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RET alternate splicing influences the interaction of activated RET with the SH2 and PTB domains of Shc, and the SH2 domain of Grb2

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Activating germline mutations of the RET receptor tyrosine kinase are found in the majority of cases of inherited cancer syndrome MEN 2, and inactivating mutations in some cases of dominantly inherited Hirschsprung disease. Using RET activated by a MEN 2 mutation, we show that both the SH2 and PTB domains of the adaptor protein Shc interact with RET, and we identify the PTB domain interaction site. Interaction with both the SH2 and PTB domains of Shc contributes to the transcriptional activation of a serum response element. *RET* alternate splicing affects the strength of interaction with both the Shc SH2 and PTB domains. In addition, a splice isoform-specific HSCR missense mutation, which does not inactivate the RET kinase activity, decreases the strength of the PTB domain interaction and the level of RET-dependent Shc phosphorylation.

Keywords: *RET*; multiple endocrine neoplasia type 2; Hirschsprung disease; Shc; Grb2

Introduction

The clinical subtypes of multiple endocrine neoplasia type 2 (MEN 2A, MEN 2B and FMTC) are caused by distinct activating germline point mutations in the RET receptor tyrosine kinase (reviewed by Ponder and Smith, 1996). MEN 2A, consisting of predisposition to medullary thyroid carcinoma (MTC), phaeochromocytoma and parathyroid hyperplasia/adenoma, is caused by mutation in one of five conserved cysteine residues in the extracellular domain (Mulligan *et al*., 1993, 1994, 1995). These mutations lead to receptor dimerisation and hence ligand-independent activation of RET (Santoro *et al*., 1995; Borello *et al*., 1995; Asai *et al*., 1995). FMTC (familial MTC), consisting of predisposition to MTC only, can be caused by mutations typical of MEN 2A and also by rarer kinase domain mutations (Eng *et al*., 1995a; Bolino *et al*., 1995). MEN 2B, which is similar to MEN 2A except without parathyroid disease, is also associated with developmental abnormalities affecting the skeleton and the peripheral nervous system. A single point mutation in the RET kinase domain causes MEN 2B (Hofstra *et al*., 1994; Eng *et al*., 1994; Carlson *et al*., 1994). This mutation leads to the ligand-independent activation of RET by an uncertain mechanism, and also to altered tyrosine phosphorylation of the receptor and altered catalytic substrate specificity (Santoro *et al*., 1995; Songyang *et al*., 1995a; Liu *et al*., 1996).

Some somatic MEN 2-type *RET* mutations have been found in the sporadic counterparts of the tumours found in MEN 2 (Eng *et al*., 1995b,c). Papillary thyroid carcinoma (PTC) is associated with RET activation by somatic rearrangement to create chimaeric proteins known as PTC-RET (reviewed by Pierotti *et al*., 1996).

Germline RET mutations are also found in some cases of dominantly inherited *H*irsch*s*prung disease of the *c*olon and *r*ectum (HSCR) (Edery *et al*., 1994; Romeo *et al*., 1994; Attie *et al*., 1995). HSCR is the congenital lack of intestinal autonomic nerve plexuses. HSCR missense mutations have been shown to inactivate the RET kinase (Pasini *et al*., 1995; Carlomagno *et al*., 1996), and many mutations lead to extreme truncations of the RET protein (Attie *et al*., 1995).

An understanding of how *RET* mutations lead to diverse phenotypes will come from a knowledge of the signal transduction pathways activated by RET. As yet little is known. Since the RET ligand has only very recently been identified (Trupp *et al*., 1996; Durbec *et al*., 1996; Treanor *et al*., 1996) studies have used either PTC-RET or an EGFR-RET chimaera as a source of activated RET in order to investigate signal transduction pathways (Borrello *et al*., 1994; Santoro *et al*., 1994; van Weering *et al*., 1995). These studies have shown that RET is able to interact with and activate the widely expressed adaptor protein Shc. Shc proteins form phosphotyrosine-dependent interactions with, and become tyrosine phosphorylated by, a number of activated growth factor and cytokine receptors and oncogenic tyrosine kinases (Crowe *et al*., 1994; Lanfrancone *et al*., 1995; Pelicci *et al*., 1992; Segatto *et al*., 1993; Yokote *et al*., 1994, reviewed by van der Geer and Pawson, 1995). The principal tyrosine phosphorylation site (Y317) of the Shc then interacts with the SH2 domain of the protein Grb2 (Buday and Downward, 1993; Crowe *et al*., 1994; Egan *et al*., 1993; Gale *et al*., 1993; Li *et al*., 1993; Rozakis-Adcock *et al*., 1992, 1993; Salcini *et al*., 1994) initiating a cascade of events which leads (via SOS, p21ras, Raf-1, MEK-1) to the activation of the MAP kinase ERK2. In cells of neuroectodermal origin, which express endogenous RET, Shc and ERK2 are activated by an EGFR-RET chimaera (van Weering *et al*., 1995). However ERK2 activation is not seen in NIH3T3 cells and ERK2 activation by RET may therefore be tissue restricted.

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Here we have used RET activated by a MEN 2A mutation to further investigate the interaction of RET with the adaptor protein Shc. We show that activated RET interacts with both the SH2 and PTB phosphotyrosine interaction domains of Shc (van der Geer *et al*., 1995; van der Geer and Pawson, 1995). We provide evidence that interaction with both the Shc PTB and Shc SH2 domains contributes to the activation of the serum response element from the c-fos promoter. The PTB interaction site (Y1062) is close to the position of an alternate splice which creates different C-termini for the RET protein (Tahira *et al*., 1990; Myers *et al*., 1995). By comparing two of the possible C-terminal isoforms of RET we show that alternate splicing is able to affect Shc interaction. In addition, we show that a splice isoform-specific HSCR missense mutation close to the Shc PTB domain interaction site does not inactivate the RET kinase, but does cause a decrease in Shc interaction *in vitro* and a decrease in Shc phosphorylation *in vivo* in transfected cells.

Results

The PTB and SH2 domains of Shc interact with activated RET in vitro*, and alternate splicing and a HSCR mutation influence the strength of interaction*

Comparison of the RET amino acid sequence and the consensus binding sequence for the Shc PTB domain (ψXNPXpY, where ψ = hydrophobic residue) (Songyang *et al*., 1995b; van der Geer *et al*., 1996) indicates a possible Shc PTB binding site at Y1062 in RET (Figure 1a) which is known to be tyrosine phosphorylated in activated RET (Liu *et al*., 1996). The site of an alternate splice (Tahira *et al*., 1990) creating RET proteins with either a nine amino acid (RET9) or 51 amino acid (RET51) C-terminus is close to the putative Shc PTB site (Figure 1a). In addition, a HSCR mutation found only in the RET51 isoform (M1064T; Attie *et al*., 1995) is close to this site. PTC-RET is known to bind to the Shc SH2 domain (Borrello *et al*., 1994) although there are no clear consensus binding sites.

Using RET proteins activated by a MEN 2A mutation (C634R) we wished to confirm binding to the Shc SH2 domain, to test binding to the Shc PTB domain and determine the interaction site, and to test if the alternate splice or the HSCR mutation influence these interactions.

RET expression constructs were transiently transfected into 293-EBNA cells, cell extracts incubated with glutathione S-transferase (GST) fusion proteins and bound RET assayed by immunoblotting with an anti-RET polyclonal antibody (Figure 1). Interaction with a GST fusion to the N terminal SH2 domain of PLCγ (GST-PLCγN) was used as a positive control. There is a consensus binding site for this SH2 domain (pYLDL; Songyang *et al*., 1993) at Y1015, and whilst this work was in progress the equivalent tyrosine in PTC-RET was reported to be the major site of interaction for this SH2 domain (Borrello *et al*., 1996). We have confirmed this as the major interaction site in MEN 2A RET (Figure 1b). 2A-RET9 and 2A-RET51 interact with GST-PLCγN. A peptide containing pY1015 competes with 2A-RET, and mutation of Y1015 to F in 2A-RET9 abolishes the interaction. In assays where we have compared different RET

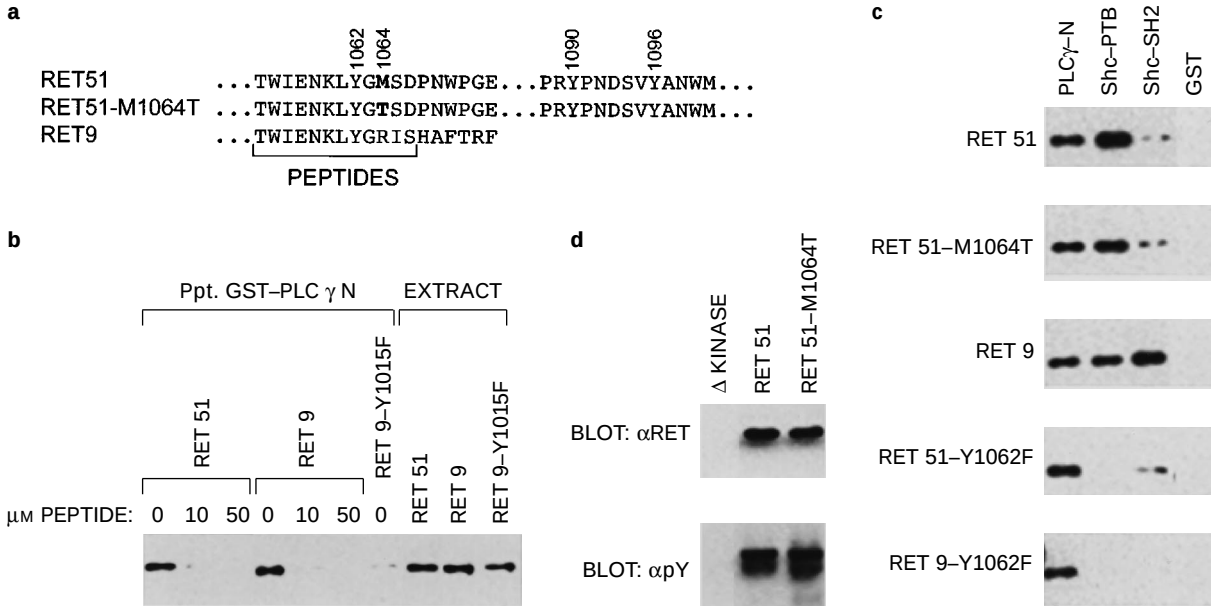

Figure 1 Alternate splicing and a HSCR mutation affect the interaction of Shc with RET. (**a**) The amino acid sequence of the C-termini of the RET9 and RET51 splice isoforms. The HSCR mutation M1064T is also illustrated. The sequence of the pY1062-containing peptides used in Figure 2 is indicated. (**b**) Extracts from 2A-RET-transfected 293-EBNA cells were incubated with equal amounts (approximately 10 μg) of GST-PLCγN immobilised on glutathione-agarose beads in the absence or presence of a peptide containing pY1015 (MVKRRDpYLDLAAS). Bound proteins were analysed by anti-RET immunoblotting following SDS–PAGE on a 7.5% gel. Whole extracts were also analysed by anti-RET immunoblotting. (**c**) Extracts from 2A-RET-transfected 293-EBNA cells were incubated with equal amounts (approximately 10 μg) of GST–PLCγN, GST–ShcPTB, GST–ShcSH2 or GST immobilised on gluathione-agarose beads. Bound proteins were analysed by anti-RET immunoblotting. (**d**) Extracts from 2A-RET-transfected 293-EBNA cells were analysed by anti-RET and anti-pY immunoblotting. ΔKinase is a control construct with the kinase domain and the anti-RET epitope deleted

constructs we have used a similar level of interaction with GST-PLCγN as an indicator of the use or expression of a similar level of activated RET.

Figure 1c shows that both 2A-RET9 and 2A-RET51 interact with GST-ShcSH2 and GST-ShcPTB fusion proteins, but not the GST negative control. Mutation of Y1062 to F in both splice isoforms abolishes interaction with GST-ShcPTB, but not GST-PLCγN indicating that pY1062 is indeed the Shc PTB domain binding site. Mutation of Y1062 to F also abolishes interaction with GST-ShcSH2 in RET 9, but not in RET51, indicating that pY1062 is a major Shc SH2 domain interaction site only in RET9.

In the assays shown in Figure 1c the level of interaction with GST-PLCγN is closely similar for all RET constructs. However, 2A-RET51 and 2A-RET9 have different abilities to bind to GST-ShcPTB and GST-ShcSH2. The RET9 isoform binds less strongly (approximately 50–60% less in several independent experiments) than RET51 to the PTB domain. As pY1062 appears to be the only PTB domain interaction site in both splice isoforms this implies that the interaction is stronger at this site in RET51. The RET51 isoform binds less strongly (approximately 70-80% less) than RET9 to the SH2 domain. As pY1062 appears to be an SH2 domain interaction site only in RET9 this suggests that the interaction with RET9 may be stronger as a result of the presence of the additional pY1062 interaction site.

The HSCR kinase domain missense mutations so far tested in activated RET have been shown to substantially reduce the autokinase activity (Pasini *et al*., 1995; Carlomagno *et al*., 1996). The extracellular domain missense mutations so far tested also lead to reduced phosphotyrosine content as a consequence of impaired protein glycosylation and transport (Carlomagno *et al*., 1996). In contrast 2A-RET51-M1064T is expressed indistinguishably from 2A-RET51 at the fully glycosylated mobility on SDS–PAGE, and does not show any decrease in the level of phosphorylation when expressed in transfected cells (Figure 1d). As a further indication of activation, 2A-RET51-M1064T binds equally well to GST-PLCγN, GST-ShcSH2 (Figure 1c) and GST-Grb2 (Figure 3). However, 2A-RET51–M1064T does show a slightly reduced capacity to bind to GST-ShcPTB (approximately 30–40% less) relative to 2A-RET51 (Figure 1c).

Interaction of pY-containing RET peptides with the Shc PTB domain in vitro

There are two possible explanations for the observations made above that RET9, RET51 and RET51-M1064T have different abilities to bind to the PTB domain of Shc. Firstly, that the PTB domain interaction site at pY1062 in RET9, RET51 and RET51-M1064T has an affinity difference at a common level of phosphorylation. Secondly, that Y1062 in these RET proteins has a different level of phosphorylation.

We have used two approaches to test for affinity differences between pY1062 in the context of the different RET proteins using pY-containing RET peptides.

Firstly, extracts from cells transiently transfected with RET constructs were incubated with GST-ShcPTB in the presence of peptides (sequences in Figure 1a) containing pY1062 in the sequence context of RET9, RET51 and RET51-M1064T. At a concentration of 25 μM, each of these peptides competes completely for binding of GST-ShcPTB to 2A-RET51 (Figure 2a). Interaction of these peptides with SchPTB is pY-dependent since pre-treatment of the peptides with phosphotyrosine phosphatases abolishes their ability to compete (not shown). As the peptide concentration is decreased to 1 μM or 5 μM it is clear that the RET51 peptide competes best, the RET51-M1064T peptide less well, and the RET9 peptide least well. These data are in accordance with the results discussed above, and suggest that the differences in binding of GST-ShcPTB to RET9, RET51 and RET51-M1064T are at least in part due to affinity differences for pY1062.

Secondly, surface plasmon resonance was used to evaluate the ability of pY1062-containing peptides to bind to GST-ShcPTB (Figure 2b). Peptide concentrations which inhibited by 50% (IC_{50}) the binding of GST-ShcPTB to a pY-containing peptide, from

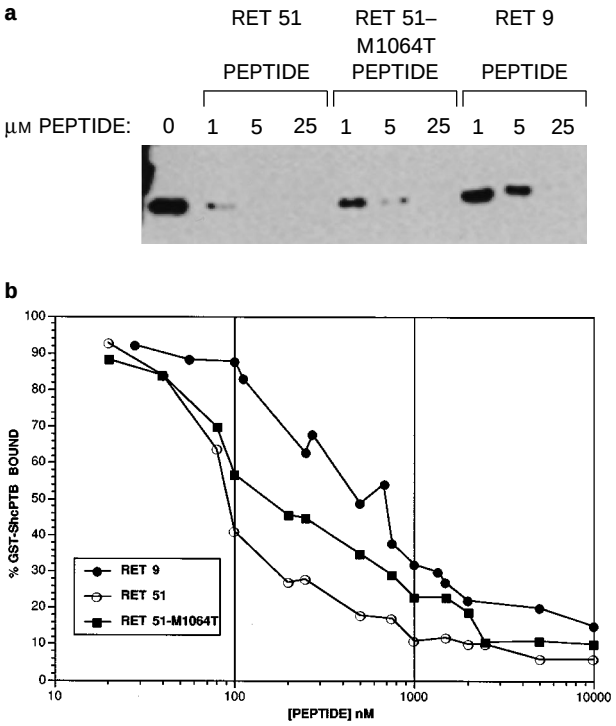

Figure 2 RET51, RET9 and RET51-M1064T peptides containing pY1062 have different affinities for the Shc PTB domain. (**a**) Extract from 2A-RET51-transfected 293-EBNA cells was incubated with equal amounts (approximately 10 μg) of GST-ShcPTB immobilised on glutathione-agarose beads in the absence or presence of the indicated concentration of pY1062-containing RET peptides. Bound proteins were analysed by anti-RET immunoblotting. Peptide sequences are indicated in Figure 1(a). (**b**) Surface plasmon resonance (SPR) (BIAcore, Pharmacia Biosensor) was used to measure binding of GST-ShcPTB to a phosphopeptide from polyoma virus middle tumour antigen immobilised on a BIAcore chip. The percentage of GST-ShcPTB bound corresponds to the SPR signal obtained at a fixed time following the injection of a 500 nM GST-ShcPTB solution containing the indicated concentration of phosphopeptide, relative to the SPR signal obtained in the absence of competing peptide

polyoma virus middle tumour antigen, are as follows: IC_{50} RET51 = 110 nM; IC_{50} RET51-HSCR = 205 nM; IC_{50} RET9 = 500 nM. These data confirm the interpretation made above. pY1062 in RET51 has greater affinity for the Shc PTB domain than the high affinity pY490 site in the NGF receptor, which has an IC_{50} of 175 nM in the same assay (van der Geer *et al*., 1996). This suggests that the interaction of RET with the Shc PTB domain is likely to be functionally significant.

The SH2 domain of Grb2 interacts with only the activated RET51 splice isoform in vitro

We have identified differences in the interaction of RET9 and RET51 with Shc *in vitro*. We also wished to compare the interaction of the SH2 domain of the adaptor protein Grb2 with the RET splice isoforms. Borrello *et al*. (1994) have found interaction between the Grb SH2 domain and both PTC-RET9 and PTC-RET51, but suggest that the Grb2 SH2 domain only binds directly to PTC-RET51 since only this splice isoform contains consensus Grb2 binding sites (pYXNX; Songyang *et al*., 1993) at Y1090 and Y1096 (Figure 1a). Liu *et al*. (1996) have identified Y1096 as the major Grb2 SH2 domain binding site in RET51, but suggest further weaker binding sites.

We have directly compared the interaction of GST-Grb2SH2 to 2A-RET9 and 2A-RET51 expressed in transiently transfected 293-EBNA cells (Figure 3). Only RET51 is able to bind to GST-Grb2SH2. Deletion of the C-terminus of 2A-RET51 (2A-RET51-Δ1089) to remove Y1090 and Y1096 completely abolishes GST-Grb2SH2 binding (but has no effect on GST-PLCγN binding) indicating that Y1090 and/or Y1096 are the only GST-Grb2SH2 binding sites. The HSCR mutation, M1064T, has no effect on GST-Grb2SH2 binding.

RET-dependent phosphorylation of Shc in transfected cells

Tyrosine phosphorylation of Shc (principally at Y317) results from its interaction with activated tyrosine kinases. In serum-starved 293-EBNA cells transiently transfected with a control construct (RET-ΔKinase) we are unable to detect Shc in anti-pY immunoprecipitates (Figure 4). This indicates that there is no detectable Shc phosphorylation. However, transfection with 2A-RET9 and 2A-RET51 leads to Shc phosphorylation.

The level of expression of active RET in transfected cells has been assessed by analysing the level of RET interaction with GST-PLCγN in anti-pY immunoprecipitates. Taking the level of active RET expression into account, Shc phosphorylation in the presence of RET9 is approximately 50% less than in the presence of RET51. Mutation of Y1062 to F in RET9, which prevents both Shc PTB domain and Shc SH2 domain interaction *in vitro*, abolishes Shc phosphorylation. Mutation of Y1062 to F in RET51, which prevents only Shc PTB domain interaction *in vitro*, reduces Shc phosphorylation by approximately 65%. This suggests that residues which are important for Shc interaction *in vitro* are important for the RET-dependent phosphorylation of Shc *in vivo* in transfected cells.

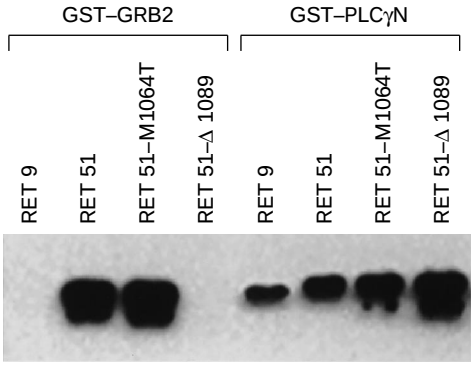

Figure 3 Alternate splicing effects the interaction of RET with the SH2 domain of Grb2. Extracts from 2A-RET-transfected 293-EBNA cells were incubated with equal amounts (approximately 10 μg) of GST-PLCγ-N or GST-Grb2SH2 immobilised on glutathione-agarose beads. Bound proteins were analysed by anti-RET immunoblotting

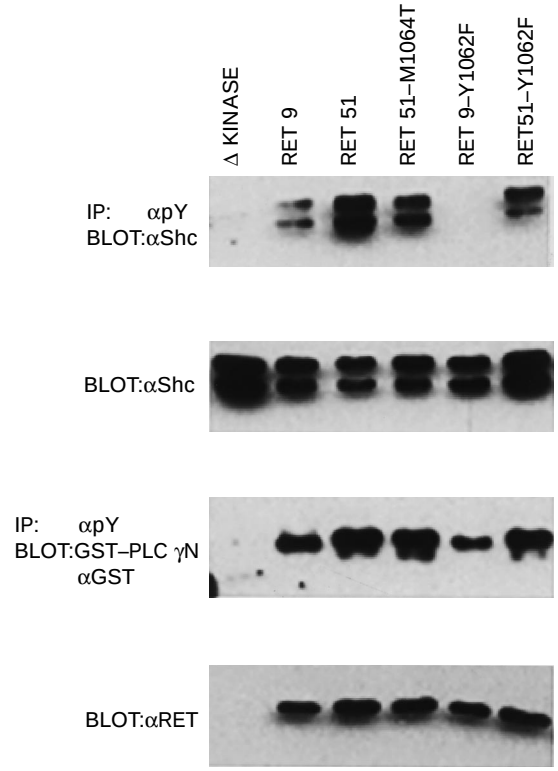

Figure 4 RET-dependent phosphorylation of Shc in transfected 293-EBNA cells. Whole extracts from 2A-RET-transfected, serum-starved 293-EBNA cells were analysed by anti-RET immunoblotting (following 7.5% SDS–PAGE) and by anti-Shc immunoblotting (following 10% SDS–PAGE). Anti-pY immunoprecipitates (IP) of extracts were analysed by anti-Shc immunoblotting and by anti-GST immunoblotting following incubation of the filter with approximately 20 μg of purified GST–PLCγN. The two bands detected by anti-Shc immunoblotting are the 52 kD (Shc-p52) and 46 kD (Shc-p46) isoforms of Shc (generated by the use of alternate initiating methionine residues). The 66 kD (Shc-p66) Shc isoform was expressed below the level of detection

The level of active RET expression is closely similar for 2A-RET51 and 2A-RET51-M1064T. However, the M1064T HSCR mutation causes an approximate 50% decrease in the level of Shc phosphorylation. This suggests that the *in vitro* difference in affinity for the Shc PTB domain, causes an *in vivo* difference in RET-dependent Shc phosphorylation in transfected cells.

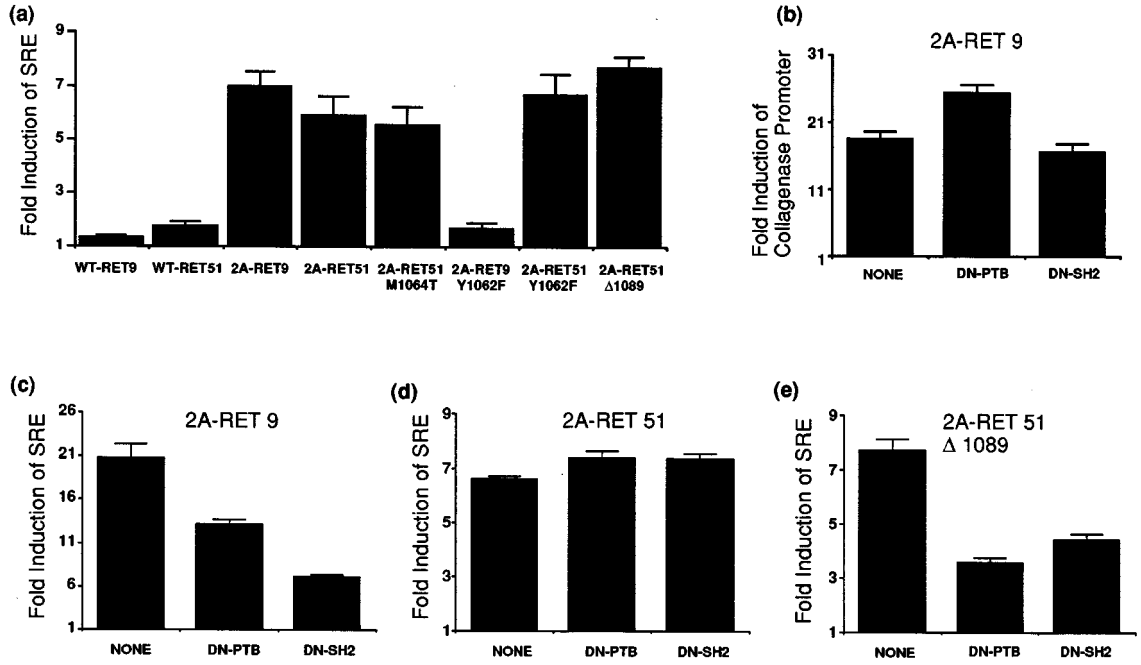

Figure 5 Dominant negative Shc SH2 and Shc PTB domains inhibit the RET-dependent activation of a serum response element (SRE). 293-EBNA cells were co-transfected with RET constructs, pSV-β-Galactosidase, SRE-luciferase or collagenase promoter-luciferase reporter constructs, and (in graphs (**b**) to (**e**)) Shc dominant negative constructs or the empty cloning vector. Shc dominant negative constructs consist of only the SH2 or only the PTB domain of Shc. In graphs (**b**) to (**e**) DN-PTB = co-transfection with dominant negative ShcPTB, DN-SH2 = co-transfection with dominant negative ShcSH2, and NONE = co-transfection with empty cloning vector. Luciferase activity and β-galactosidase activity were measured in the same extract of serum-starved cells, and the luciferase activity standardised for transfection efficiency by dividing the luciferase activity by β-galactosidase activity. Fold induction (arbitary units) was calculated by dividing standardised luciferase activity by the mean activity in control transfections with the RET ΔKinase construct, which lacks the kinase domain. The combined results of at least three different transfection experiments with standard error is shown

Dominant negative Shc PTB and SH2 domains inhibit the RET-dependent activation of a serum response element

Tyrosine phosphorylated Shc recruits and activates Grb2, initiating a signal transduction cascade leading to the activation of ERK2. Active ERK2 is able to phosphorylate the transcription factor accessory protein Elk-1 leading to the activation of the c-*fos* promoter serum response element (SRE) (reviewed in Johansen and Prywes, 1995). In Figure 5a we show that 2A-RET9 and 2A-RET51 (expressed in transiently transfected 293-EBNA cells) are able to activate a basal promoter-luciferase reporter linked to the c-*fos* promoter SRE. No activation via the basal promoter alone is seen (not shown). This provides indirect evidence that active RET is able to activate ERK2 in 293-EBNA as well as neuroblastoma cells (van Weering *et al*., 1995).

Mutation of Y1062 to F in 2A-RET9 greatly reduces the activation of the SRE (Figure 5a) probably as a consequence of the loss of Shc PTB and SH2 domain interaction and Shc phosphorylation. Mutations in 2A-RET51 (Y1062F, Δ1089 and M1064T) have no significant effect on SRE activation although they do reduce Shc or Grb2 interaction. This suggests that (at this level of 2A-RET expression) activation of the pathway leading to the SRE in saturated even with the lower mutant levels of Shc/Grb2 interaction.

We wished to investigate whether interaction with both the SH2 and PTB domains of Shc contributes to the activation of the SRE. In order to do this we made constructs which express only the PTB or SH2 domains of Shc, without the Y317 phosphorylation site. Constructs contained a C-terminal HA epitope tag and a monoclonal antibody against the epitope was used to confirm expression (not shown). These dominant negatives were designed to interact competitively with endogenous Shc at pY binding sites in RET, and hence to cause a reduction in Shc activation. There are precedents for the use of Shc SH2 domain dominant negatives (e.g. Gotoh *et al*., 1995). As a specificity control, the Shc dominant negatives do not cause a reduction in the RET-dependent activation of the human collagenase promoter (Figure 5b).

Both dominant negative (DN-) PTB and DN-SH2 domains cause a significant reduction in the level of SRE activation by 2A-RET9 (Figure 5c). As Y1062 is both a Shc PTB and SH2 domain interaction site in RET9, these data do not provide evidence that both the PTB and SH2 domain interactions contribute to SRE activation.

Neither DN-SH2 nor DN-PTB is able to cause a reduction in the level of SRE activation by 2A-RET51 (Figure 5d). 2A-RET51 contains Grb2 interaction site(s). We reasoned that the lack of effect of Shc dominant negatives may be because the pathway leading to SRE activation is saturated by direct interaction with and activation of Grb2, even when Shc interactions are being blocked. Consistent with this, both DN-PTB and DN-SH2 cause a significant reduction in SRE activation by 2A-RET51-Δ1089

which has the Grb2 interaction site(s) deleted. The PTB and SH2 domain interaction sites in RET51 are distinct. Therefore, these data provide evidence that RET51 interacts with both the PTB and SH2 domains of Shc *in vivo*, and that these interactions contribute to the activation of the SRE.

Discussion

Two C-terminal splice isoforms of RET, RET9 and RET51, activated by a MEN 2A mutation are able to interact with both the SH2 and PTB phosphotyrosine interaction domains of Shc *in vitro*. Both isoforms cause the phosphorylation of Shc *in vivo* in transfected cells. The RET-dependent activation of a serum response element *in vivo* in transfected cells is partially dependent on the interaction of RET with both the Shc PTB and SH2 domains. Mutation of Y1062 to F and peptide competition analysis identifies the Shc PTB domain interaction site in both RET9 and RET51 as pY1062. Residues N-terminal to pY are most important in Shc PTB domain interaction, and the consensus PTB site (NPXpY) (Songyang *et al*., 1995b; van der Geer *et al*., 1996) resembles the sequence around pY1062 in RET9 and RET51 (NKLpY). In addition non-polar residues 5 and 6 residues N terminal to pY are important for Shc PTB binding, and RET has I and W at these positions. In fact, a RET51 pY1062-containing peptide interacts more strongly with the Shc PTB domain than the high affinity site pY490 site in the NGF receptor. This indicates that the P two residues N-terminal to pY in the consensus Shc PTB site is not essential for high affinity binding in all cases, as previously suggested by Laminet *et al*. (1996). pY1062 is also a Shc SH2 domain interaction site in RET9 only. Residues C-terminal to the pY residue are most important in Shc SH2 domain interaction, and the consensus SH2 site (pY(I/E/Y)X(I/L/Y)) Songyang *et al*., 1993) resembles the sequence around pY1062 in RET9 (pYGRI), but not around pY1062 in RET51 (pYGMS). RET51 interacts with the Shc SH2 domain, but much less strongly than RET9, presumably because RET9 has an additional strong interaction site at pY1062. We have not defined the Shc SH2 domain interaction sites in RET51, but further Y to F mutants and peptide competition experiments have indicated that there are multiple weak interaction sites (not shown). Such a situation is not uncommon, and is the case, for example, with the PDGF receptor (Yokote *et al*., 1994). Using interaction with the N-terminal SH2 domain of PLCγ at pY1015 as a control to ensure that equal amounts of activated RET are used in assays we have shown that RET51 interacts approximately twice as strongly as RET9 with the Shc PTB domain. Interaction of the Shc PTB domain at pY1062 in RET9 will presumably be reduced further *in vivo* by competition with the Shc SH2 domain. Experiments using pY1062-containing peptides imply that the difference in PTB binding is accounted for (at least in part) by a stronger affinity of pY1062 in the sequence context of RET51 for the PTB domain. RET9 and RET51 diverge in sequence at residue 1064, indicating that although residues N terminal to pY1062 may be more important in Shc PTB binding, residues C-terminal to pY1062 can also influence the strength of interaction. This is unexpected given that the nuclear magnetic resonance structure of the PTB domain of Shc complexed with a pY-containing peptide ligand indicates that residues C-terminal to the pY residue do not form intermolecular interactions with the Shc PTB domain (Zhou *et al*., 1995). However, deletion analysis from the C-terminus of a pY-containing peptide ligand for the Shc PTB domain has been shown to result in increased affinity for the Shc PTB domain (Trub *et al*., 1995), also indicating that residues C-terminal to the pY residue can influence Shc PTB binding.

The EGF receptor, for example, interacts with both the PTB and SH2 domains of Shc via distinct pY residues (van der Geer *et al*., 1995; van der Geer and Pawson, 1995). It has been suggested that interaction via both domains together may lead to stronger activation of Shc by phosphorylation. This could be consistent with the greater phosphorylation of Shc by RET51 which has distinct PTB and SH2 binding sites. In addition it has been suggested that interaction of the receptor with one Shc pY interaction domain may allow the second pY interaction domain to recruit other pY-containing proteins into a complex with the receptor and Shc. Numerous proteins have been shown or predicted to interact with the Shc SH2 and PTB domains (Songyang *et al*., 1993, 1995b; van der Geer and Pawson, 1995; Kavanaugh *et al*., 1996). The Shc PTB domain can also interact with acidic phospholipids (Zhou *et al*., 1995). The difference in affinity between RET9 and RET51 for the Shc PTB and SH2 domains may result in different abilities of RET9 and RET51 to recruit multiple Shc interacting phosphoproteins or phospholipids into a complex with RET. It is not apparent why the two splice isoforms may differ in these ways. No tissue-specific or developmental stage-specific differences in RET9 and RET51 expression have been reported, although a detailed examination of this has not been described.

A further difference between the two splice isoforms is that only RET51 and not RET9 interacts with the SH2 domain of Grb2 *in vitro*. Interaction is mediated by either one or both of two RET51-specific tyrosine residues (Y1090 and Y1096) both of which resemble the Grb2 SH2 domain consensus binding site. It seems likely, therefore, that RET51 only can activate Grb2 directly as well as indirectly via Shc.

The effect of three different kinase domain HSCR missense mutations has been tested in PTC–RET (Pasini *et al*., 1995), and one of these mutations also tested in MEN 2A RET (Carlomagno *et al*., 1996). Each of these mutations substantially or completely abolishes the RET autokinase activity. Extracellular domain HSCR missense mutations have been shown to impair the kinase activity of RET by affecting its glycosylation and transport to the cell surface (Carlomagno *et al*., 1996). RET mutations associated with HSCR also cause extreme truncations or whole gene deletions (Attie *et al*., 1995) and are therefore clearly inactivating. Hence partial or complete loss of the activity of one RET allele can cause abnormal development of enteric neurons leading to HSCR.

The familial M1064T HSCR mutation (Attie *et al*., 1995) which is RET51-specific occurs in three members of a family, two of which have short segment HSCR (a milder form of the disorder). Mutant RET HSCR

alleles are not fully penetrant, which is consistent with one mutant gene carrier in the family without the disorder. The possibility does, however, exist that M1064T is a polymorphism rather than a mutation. The most compelling evidence against this possibility is that the only report of this sequence variant is in Attie *et al*. (1995), despite extensive RET mutation analysis by many laboratories. Attie *et al*. (1995) reported that this sequence variant was absent from 100 randomly selected control chromosomes (50 individuals) and our own laboratory has not seen this variant whilst sequencing RET in at least a further 100 chromosomes. Consequently this sequence variant would represent an extremely rare polymorphism. In addition the residue M1064 is conserved in man, mouse and chicken RET (Schudardt *et al*., 1995) supporting the suggestion that it is functionally significant. The M1064T splice isoform-specific mutation does not detectably inactivate the autokinase activity of RET51 activated by a MEN 2A mutation. Consequently we decided to try and identify a specific defect in RET activity associated with this mutation, as this may pin-point a particular RET function which is essential for the development of enteric neurons. We have tested the binding of the PLCγ N terminal SH2 domain, the Grb2 SH2 domain, the Shc SH2 domain and the Shc PTB domain to RET51 with the M1064T mutation *in vitro*. This mutation specifically causes a reduction (approximately 30–40%) in Shc PTB domain binding to pY1062 *in vitro* and in addition causes a reduction (approximately 50%) in RET-dependent Shc phosphorylation *in vivo* in transfected cells. Experiments using pY1062 peptides imply that the difference in PTB binding is accounted for (at least in part) by a lower affinity of pY1062 in RET51–M1064T for the PTB domain.

Our data suggests that disruption of the interaction of RET51 with the PTB domain of Shc may cause HSCR. This disruption leads to a reduction in the level of Shc phosphorylation *in vivo* in transfected cells. Shc phosphorylation leads to the activation of Grb2. Our preliminary data (MJL, DPS, C Eng; unpublished) suggests that both RET9 and RET51 are expressed in the developing intestine. Consequently in RET51–M1064T gene carriers Grb2 will be normally and directly activated through two RET51 alleles and normally and indirectly activated (via Shc) through two RET9 alleles and the wild-type RET51 allele. It seems unlikely, therefore, that the M1064T mutation causes a significant reduction in Grb2 activation. Perhaps a more likely significant effect of the M1064T mutation is that the disruption of the strong RET51 interaction with the Shc PTB domain causes a reduction in the recruitment of other phosphoproteins into a complex with RET through the Shc SH2 domain.

Materials and methods

RET and Shc expression constructs

RET9 cDNAs have been described elsewhere (Borrello *et al*., 1995). RET51 cDNAs were made by PCR amplification of a fragment encompassing nucleotide 2986 (at a unique AvrII site in the intracellular domain) to nucleotide 3587 (in the 3′ untranslated region) of the published RET51 sequence (Tahira *et al*., 1990). This fragment was cloned, sequenced, and used to replace a corresponding fragment in RET9. The MEN 2A mutation used is C634R. Y to F mutants, and the M1064T HSCR mutant were made using the Clontech Transformer site-directed mutagenesis kit. Deletion of an EcoRV to HpaI fragment was used to make the construct RET51Δ1089, which has amino acids 1090 to the C-terminus deleted. The RETΔKinase (for kinase deleted) control cDNA consists of the wild-type RET9 cDNA except with a frame-shift mutation (absent base 2347), and encodes a protein truncated within the first kinase domain. Two expression vectors were used: pCEP9β (Borrello *et al*., 1995) and pMJL1 which is the vector pMEX (Oskam *et al*., 1988) except with the polylinker adapted to accept RET cDNAs. The Shc SH2 domain dominant negative construct consists of the coding sequence for amino acids 318 to 473 of Shc (isolated by PCR amplification) with an ATG translation start codon at the 5′-end and the coding sequence for the HA epitope tag at the 3′-end preceding a stop codon cloned into the vector pCEP9β. The Shc PTB domain dominant negative construct consists of the coding sequence for amino acids 1 to 225 of Shc (isolated by PCR amplification) with the coding sequence for the HA epitope tag at the 3′-end preceding a stop codon cloned into the vector pCEP9β.

GST fusion proteins and interaction assays

GST-ShcPTB is the GST-ShcB construct described by van der Geer *et al*. (1995). GST-ShcSH2 consists of the coding sequence for amino acids 366 to 473 of human Shc in the vector pGEX2T. GST-PLCγN consists of the coding sequence for amino acids 547 to 659 of bovine PLCγ encompassing the N terminal SH2 domain. GST-Grb2SH2 consists of the coding sequence for amino acids 59 to 159 of human Grb2. Fusion proteins were prepared as described by van der Geer *et al*. (1995) and interaction with RET assayed as described by van der Geer *et al*. (1995).

Immunoprecipitation and immunoblotting

Immunoprecipitation was carried out as described by van der Geer *et al*. (1995a). For immunoblotting proteins were transferred to Amersham Hybond-ECL membranes using a Biorad semi-dry blotting apparatus following the manufacturer's instructions. Membranes were blocked for at least 1 h in 3% BSA in TBS-highT (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% Tween-20). Primary antibodies were incubated in TBST (10 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.05% Tween-20) overnight at 4°C, and HRP secondary antibodies in TBST for 1 h at room temperature. Membranes were washed in four changes of TBST for 1 h between antibody incubations. Reactive proteins were visualised with Amersham ECL reagents following the manufacturer's instructions. The anti-RET rabbit polyclonal antibody was developed in our own laboratory against the synthetic peptide described by Bongarzone *et al*. (1993) to raise the 'anti-common peptide serum' and was used at a 1 : 1000 dilution. Other antisera were from the commerical sources indicated and used as recommended by the manufacturer: rabbit polyclonal anti-Shc from Transduction Labs; mouse monoclonal anti-GST from Santa Cruz; mouse monoclonal anti-phosphotyrosine (clone PY20) from Transduction labs.

Cell line and transfections

The cell line 293-EBNA was purchased from In Vitrogen and maintained as recommended. For luciferase assays

approximately 200 000 cells in a 2.5 cm diameter dish were transfected by the calcium phosphate method with expression, reporter and control DNAs maintained for 24 h and then serum-starved for 16 h before harvesting. For GST fusion protein assays, immunoprecipitations and immunoblots approximately 2 000 000 cells in a 25 cm^2 flask were transfected by the calcium phosphate method with 5 μg of expression construct and maintained for 48 h before harvesting. RET constructs in the vector pMJL1 were used in transfections for luciferase assays. RET constructs in the vector pCEP9β were used in transfections for GST fusion protein assays, immunoprecipitations and immunoblots.

Luciferase reporter constructs and assays

RET and Shc expression constructs are described above. The control β-galactosidase construct is pSV-β-Galactosidase (Promega). To construct the serum response element luciferase reporter the SV40 promoter in the vector pGL3-Promoter (Promega) was excised with BglII and HindIII and replaced by a minimal HSV TK promoter (obtained by PCR amplification) containing nucleotides -109 to $+17$ flanked by BglII and HindIII sites. A double stranded oligonucleotide containing the serum response element from the c-fos promoter (caggatgtccatattaggacatc) flanked by Asp 718 and BglII sites was then cloned into this vector. The Col-luc luciferase reporter containing the human collagenase promoter (-517 to $+63$) is described by Guiton *et al*. (1994). Cells (as described above) were typically transfected with 0.2 μg of RET expression construct, 1.0 μg of β-galactosidase control construct, 0.2 μg of luciferase reporter construct and 0.2 μg of Shc expression construct or the equivalent amount of empty vector. Following serum-starvation cells were harvested and luciferase and β-galactosidase assays performed using Promega reagents as recommended by the manufacturer.

Peptide synthesis and surface plasmon resonance measurements

Peptides were synthesized using Fmoc (9-fluorenyl methoxycarbonyl) solid phase chemistry on an Applied Biosystems 431 peptide synthesizer. Phosphotyrosine was directly incorporated into the peptides using the N-fluorenylmethyloxycarbonyl-O-phosphono-L-tyrosine derivative. Cleavage of the peptide from the resin and deprotection were achieved through a 90 min incubation at room temperature in trifluoroacetic acid containing a scavenger mixture of thioanisol,1,2-ethanedithiol, and water (1.0 : 0.1 : 2.0% by volume). The product was precipitated with cold-t-butylethyl ether, collected by centrifugation and purified using reverse phase HPLC. The authenticity of the phosphopeptides were confirmed by amino acid analysis and mass spectrometry.

Surface plasmon resonance experiments used to measure the affinity of phosphotyrosine-containing peptides for the GST–ShcPTB domain were carried out using a BIAcore instrument (Pharmacia BioSensor). The assay was performed essentially as described by van der Geer *et al*. (1996). Briefly, the Shc PTB domain binding phosphopeptide (based on a sequence from polyoma virus middle tumour antigen-T.L.S.L.L.S.N.P.T.pY.S.V.M.R.S.K) was immobilized on a biosensor chip by the injection of a 0.5 mM solution of the phosphopeptide (in 50 mM HEPES pH 7.5, 2 M NaCl) across the surface of a chip previously activated following procedures outlined by the manufacturer. Injection of anti-phosphotyrosine antibody was used to confirm that successful immobilization of the peptide was achieved. Peptide inhibition experiments were performed by injecting solutions (100 μl) containing 500 nM GST–ShcPTB domain and a known concentration of soluble phosphopeptide across the Biosensor surface. The amount of bound GST–ShcPTB domain was estimated from the surface plasmon resonance signal at a fixed time following the end of the injection, and the percentage bound, relative to injection of GST–Shc PTB domain alone, calculated. Following each injection the chip surface was regenerated using 2 M guanidinium HCl.

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