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ORIGINAL ARTICLE

Self-association of the transmembrane domain of RET underlies oncogenic activation by MEN2A mutations

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In patients with medullary thyroid carcinoma (MTC) and type 2A multiple endocrine neoplasia (MEN2A), mutations of cysteine residues in the extracellular juxtamembrane region of the RET receptor tyrosine kinase cause the formation of covalent receptor dimers linked by intermolecular disulfide bonds between unpaired cysteines, followed by oncogenic activation of the RET kinase. The close proximity to the plasma membrane of the affected cysteine residues prompted us to investigate the possible role of the transmembrane (TM) domain of RET (RET-TM) in receptor–receptor interactions underlying dimer formation. Strong self-association of the RET-TM was observed in a biological membrane. Mutagenesis studies indicated the involvement of the evolutionary conserved residues Ser-649 and Ser-653 in RET-TM oligomerization. Unexpectedly, RET-TM interactions were also abrogated in the A639G/A641R double mutant, first identified in a sporadic case of MTC. In agreement with this, no transforming activity could be detected in full-length RET carrying the A639G and A641R mutations, which remained fully responsive to glial cell-line-derived neurotrophic factor (GDNF) stimulation. When introduced in the context of C634R – a cysteine replacement that is prevalent in MEN2A cases – the A639G/A641R mutations significantly reduced dimer formation and transforming activity in this otherwise highly oncogenic RET variant. These data suggest that a strong propensity to self-association in the RET-TM underlies – and may be required for – dimer formation and oncogenic activation of juxtamembrane cysteine mutants of RET, and explains the close proximity to the plasma membrane of cysteine residues implicated in MEN2A and MTC syndromes.
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Introduction

The receptor tyrosine kinase RET is the signaling subunit of the receptor complex for members of the glial cell-line-derived neurotrophic factor (GDNF) family of ligands, a small family of neurotrophic growth factors, which in addition to GDNF, also includes neurturin, artemin and persephin. In the receptor complex, RET does not bind the ligand directly but recognizes it in complex with members of a family of glycosyl-phosphatidyl inositol-anchored receptors termed GDNF family receptor (GFR)α1–4, which function as the ligand-binding subunit of the complex (Airaksinen and Saarma, 2002). RET signaling is essential for kidney development, and for the development and function of the sympathetic, parasympathetic, sensory and enteric nervous systems (Manie *et al*., 2001; Airaksinen and Saarma, 2002). RET was originally discovered as an oncogene (Takahashi and Cooper, 1987), and both loss- and gain-of-function mutations in the *Ret* gene (Manie *et al*., 2001; Airaksinen and Saarma, 2002) led to different forms of human disease (van Heyningen, 1994). Gain-of-function dominant *Ret* mutations are found in patients with sporadic or inherited forms of medullary thyroid carcinoma (MTC or FMTC, respectively), as well as types 2A and 2B multiple endocrine neoplasias (MEN2A and MEN2B) (Donis-Keller *et al*., 1993; Mulligan *et al*., 1993, 1994). MEN2A is characterized by MTC as well as pheochromocytomas, whereas MEN2B comprises, in addition to the MEN2A clinical phenotype, oral neuromas, ganglioneuromatosis as well as skeletal abnormalities. In contrast, loss-of-function *Ret* alleles constitute the major genetic component of Hirschsprung disease (HSCR), a congenital developmental condition affecting 1/5000 newborns, which is characterized by incomplete innervation of the distal part of the gut (Edery *et al*., 1994).

The underlying molecular mechanisms leading to this diverse array of diseases are only partially understood. MEN2A and FMTC cases are typically caused by mutation of one of six extracellular cysteine residues – that is, 609, 611, 618, 620, 630 and 634, respectively – located in the juxtamembrane region of the receptor, very close to the plasma membrane. Whereas MEN2A is nearly always caused by mutations affecting cysteine codons in the *Ret* gene, this is less often so in MTC, where a substantial number of cases cannot be

accounted for by mutations in the *Ret* gene (Marx, 2005). The mutation of a cysteine residue leaves the unpaired partner cysteine free to form intermolecular disulfide bonds, thereby generating covalent receptor dimers that display constitutive activity (Asai *et al*., 1995; Santoro *et al*., 1995). Mutations affecting Cys-634 are the most highly transforming, and 85% of patients with MEN2A carry mutations in this codon (Eng *et al*., 1996). Although insertions or deletions affecting non-cysteine residues have also been found in a few unique MEN2A cases, these are likely to affect native disulfide formation in the cysteine-rich domain (CRD) of RET (Jhiang *et al*., 1996; Hoppner and Ritter, 1997; Hoppner *et al*., 1998). In addition, several non-cysteine mutations are known for sporadic MTC/FMTC and rare cases of MEN2A. These affected residues are present in the tyrosine kinase domain and include Glu-768, Leu-790, Tyr-791, Val-804 and Ser-891. In general, these mutations have a lower transforming ability than those involving cysteines in the extracellular domain (Iwashita *et al*., 1999). Finally, MEN2B, the most aggressive form of the *Ret*-associated cancers, is usually caused by a M918T mutation in the kinase domain, although in rare cases, A883F as well as a double V804M/Y806C mutation have also been found in MEN2B patients (Gimm *et al*., 1997; Miyauchi *et al*., 1999). Unlike cysteine mutants or FMTC-associated kinase mutants, kinase activation in MEN2B is independent of RET dimerization and phosphorylation of Tyr-905 in the activation loop (Iwashita *et al*., 1999), and thus mechanistically distinct from RET activation by MEN2A mutations or ligand binding.

HSCR mutations leading to RET loss-of-function fall into three major categories: (i) intracellular mutations that impair RET kinase activity (Carlomagno *et al*., 1996; Iwashita *et al*., 2001); (ii) intracellular mutations that affect the binding of downstream effector molecules (Geneste *et al*., 1999) and (iii) extracellular mutations that affect the folding of the RET extracellular domain, thereby preventing RET from reaching the plasma membrane (Carlomagno *et al*., 1996; Ellgaard and Helenius, 2003; Kjaer and Ibáñez, 2003). Interestingly, mutation of either Cys-609, Cys-611, Cys-618 or Cys-620 can lead to a dual loss- and gain-of-function phenotype with impaired surface localization of RET – resulting in loss of ligand responsiveness and HSCR – and constitutive kinase activation leading to FMTC or MEN2A. Mutation of either Cys-630 or Cys-634 does not affect cell surface expression of RET, and have a higher transforming capacity (Chappuis-Flament *et al*., 1998; Takahashi *et al*., 1999; Arighi *et al*., 2004).

Why would mutant RET molecules carrying an unpaired cysteine residue dimerize with each other rather than with other cellular proteins? Although the RET CRD extends over 120 residues and contains 16 conserved cysteines, all known mutations leading to constitutive disulfide-mediated receptor dimerization have been found within 25 residues of the plasma membrane. This suggested the possible involvement of the RET transmembrane (TM) domain (RET-TM) in non-covalent receptor–receptor interactions that may contribute to keep receptor molecules in proximity of each other and allow RET homodimers to be formed by either cysteine mutations or ligand binding.

Results

Only mutations in extracellular cysteine residues close to the plasma membrane lead to disulfide-linked RET dimer formation

The CRD of human RET contains 16 of the 28 cysteine residues found in the RET extracellular domain. However, all disease-causing mutations are located within 25 residues of the RET-TM (spanning residues 636–657) (Figure 1). This suggested that proximity to the TM domain could be important for the correct alignment that is required for disulfide formation. In agreement with this notion, a number of mutations involving cysteine residues located relatively far from the RET-TM have been found in HSCR patients, including R77C, Y96C, C142S, C157S/W/Y, C197Y, W324C and C570W, none of which lead to aberrant dimerization of RET molecules (Kashuk *et al*., 2005). Both close spatial proximity – that is, <3 Å (Kubatzky *et al*., 2005) – and correct relative orientation are required for the oxidation reaction of the sulfhydryl groups of two unpaired cysteines to occur. From examination of the disease databases (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM), it was furthermore noted that mutations in Cys-630 and Cys-634 are exclusively found in patients with MEN2A or MTC, but never in patients presenting a dual MEN2A/HSCR phenotype, suggesting that these two cysteine residues may normally be pairing with each other.

Self-association of the RET-TM in a biological membrane

In order to test the possibility that the RET-TM may be able to associate with itself and thus contribute to stabilize receptor–receptor interactions, we employed the ToxCAT system (Russ and Engelman, 1999), a modified version of the ToxR assay (Langosch *et al*., 1996), which allows to monitor and assess the efficiency of TM–TM interactions in a biological membrane. The reporter system exploits the ability of the ToxR transcription activator of the *Vibrio cholerae* pathogen to bind the cholera toxin (*ctx*) gene promoter only when dimerized. A TM segment of interest fused to ToxR is delivered to the bacterial inner membrane by fusion to maltose-binding protein (MBP). Varying amounts of ToxR dimers will be formed in the cytosol in direct proportion to the oligomerization ability of the TM domain. Binding of dimerized ToxR to the *ctx* DNA element triggers expression of a chloramphenicol transferase (*cat*) gene reporter and production of CAT protein, which can then be quantified by enzyme-linked immunosorbent assay (ELISA). The ToxR and ToxCAT systems have been previously applied to demonstrate self-association of a range of TM domains from glycophorin A (GpA) (Russ and Engelman, 1999, 2000;

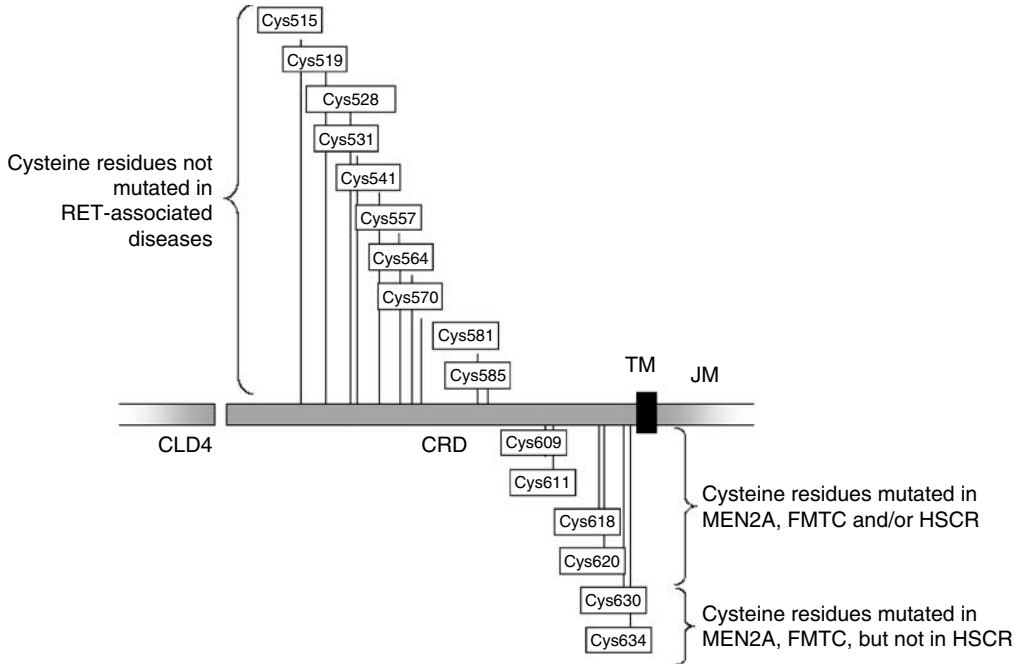

Figure 1 Only mutations in juxtamembrane extracellular cysteine residues lead to disulfide-linked RET dimer formation. Representation of cysteine residues present in the CRD of RET. The cysteines are evolutionarily conserved with the exception of Cys-515 and Cys-531, both of which are absent in RET sequences from Pufferfish (Fugo) (Accession no. AAD10845), Green Pufferfish (Accession no. CAG04714) and Zebrafish (Accession no. CAA64146) (Kashuk *et al*., 2005). Cysteines not mutated in RET-associated genetic diseases (HSCR, MTC, MEN2A or MEN2B) are listed above the bar. Cysteines implicated in MEN2A, FMTC or HSCR are shown below the bar (from OMIM 164761). The disease-associated cysteines are further distinguished between those that may lead to dual gain- and loss-of-function phenotypes (i.e. Cys-609, Cys-611, Cys-618 and Cys-620), and those only found in patients with MEN2A or FMTC (Cys-630 and Cys-634). CLD4, cadherin-like domain 4.

Kubatzky *et al*., 2001), integrin α_{IIb} (Li *et al*., 2004), ErbB1–4 (Mendrola *et al*., 2002) and the Epo receptor (Kubatzky *et al*., 2001). Other studies have shown good agreement between ToxCAT values and biophysical measurements of TM–TM interactions in both bacterial and mammalian membranes (Kubatzky *et al*., 2001; Sulistijo *et al*., 2003; Li *et al*., 2004).

The importance of the length of the inserted TM domain has been indicated in several studies using the ToxCAT system (Li *et al*., 2004; Roy *et al*., 2004). In our case, RET-TM constructs were chosen to encode residues 635–657 based on computer algorithms (http://www.ch.embnet.org/software/TMPRED_form.html), using alignments of RET-TM domains from a range of different species (Figure 2). Arg-635 was included to act as a stop signal at the N-terminal side of the TM domain. The RET-TM was predicted to end at Ile-657; exclusion of His-658 is in agreement with the very low frequency of this residue in native RET-TM domains (Gratkowski *et al*., 2001; Zhou *et al*., 2001). RET-TM length was 22 residues, well within the typical TM range of 18–24 residues (Senes *et al*., 2000). A series of RET-TM constructs were made in pccKAN (see Materials and methods) as indicated in Figure 3a. The pccGpA-WT and pccGpA-G83I plasmid express wild-type and mutant forms of the TM domain of GpA, and serve as positive and negative controls, respectively (Russ and Engelman, 1999). The GpA TM domain is well known to self-associate primarily through its ^{79}GxxxG^{83} sequence, a motif that is overrepresented in TM domains of many proteins (Russ and Engelman, 2000; Senes *et al*., 2000). As shown in Figure 3b, the ToxR-RET-TM-MBP construct was well expressed in *malE*$^-$ MM39 cells, at levels comparable to those observed with GpA-TM domains. In addition, correct insertion of all TM constructs in the bacterial inner membrane was confirmed in maltose-complementation assays (data not shown). The construct containing human RET-TM produced a significant amount of CAT as measured by ELISA (Figure 3c), corresponding to about 60% of the value obtained with the strongly dimerizing GpA-TM sequence, indicating that the human RET-TM has a strong propensity for self-association in a biological membrane. Self-association of a comparable strength has previously been reported for the TM domains of ErbB receptors, which – like GpA – carry a typical GxxxG motif (Mendrola *et al*., 2002). To exclude the possibility that the observed RET-TM interaction was owing to disulfide bond formation through Cys-656, we expressed a ToxR-RET-TM-MBP construct carrying a C656S mutation and obtained similar results (Figure 3c).

The TM domain of Drosophila *RET self-associates with intermediate strength*

The predicted TM domain of Drosophila RET (dRET) extends from residues 697 to 716 and is highly divergent

compared to its vertebrate counterparts (Figure 2). It was therefore of interest to investigate whether it would also be capable of self-association in the ToxCAT assay. It has recently been shown that a C695R mutation in dRET – that is, analogous to the C634R mutation found in MEN2A cases – results in constitutive activation of the dRET kinase (Vidal *et al*., 2005), suggesting that dRET may also be activated through a dimerization mechanism. As shown in Figure 3c, dRET-TM was able to self-associate at 40% of the level of the GpA-TM domain. A heptad of leucine residues towards the C-terminal portion of this domain may be responsible for this effect through a 'leucine-zipper' type of mechanism as previously reported for other TM domains (Gurezka *et al*., 1999).

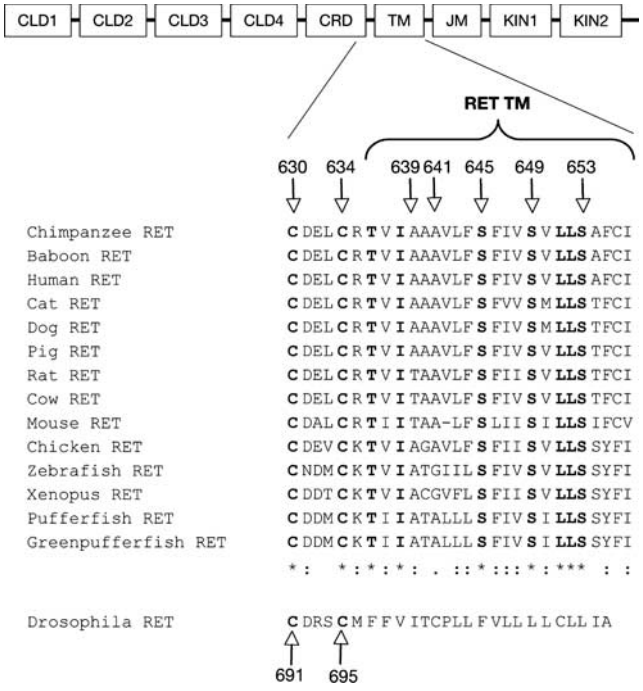

Figure 2 Sequence alignment of RET-TM domains. The sequences of the TM domain and juxtamembrane extracellular cysteines of RET proteins from a range of different species were aligned using Clustal X (Thompson *et al*., 1997). The TM domain sequence of the RET homolog from *Drosophila melanogaster* is listed (Hahn and Bishop, 2001). Selected residues discussed in the present study are indicated by arrows. Conserved residues (asterisk below alignment) are denoted in boldface. CLD, cadherin-like domain; JM, juxta membrane.

Contribution of serine residues to self-association of the RET-TM

The lack of a *bona fide* GxxxG motif in the RET-TM prompted a search for alternative determinants of TM–TM interactions in this domain. Across vertebrate sequences, only seven out of the 22 residues are conserved in the RET-TM (Figure 2). From these, we focused our attention on the serine triad consisting of Ser-645, Ser-649 and Ser-653 because of their polar nature and relative spacing – that is, $i+4$ – which would approximately place them on the same side of a membrane-spanning α-helix. In addition, serine/threonine motifs conforming to the SxxSSxxT or SxxxSSxxT consensus have previously been selected by applying the ToxCAT method to combinatorial libraries of TM domains engineered to lack GxxxG motifs (Dawson *et al*., 2002), suggesting the possible importance of such residues for TM–TM interactions. We introduced the S645A, S649A and S653A replacements in the RET-TM individually or together and tested the resulting constructs using the ToxCAT assay. As shown in Figure 4a, mutant and wild-type RET-TM fusion proteins were expressed at comparable levels. Interestingly, replacement of the central Ser-649 residue resulted in 50%

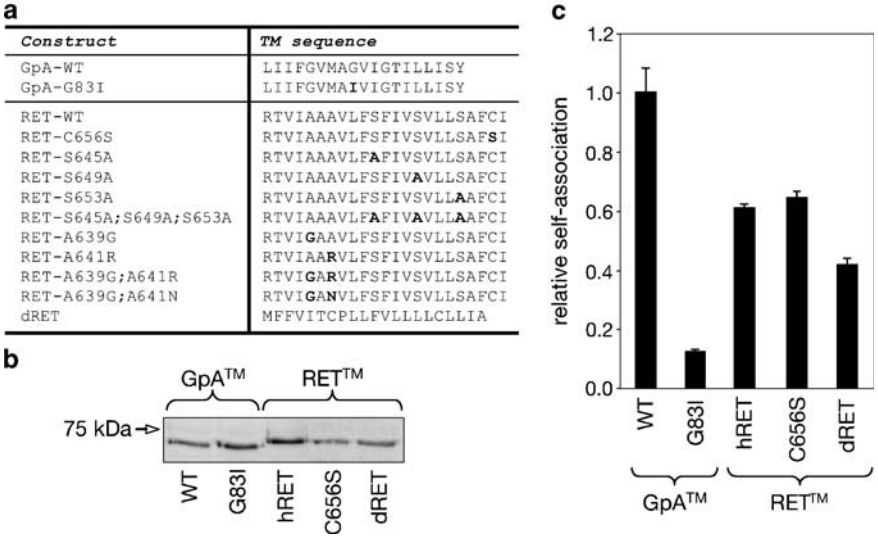

Figure 3 Self-association of the RET-TM in a biological membrane. (**a**) Sequences of GpA and RET-TM domains cloned into the pccKAN vector. Mutations are indicated in boldface. (**b**) Levels of expression of selected ToxR-TM-MBP constructs as analysed by immunoblotting. (**c**) Relative self-association of the indicated TM domain variants as measured by CAT-ELISA normalized to the wild-type GpA TM domain. Average $\pm$ s.d. of results from three independent experiments performed in triplicate are shown.

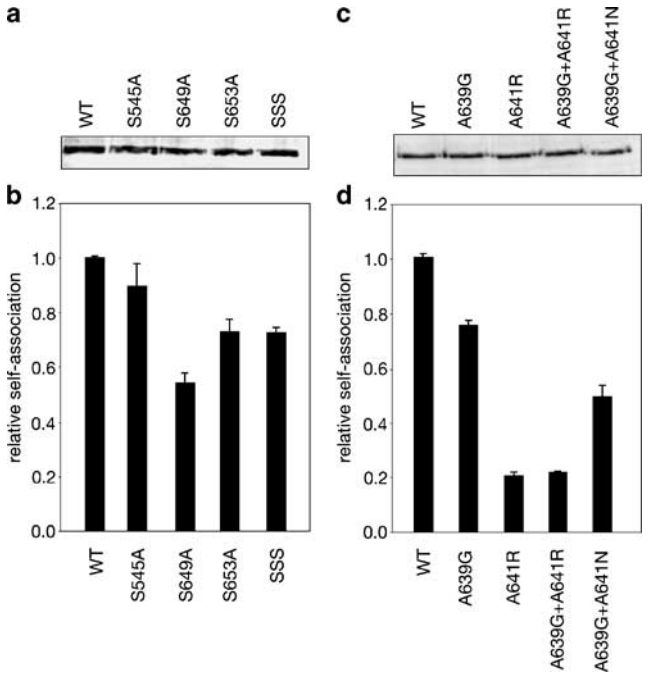

Figure 4 Determinants of self-association of the RET-TM. (**a**) Levels of expression of wild-type and mutant ToxR-RET-TM-MBP constructs as analysed by immunoblotting. SSS denotes the serine triple mutant. (**b**) Relative self-association of wild-type and mutant RET-TM variants as measured by CAT-ELISA normalized to wild-type (WT) RET-TM. Average $\pm$ s.d. of results from three independent experiments performed in triplicate is shown. (**c**) Levels of expression of wild-type (WT) and mutant ToxR-RET-TM-MBP constructs as analysed by immunoblotting. (**d**) Relative self-association of wild-type and mutant RET-TM variants as measured by CAT-ELISA normalized to wild-type RET-TM. Average $\pm$ s.d. of results from three independent experiments performed in triplicate are shown.

reduction in TM self-association compared to wild-type RET-TM (Figure 4b). Mutation of Ser-653 had an intermediate effect (30% reduction), whereas the S645A replacement had no effect (Figure 4b). Intriguingly, no additive effects were observed upon combination of the three mutations (Figure 4b). It is possible that a more favorable packing of the three introduced alanine residues may have offset the negative effects of the individual replacements, a phenomenon that has also been observed in previous structure–function analyses of TM domains (Schneider and Engelman, 2004; Senes *et al*., 2004). Together, these data suggest that Ser-649 and Ser-653 may play an important role in the self-association of the RET-TM, possibly through intermolecular hydrogen bonding within the plasma membrane.

MTC mutation A641R disrupts RET-TM self-association

A double mutation in the RET-TM – that is, A639G/A641R – has been identified in a patient with sporadic MTC (Kalinin *et al*., 2001). However, the possible effects of these replacements on RET function, and hence to what extent they may be related to tumor formation in MTC, are unknown. We generated both mutants, alone and in combination, in the context of the ToxR-RET-TM-MBP fusion construct. All mutants were well expressed as assessed by immunoblotting (Figure 4c). Unexpectedly, the A641R mutation drastically reduced RET-TM self-association in the ToxCAT assay to background levels (Figure 4d). The A639G replacement had only a minor effect, whereas the double mutant was not different from A641R alone (Figure 4d). A double mutant containing Asn instead of Arg at position 641 was also generated. This construct showed a partial recovery in dimerizing activity (Figure 4d), indicating that the positive charge of the Arg side chain – rather than the loss of the alanine – may be responsible for the disruptive effects of the A641R mutation.

Full-length RET carrying the A639G/A641R mutation is correctly expressed and activated by GDNF in mammalian cells

In order to assess the possible functional consequences of the A639G/A641R mutation, a fibroblast cell line stably expressing full-length human RET (long isoform) carrying the double replacement was generated. For this purpose, we utilized a cell line derived from MG87 fibroblasts stably expressing the GDNF co-receptor GFRα1 (termed MG87-α1 cells) to facilitate ligand stimulation of RET tyrosine phosphorylation (Besset *et al*., 2000). Full-length mutant RET was expressed at levels comparable to wild-type and displayed similar low levels of basal tyrosine phosphorylation in the absence of ligand (Figure 5a). The mutant also showed a normal ratio between mature and immature RET species (solid and open arrowheads, respectively, in Figure 5a), suggesting that processing and membrane transport were not affected by the mutations, as previously observed in other RET mutants (Kjaer and Ibáñez, 2003). Importantly, and similar to the wild-type receptor, tyrosine phosphorylation could be stimulated upon GDNF treatment in the A639G/A641R double mutant (Figure 5a). In addition, the kinetics of activation of the A639G/A641R mutant in response to ligand was indistinguishable compared to the wild-type receptor (Figure 5b), demonstrating normal ligand responsiveness and interaction with the GFRα1 co-receptor. Thus, and despite its disruptive effect on TM–TM interaction, the A639G/A641R mutation did not affect the ability of RET to be activated by GDNF and GFRα1. On the other hand, the lack of spontaneous activity in the double mutant rules out the involvement of these mutations in MTC.

Disruption of RET-TM self-association reduces homodimerization and attenuates oncogenic activation of MEN2A RET

Although ligand stimulation appeared to be able to overcome disfavorable TM–TM interactions, we tested whether the A639G/A641R mutation had any effect on RET homodimerization and activation mediated by the cysteine mutation C634R, a strongly oncogenic mutation found in most cases of MEN2A. For this purpose, MG87-α1 cells stably expressing full-length human RET

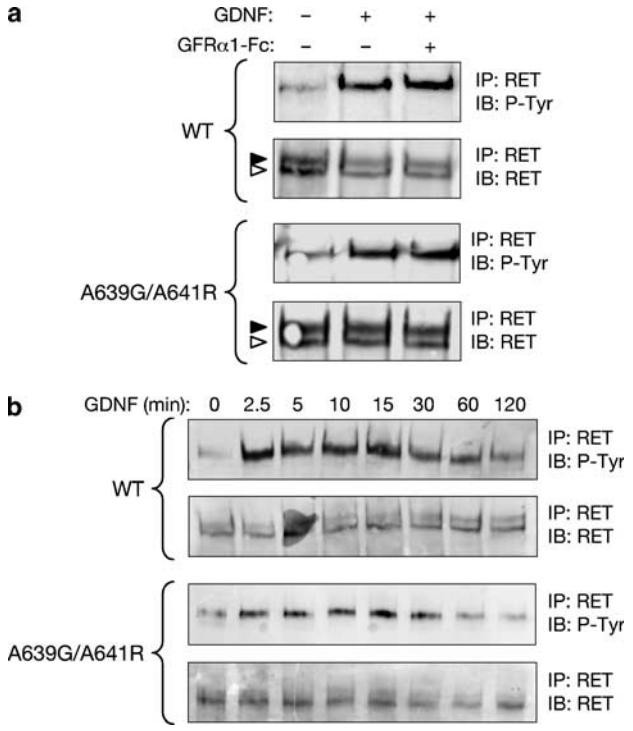

Figure 5 Full-length RET carrying the A639G/A641R mutation is correctly expressed and activated by GDNF in mammalian cells. (**a**) MG87-α1 cells expressing wild-type or A639G/A641R mutant full-length RET were stimulated with GDNF in the presence or absence of soluble GFRα1-Fc as indicated, and analysed for RET tyrosine phosphorylation by immunoprecipitation (IP) and immunoblotting (IB). Filters were then stripped and reprobed with anti-RET antibodies (lower panels). Solid and open arrowheads indicate mature and inmature RET forms, respectively. (**b**) Time course of wild-type and A639G/A641R RET activation in response to GDNF.

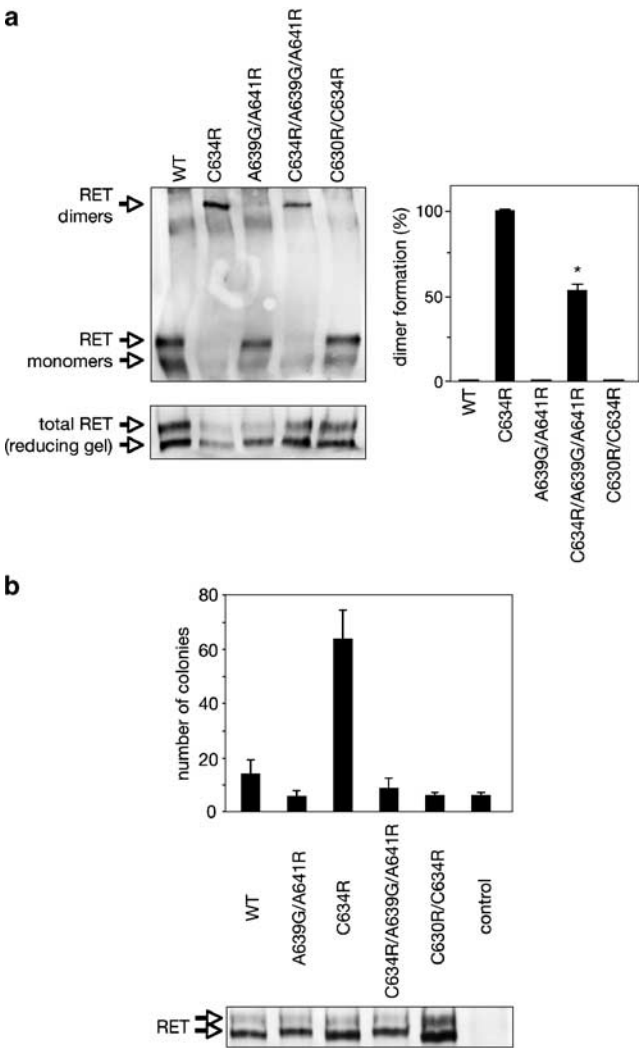

Figure 6 Disruption of RET-TM self-association prevents homodimerization and oncogenic activation of MEN2A RET. (**a**) Analysis of homodimerization of full-length RET in MG87-α1 cells transiently transfected with the indicated constructs. Cell lysates were immunoprecipitated with anti-RET antibodies and separated in non-reducing (top panel) or reducing (bottom panel) SDS/PAGE, and subjected to immunoblotting. The migration of RET monomers and dimers in non-reducing conditions are indicated. The histogram shows the quantification of dimer formation relative to the C634R mutant ($n=3$; $^*P<0.005$). (**b**) The transforming potential of MG87-α1 cells transiently transfected with different wild-type (WT) and mutant RET constructs was assessed by colony formation assay in soft agar plates. Control indicates vector-transfected cells. Average ± s.d. of the number of colonies obtained with each construct in two independent experiments performed in triplicate are shown. The levels of RET protein expressed by MG87-α1 in each condition were verified by immunoblotting (panel below histogram).

carrying all three mutations were generated. RET homodimerization was assessed by non-reducing SDS/PAGE of cell lysates. As shown in Figure 6a, high molecular weight species could be detected in RET carrying the C634R mutation but not in wild-type RET or in the A639G/A641R double mutant. Dimer formation in the C634R mutant occurred at the expense of monomeric RET species (Figure 6a), as observed previously (Bongarzone *et al*., 1998; Asai *et al*., 1999). Interestingly, RET dimer formation was significantly attenuated in the triple mutant ($53.4\pm4.7\%$, $n=3$, $P<0.005$), demonstrating the importance of TM–TM interactions for disulfide bridge formation in MEN2A RET (Figure 6a). Dimer formation was completely abrogated in the C630R/C634R double mutant (Figure 6a), in agreement with these two cysteine residues being involved in intramolecular disulfide bonding, as indicated in previous studies (Asai *et al*., 1999).

The transforming activity of mutant RET molecules was assessed by their ability to promote anchorage-independent growth of MG87-α1 cells in soft agar. As shown in Figure 6b, the ability of the different RET molecules to induce foci formation in soft agar mirrored their homodimerization capacity. In particular, strong transforming activity was observed in C634R RET, which was significantly diminished in the triple mutant and in the double cysteine mutant (Figure 6b). No foci formation activity above background was detected in the A639G/A641R double mutant (Figure 6b), confirming its lack of oncogenic activity. All constructs were expressed at comparable levels in MG87-α1 cells (Figure 6c).

Discussion

Although homodimerization has been hailed as the universal activation mechanism for growth factor receptors (Schlessinger, 2000), recent studies have demonstrated the existence of dimeric – yet inactive – complexes in a number of receptor systems (Jiang and Hunter, 1999; Livnah *et al*., 1999), indicating that a dimeric organization does not necessarily lead to receptor activation and downstream signaling. Studies on receptors for erythropoietin, epidermal growth factor, neuregulins, growth hormone and several other ligands have suggested models in which activation of preformed, inactive receptor dimers is achieved via rotational changes of TM and intracellular domains that bring kinases into a preferred orientation for signaling (Burke and Stern, 1998; Moriki *et al*., 2001; Seubert *et al*., 2003; Kubatzky *et al*., 2005). Experimental approaches based on chemical crosslinking and cysteine-scanning mutagenesis have been utilized to probe the relationship between protomer orientation and activation in receptor dimers (Burke and Stern, 1998; Horenstein *et al*., 2001; Moriki *et al*., 2001). In the case of RET, the presence of a CRD and the occurrence of cysteine mutations in patients with sporadic MTC/FMTC and MEN2A has provided a natural cysteine-scanning mutagenesis collection of RET molecules. In this study, we have tapped into the RET disease database resource (OMIM 164761) and noted that membrane proximity of the cysteine mutations is an important determinant of disulfide bridge formation among mutated RET molecules. Interestingly, a soluble RET extracellular domain carrying the C634R MEN2A mutation does not form disulfide-linked dimers (Cerchia *et al*., 2003), suggesting that membrane anchorage is required for the formation of intermolecular disulfide bonds between two mutant RET protomers.

The ability of the RET-TM to self-associate may provide an explanation for the occurrence of dimerizing, disease-causing mutations in cysteine residues that are close to the plasma membrane. The present analysis has begun to delineate a surface in the RET-TM that may contribute to receptor–receptor interactions. Assuming a α-helical conformation for the RET-TM and its immediately adjacent residues, the serine triad formed by Ser-645, Ser-649 and Ser-653 would be located in the same face of the RET-TM (Figure 7a). Cys-634 and Cys-630 appear right on top of each other on the extracellular side, in agreement with their taking part in an intramolecular disulfide bridge. Interestingly, this model presents both cysteines aligned on the same face with Ser-649, the most important residue in the serine triad revealed by our ToxCAT

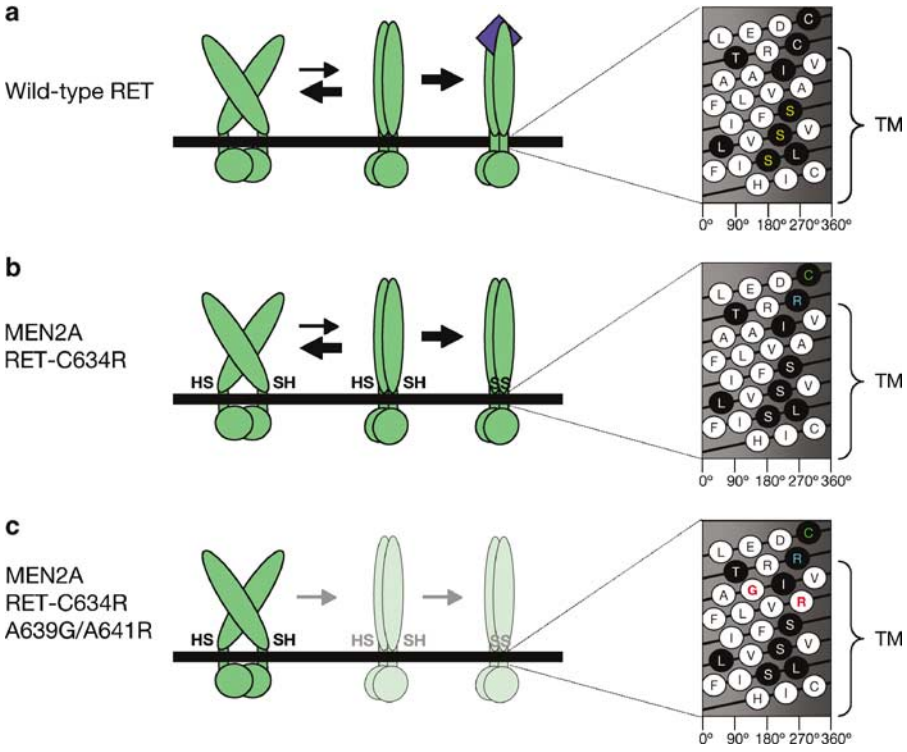

Figure 7 Model of TM–TM interactions in wild-type and MEN2A RET. Schematic representations of hypothetical equilibrium states between inactive and active RET dimers for wild-type RET (**a**), MEN2A RET (**b**) and MEN2A RET with TM double mutation A639G/A641R (**c**). The GDNF/GFRα1 complex is represented by a blue diamond in (**a**). Helical representations of the TM domain and upstream juxtamembrane residues are shown to the right. Note the alignment of Cys-630, Cys-634, Ser-649 and Ala-641 in the same face of the helix. Conserved residues are highlighted in black circles. The serine triad is indicated in yellow in (**a**). Arg-634 in MEN2A RET (**b**) is indicated in blue, and Cys-630 – involved in intermolecular disulfide bonding – is indicated in green. Mutations that destabilized TM–TM interactions are shown in red in (**c**). See text for details.

experiments, as well as A641, which when mutated into Arg completely disrupted TM–TM interactions in the ToxCAT assay. Together, these observations support an active role for the RET-TM interface delineated by Ala-641, Ser-649 and other highly conserved residues – such as Ile-638 and Leu-648 – in receptor homodimerization by disulfide bond formation through either Cys-630 or Cys-634. Although our experiments did not directly addressed whether the configuration of RET-TM oligomers revealed by the ToxCAT assay corresponds to that found in active full-length RET dimers, the fact that mutation of either Cys-630 or Cys-634 results in constitutively active receptors suggests that the RET-TM interface identified in this study corresponds to an active RET dimer conformation.

Our data do not support a causative role for the A639G/A641R mutation in MTC tumor formation. Other mutations, independently or together with A639/A641 changes, may account for the formation of such tumors. The A639G/A641R mutation may have simply been fortuitously found in a sporadic MTC tumor, or else be only weakly linked to tumor formation.

Unlike GpA, for which most of the dimerization binding energy is actually provided by the TM domain (Young and Tanner, 2003), disruption of TM–TM interactions in RET did not prevent ligand-mediated receptor activation, suggesting that the energy provided by TM interactions has a relatively smaller contribution in comparison to that provided by ligand binding, which is mainly driven by contacts in the extracellualr domain. This is in agreement with previous work on TM domains from ErbB and fibroblast growth factor receptors (Li *et al*., 2005; Stanley and Fleming, 2005), which indicated that TM–TM interactions provide contacts of a more transient nature, but which may nevertheless be important to fine-tune the arrangement of intracellular kinase domains in a productive orientation. In contrast, TM–TM interactions appeared to be critical for RET activation by dimerizing oncogenic mutations found in MEN2A. This suggests that the existence of an equilibrium between active and inactive states is, to some extent, affected by TM–TM interactions (Figure 7a). Normally, very few wild-type molecules would populate the active state in the absence of ligand. The C634R mutation allows some of these transient, active states to be trapped covalently by irreversible disulfide formation, thereby displacing the equilibrium towards an unusually high proportion of active dimers (Figure 7b). Mutations affecting RET-TM self-association – such as A641R – disrupt the equilibrium and thus prevent capture of active RET dimers by disulfide formation (Figure 7c), underlying the importance of TM–TM interactions despite their modest energetic contribution. From a therapeutic point of view, our findings suggest that lipid-soluble, small molecular weight compounds affecting RET-TM self-association could disrupt homodimer formation of mutant RET molecules without affecting ligand-mediated activities, and may thus be beneficial for the treatment of MEN2A and FMTC.

Materials and methods

DNA constructs

The TM domains of human (residues 635–657) and *Drosophila* (residues 697–716) RET were amplified by the polymerase chain reaction (PCR) with primers containing *Nhe*I restriction sites (underlined) for the sense primer (5′-ccg cag gct agc ggc cgc acg gtg atc g-3′) and a *Bam*HI site (underlined) for the antisense primer (5′-ggc gca atg gat ccc gat gca gaa ggc aga cag-3′). dRET-TM was amplified using sense primer: 5′-ccg cag gct agc ttc ttc gtg atc acg tgc cct cta ttg ttc gtt-3′; and antisense primer: 5′-ggc gca atg gat ccc cgc aat cag caa aca gag-3′. Upon digestion, PCR products were cloned into the pccKAN vector (kindly provided by Dr Donald Engelman). A639G, A641R, C630R and C634R mutations, as well as combinations thereof, were introduced into the human *Ret* gene (long isoform) cloned in the pcDNA3 vector (Invitrogen, Rockville, MD, USA) using Quick-Change mutagenesis (Stratagene, La Jolla, CA, USA). All DNA constructs were validated by automated DNA sequencing.

Antibodies

The expression levels of chimeric ToxR-TM-MBP constructs were assessed by Western blotting using anti-MBP antibodies from New England Biolabs, Beverly, MA, USA. Full-length RET expression in mammalian cells was determined using C-20 and T-20 anti-RET antibodies from Santa-Cruz, CA, USA, or a monoclonal anti-RET antibody raised against a peptide derived from the RET kinase activation loop as described previously (Abrescia *et al*., 2005). Phosphotyrosine residues were detected by the monoclonal anti-pTyr pY99 antibody from Santa-Cruz. Secondary antibodies were from Amersham Biosciences, Buckinghamshire, UK and used in conjunction with the ECF kit.

ToxCAT assay of TM self-association

Maltose-complementation assay was performed as described (Russ and Engelman, 1999) in order to determine the correct orientation and insertion in the inner membrane of constructs containing TM domains. Briefly, MalE$^-$ bacteria (*Escherichia coli* strain MM39) harboring different pccRET-TM constructs were grown in LB-Amp medium to mid-log phase, washed twice in phosphate-buffered saline (PBS) and streaked onto M9-Agar plates containing either 1% (w/v) glucose or 0.4% (w/v) maltose as the sole carbon source. Only MalE$^-$ *E. coli* with the ToxR-TM-MBP chimeras inserted such that the MBP is located in the periplasmic space will be able to grow on M9-Agar-Maltose plates. The plates were incubated for 48 h at 37°C and subsequently analysed by visual inspection. The expression levels of the ToxR-TM-MBP chimeras were determined by Western blotting. Reducing sodium dodecyl sulfate (SDS)-sample buffer was added to 20 μl of mid-log phase MM39 *E. coli* cultures transformed with ToxR-TM-MBP plasmids and boiled. The lysates were resolved on 10% SDS–polyacrylamide gel electrophoresis (SDS–PAGE) gels and transferred to polyvinylidine difluoride membranes by electroblotting. The membranes were blocked in 4% (w/v) skimmed milk powder in PBS. The MBP protein was detected by a 1:2000 dilution of anti-MBP rabbit antibodies (New England Biolabs) followed by anti-rabbit-AP-conjugated antibodies (Amersham). The blot was developed and scanned in a STORM phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA). A CAT-ELISA kit (Roche Diagnostics, Mannheim, Germany) was used to measure the amount of CAT enzyme produced in MM39 *E. coli* strains as described by McClain *et al*. (2003). Cultures

of pccRET-TM-transformed bacteria were grown to 0.5 OD_{600}. Bacteria were lysed with a drop of toluene added to 200 μl of culture followed by 30 min incubation at 30°C. Subsequent freeze–thawing cycles ensured complete lysis of the culture. Bacterial lysates were diluted 30-fold in assay buffer before CAT ELISA according to the manufacturer's instructions.

Generation of stable RET-expressing fibroblast cell lines
MG87 fibroblasts stably transfected with GFRα-1 expression plasmids (Besset *et al*., 2000) (MG87-α1) were maintained under standard conditions. Stable expression of wild-type or mutant RET receptors in MG87-α1 cells was achieved by transfection with pcDNA3-based RET constructs using Fugene6 (Roche Diagnostics) followed by selection with 500 μg/ml G418. Positive clones were identified by Western blotting using anti-RET antibodies and expanded.

Analysis of RET phosphorylation
RET phosphorylation upon ligand stimulation was examined using MG87-α1 cells expressing wild-type or mutant RET constructs. Cells were stimulated for 15 min with 50 ng/ml GDNF alone or together with 150 ng/ml GFRα-1-Fc (R&D systems, Minneapolis, MN, USA). The cells were subsequently lysed in 1% Triton X-100, TNE-buffer (50 mM Tris pH 7.5, 150 mM NaCl, 2 mM ethylenediamine tetraacetic acid (EDTA)). The lysis buffer contained phosphatase inhibitors (50 mM NaF, 10 mM β-glycerophosphate, 2 mM Na_3VO_4), as well as complete EDTA-free protease inhibitors (Roche Diagnostics) and 1 mM phenylmethylsulfonyl fluoride. For the detection of dimer formation, 10 mM iodoacetamide was added to the cell lysis and SDS-sample buffers. The level of tyrosine phosphorylation was examined by RET immunoprecipitation using C-20 and T-20 antibodies, followed by immunoblotting with anti-phosphotyrosine antibodies. Total RET levels were assessed by reprobing with an anti-RET kinase monoclonal antibody. The beads were washed, resuspended in SDS-containing sample buffer – without reducing agent for detection of RET dimers – and boiled. Samples were resolved on 6 and 7.5% SDS–PAGE gels.

Transformation assay
MG87-α1 cells were cultured as above. Sub-confluent 10 cm plates were transfected with 8 μg of RET constructs in pcDNA3. Two days after transfection, cells were plated in 0.4% (w/v) soft agar. After 2 weeks, the number of colonies was counted. The experiments were performed in triplicates and the levels of RET protein expression were confirmed by Western blotting using material from a 10 cm plate transfected in parallel.

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