3 and ERK1/2 pathways by oncogenic RET

studies (5), suggest that activation of both the ERK1/2 and STAT3 pathways occurs in vivo in MTCs.

To obtain some insight in the biological consequence of STAT3 Ser727 phosphorylation, we performed proliferation assays using MTC-TT cells (expressing MEN2A mutant RETC634W), treated with increasing amounts of the MEK1/2 inhibitor U0126. Inhibition of ERK1/2 resulted in both a loss of STAT3 Ser727 phosphorylation and a dose dependent inhibition of MTC-TT cell proliferation (Fig.5).

In conclusion, our data show that aberrant activation of STAT3 by oncogenic RET involves not only the constitutive activation and nuclear translocation of STAT3 by Tyr705 phosphorylation but that these mutants further enhance the transcriptional activity of STAT3 by phosphorylation of Ser727 through a RAS-RAF-MEK1/2-ERK1/2 pathway (Fig. 6). These data suggest that dysregulation of both the STAT3 and the Ras/MAPK pathways by oncogenic RET converge on STAT3 at Ser727, which could play an important role in the development of Medullary Thyroid Carcinomas and gives new insights in the signalling networks causing this type of cancer.

**Figure 6**

A model for constitutive oncogenic STAT3 activation by RET

Oncogenic RET induces Tyr705 STAT3 phosphorylation, STAT3 transcriptional activity is further enhanced by Ser727 phosphorylation via a RAS/ERK1/2 pathway. In contrast, wild type RET does not trigger activation of STAT3 by Tyr705 phosphorylation, but does, in response to GDNF, promote Ser727 STAT3 phosphorylation by the RAS/MAPK pathway.
REFERENCES


