

Distinct Turnover of Alternatively Spliced Isoforms of the RET Kinase Receptor Mediated by Differential Recruitment of the Cbl Ubiquitin Ligase*

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Alternative splicing of transcripts encoding the RET kinase receptor leads to isoforms differing in their cytoplasmic tail. Although *in vitro* studies have demonstrated a higher transforming activity of the long RET isoform (RET51), only the short isoform (RET9) can rescue the effects of a RET null mutation in the enteric nervous system and kidney development. The molecular basis underlying the distinct functions of the two RET isoforms is not understood. Here we demonstrated that activated RET51 associated more strongly with the ubiquitin ligase Cbl than did RET9, leading to increased ubiquitylation and faster turnover of RET51. The association of Cbl with RET was indirect and was mediated through Grb2. A constitutive complex of Grb2 and Cbl could be recruited to both receptor isoforms via docking of Shc to phosphorylated Tyr-1062 in RET. A mutant Shc protein unable to recruit the Grb2·Cbl complex decreased the turnover and prolonged the half-life of RET9, thus ascribing a previously unknown negative role to the Shc adaptor molecule. In addition, phosphorylation of Tyr-1096, which is present in RET51 but absent in RET9, endowed the longer isoform with a second route to recruit the Grb2·Cbl complex. These findings establish a mechanism for the differential down-regulation of RET9 and RET51 signaling that could explain the apparently paradoxical activities of these two RET isoforms. More generally, these results illustrate how alternative splicing can regulate the half-life and function of a growth factor receptor.

The *RET* proto-oncogene encodes a transmembrane tyrosine kinase receptor that plays a crucial role in the development of the neural crest and the excretory system (1, 2). The RET protein is activated by engaging members of a family of structurally related ligands consisting of glial cell line-derived neurotrophic factor (GDNF),¹ neurturin, artemin, and persephin,

in the presence of specific auxiliary receptors called GFR α 1–4 (GDNF family receptor alphas). Targeted deletion of the *RET* gene leads to severely impaired development or complete loss of kidneys (44, 45). RET is expressed in the ureteric bud and mediates the growth and branching morphogenesis of this structure. The role of RET in neural crest development is highlighted in the establishment of the enteric nervous system. Neural crest progenitors that migrate to the foregut give rise to enteric neurons and glial cells. In the absence of RET, the rostrocaudal migration of enteric neural progenitors fails, leading to a lack of innervation of the intestinal tract posterior to the stomach (3). Survival and proliferative effects of RET signaling on progenitor cells of the enteric nervous system have also been proposed (4). Characteristic aganglionosis of the colon in Hirschsprung disease has also been linked to inactivating mutations of *RET* (5).

The alternative splicing of the 3' exons of the *RET* gene can give rise to three distinct protein products (6, 7). The isoforms RET9, RET43, and RET51 have different sizes because of distinct amino acid sequences in the extreme C-terminal end but are otherwise identical in upstream sequences until residue Tyr-1062. The mRNAs for RET9 and RET51 are often co-expressed and are more predominant than the RET43 transcript. The C-terminal sequences of RET9 and RET51 proteins are also highly conserved across species, whereas RET43 is not (8, 9). Functional differences between the two predominant isoforms are just beginning to be understood, and the underlying signaling mechanisms are yet to be elucidated.

The clearest functional distinction between RET9 and RET51 has so far been provided by a seminal study using mice engineered to express either one of these two major isoforms (10). Embryonic and postnatal development were normal and indistinguishable in mice monomorphic for RET9. On the other hand, mice expressing only RET51 displayed several deficits in the enteric nervous system and kidneys similar to, albeit milder than, those observed in *RET* null mice. Moreover, a transgene for RET9 but not RET51 was able to fully rescue the effects of a null mutation in the *RET* gene. It was concluded in that study that RET51 represents a hypomorphic allele of this receptor.

Activating mutations in *RET* have been implicated in a number of neoplastic syndromes involving tissues arising from the neural crest (1, 11). These mutations fall into four categories with varying severities. Papillary thyroid carcinoma and famil-

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¹ The abbreviations used are: GDNF, glial cell line-derived neurotrophic factor; GFR α , GDNF family receptor alpha; MEN2, multiple en-

docrine neoplasia type 2; GST, glutathione S-transferase; HA, hemagglutinin; EGFR, epidermal growth factor receptor; TKB, tyrosine kinase binding; RF, ring finger; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; CHX, cycloheximide; DMEM, Dulbecco's modified Eagle's medium; SH2, Src homology 2; SH3, Src homology 3.

ial medullary thyroid carcinoma affect only the thyroid gland. Multiple endocrine neoplasia types 2A and 2B (MEN2A and MEN2B) are more progressive diseases characterized with additional adrenal tumors. Oncoproteins of the RET isoforms have been reported to have different transforming activities. A constitutively active RET43 is only weakly transforming in NIH3T3 fibroblasts (12). RET51 oncoproteins on the other hand are either equally or more transforming than RET9 depending on the exact mutation and the cell context. Familial medullary thyroid carcinoma and MEN2B mutations particularly are more transforming in RET51 and can induce more neurite outgrowth in pheochromocytoma PC12 cells than the corresponding active mutants of RET9 (13–15). This raises an apparent paradox: how can RET51 be hypomorphic during embryogenesis and yet have a higher oncogenic potential than RET9?

In this study, we have described negative regulatory mechanisms by which RET isoforms differed from each other. We demonstrated the association of the ubiquitin ligase Cbl on activated complexes of RET9 and RET51. The stronger interaction of Cbl with RET51 resulted in a higher level of ubiquitylation and consequent down-regulation than with RET9. We propose that signaling mediated by RET51 is not necessarily weaker than RET9 but is perhaps attenuated more effectively.

EXPERIMENTAL PROCEDURES

Antibodies and Other Reagents—Antibodies against Cbl, Grb2, and isoform-specific antibodies against RET9 and RET51 were from Santa Cruz Biotechnology. Pan-RET polyclonal antibodies were either from R&D Systems or Santa Cruz Biotechnology. A pan-RET monoclonal antibody raised against an epitope within the kinase domain of RET was generously provided by Anatoly Sharipo (Kirchenstein Institute of Microbiology and Virology, Riga, Latvia). Anti-hemagglutinin (HA) monoclonal antibody was purchased from Covance. A polyclonal antibody against Shc was from Upstate Biotechnology. Anti-glutathione S-transferase (GST) antibody was purchased from Amersham Biosciences. Cycloheximide (CHX) was from Sigma-Aldrich, and proteasome inhibitor I was from Calbiochem.

DNA Constructs and Mutagenesis—Expression constructs for MEN2A variants of human RET9 and RET51 cDNA in pJ7 μ vector were from M. Billaud (Université Claude Bernard). An HA-tagged human Cbl expression construct was obtained from Y. Yarden (Weizmann Institute). GST-Grb2 and GST-Shc constructs were from J. Bliska (State University of New York, Stony Brook, NY) and R. Bradshaw (UCLA), respectively. The HA-tagged ubiquitin expression construct was from D. Bohmann (University of Rochester). GST fusion constructs for Cbl were made by subcloning *Pfu*-amplified fragments in pGEX6P1 vector (Amersham Biosciences). Site-directed mutagenesis of various constructs was done using the QuikChange method (Stratagene).

Cell Culture—COS cells were grown in 10% serum in DMEM plus 1 mM glutamine. MN1 cells were grown in 7.5% serum in DMEM buffered with 10 mM HEPES. Transfection was done using polyethylenimine (PEI, 25 kDa, Sigma-Aldrich). Briefly, 10–15 μ g of DNA/10-cm tissue culture plate was diluted in 750 μ l of DMEM at room temperature. Subsequently, 50 μ g of polyethylenimine was added and mixed by vortexing, and the mixture was immediately dropped onto cells. Twelve to 16 h prior to lysis, cells were deprived of serum by incubation in DMEM buffered with 10 mM HEPES supplemented with glutamine and 0.1% bovine serum albumin. Ligand-dependent RET activation was induced by the addition of recombinant GDNF (R&D Systems) at 50 ng/ml.

Analysis of Protein Turnover—MN1 cells were serum-starved overnight prior to stimulation. To characterize the decay of RET isoforms, cells were treated in serum-free medium with cycloheximide (100 μ g/ml) with or without GDNF for varying time periods. RET9 and RET51 levels were then assessed by immunoprecipitation with isoform-specific antibodies and Western blotting.

Expression and Purification of GST Fusion Proteins—GST fusions of Cbl and Grb2 were expressed in *Escherichia coli* BL21 strain. Logarithmic phase cells were induced with 1 mM isopropyl 1-thio- β -D-galactopyranoside for 4 h at 37 °C and the cell pellets lysed by sonication. Fusion proteins were affinity captured on glutathione beads (Amersham Biosciences), eluted with reduced glutathione, and dialyzed against phosphate-buffered saline.

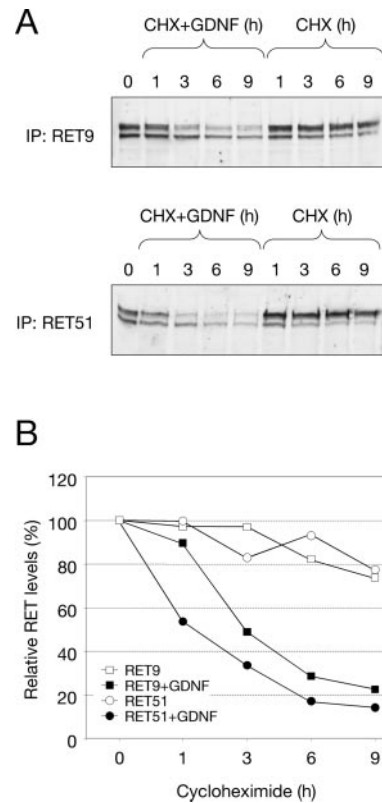


FIG. 1. RET51 has a faster turnover than RET9. *A*, MN1 cells were treated with CHX in the presence or absence of GDNF for the indicated periods of time. RET9 and RET51 were selectively immunoprecipitated (IP) from normalized cell lysates, and receptor levels were assessed by Western blotting using a pan-RET antibody. *B*, quantification of RET decay over time in the presence or absence of ligand. Data points correspond to averages of three independent experiments normalized to total levels of protein.

Immunoprecipitation, Pull-down Assays, and Protein Immunoblots—Cultured cells were lysed in modified radioimmune precipitation assay buffer (50 mM Tris-HCl, pH7.4, 100 mM NaCl, 50 mM NaF, 1 mM EDTA, 10 mM 2-glycerolphosphate, 2 mM Na₃VO₄, 1% (v/v) IGEPAL CA-630, 0.25% (w/v) sodium deoxycholate, 10% (v/v) glycerol) and supplemented with Complete protease inhibitor mixture (Roche Applied Bioscience). Immunoprecipitations were done overnight on pre-cleared lysates using the appropriate antibodies and protein G-Sepharose beads. GST pull-down assays were done using the appropriate GST fusion protein bait immobilized on glutathione-Sepharose beads. Immune and affinity pull-down complexes were eluted for protein gel analysis by boiling in reducing Laemmli buffer. polyvinylidene difluoride membranes were used for immobilization of gel-fractionated proteins, and Western blot detection was performed by enhanced chemifluorescence method using a Storm PhosphorImager (Amersham Biosciences).

RESULTS

RET51 Has a Faster Turnover and Is More Ubiquitylated than RET9—We hypothesized that the disparate biological activities of the receptor isoforms RET9 and RET51 might correlate with different patterns of receptor down-regulation following ligand activation. This possibility was investigated in the motor neuron cell line MN1, which expresses endogenously RET9 and RET51 isoforms as well as the ligand-binding receptors GFR α 1 and GFR α 2 (16). MN1 cells were treated with GDNF for different periods of time in the presence or absence of the protein synthesis inhibitor cycloheximide. Using isoform-specific antibodies, we selectively immunoprecipitated RET9 and RET51 and then assessed the decline of receptor protein levels in the absence of ongoing protein synthesis by Western blotting. We found that the levels of RET51 were down-regulated faster in response to ligand stimulation than those of RET9 (Fig. 1A). In three independent experiments, the half-

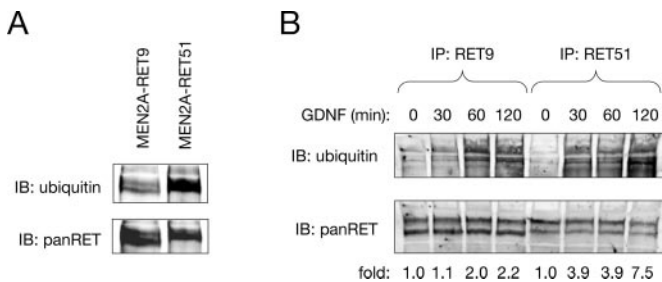


FIG. 2. RET51 is more ubiquitylated than RET9. *A*, steady state ubiquitylation of RET9 and RET51 oncoproteins (MEN2A-RET9 and MEN2A-RET51, respectively) expressed in COS cells was assessed by Western blotting (*IB*) of pan-RET immunoprecipitates with antibodies against ubiquitin and RET. *B*, GDNF-dependent ubiquitylation of RET9 and RET51 in MN1 cells. Endogenous RET9 and RET51 were selectively immunoprecipitated (*IP*) from lysates of proteasome inhibitor I-treated MN1 cells after GDNF stimulation over the indicated periods of time. Ubiquitylation was assessed by Western blotting with anti-ubiquitin. Blots were reprobed with anti-pan-RET antibodies. Levels of RET ubiquitylation were quantified and normalized to the total levels of RET protein and are expressed relative to control conditions (*fold*).

lives of RET51 and RET9 were ~1 and 3 h, respectively (Fig. 1*B*), indicating a 3-fold higher turnover for the longer RET isoform. The relative turnover of the two isoforms was comparable in the absence of GDNF (Fig. 1*B*).

Because the targeting of proteins for recycling and degradation is initiated by the covalent conjugation of ubiquitin (17, 18), we wondered whether the faster turnover of RET51 relative to RET9 could be explained by an increased level of ubiquitylation. We examined ubiquitylation patterns of RET9 and RET51 by immunoblotting using antibodies against ubiquitin. Steady state ubiquitylation levels of the receptor isoforms were assessed in transfected MEN2A receptor variants (carrying the substitution C634R) that are constitutively active. This analysis showed that MEN2A-RET51 was more ubiquitylated than MEN2A-RET9 in transfected COS cells (Fig. 2*A*). We also assessed the time course of ligand-dependent ubiquitylation of the two RET isoforms in MN1 cells treated with the proteasomal inhibitor PSI. Ligand treatment stimulated ubiquitylation of both RET isoforms (Fig. 2*B*). However, ubiquitylation of RET51 was found to occur more rapidly and to higher levels than that of RET9 (Fig. 2*B*).

The differential down-regulation of the two RET isoforms was at first surprising, because we expected that formation of heteromeric receptor complexes between RET9 and RET51 would result in a common fate for the two receptor variants after ligand stimulation. We therefore investigated whether RET9 and RET51 were actually associated in the same complex upon ligand binding. Co-immunoprecipitation assays were performed in MN1 cells in the presence or absence of GDNF using isoform-specific antibodies. Contrary to our expectations, the two RET isoforms were not found to co-immunoprecipitate with each other (Fig. 3). In agreement with these data, neither has heterodimerization of RET9 and RET51 been observed in GDNF-stimulated sympathetic neurons (19). Although these analyses cannot at present exclude the possibility that heteromeric complexes of RET isoforms may have been lost under the conditions used for co-immunoprecipitation, these results suggest that RET9 and RET51 may be physically segregated in the cell and form distinct signaling complexes, so that the fate of one isoform is independent of the other.

Ubiquitylation of RET Is Mediated by Cbl—The 120-kDa ubiquitin protein ligase Cbl has been shown to play a crucial role in the ubiquitylation and down-regulation of a growing number of receptor and non-receptor tyrosine kinases includ-

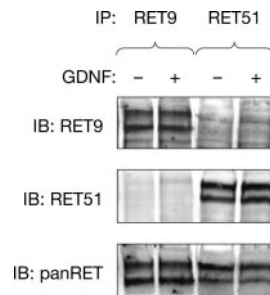


FIG. 3. RET9 and RET51 form independent complexes. RET9 and RET51 were selectively immunoprecipitated (*IP*) from GDNF-treated and untreated MN1 cells, and co-immunoprecipitation of the other receptor isoform was assessed by Western blotting (*IB*). The *upper two panels* show probing with isoform-specific antibodies, and the *bottom panel* is a reprobing with a pan-RET antibody.

ing the epidermal growth factor receptor (EGFR), platelet-derived growth factor receptor, and Src family kinases (20, 21). We were therefore interested in determining whether Cbl associated with RET. We co-transfected MEN2A variants of RET9 and RET51, or a kinase-defective mutant of RET51 (K758M), with a HA-tagged Cbl construct in COS cells. Both MEN2A-RET9 and MEN2A-RET51 but not the kinase-dead receptor mutant were found in immunoprecipitation complexes of Cbl (Fig. 4*A*). Interestingly, constitutively active RET51 associated more strongly with Cbl compared with RET9 (Fig. 4*A*), consistent with the patterns of receptor ubiquitylation of the two isoforms. The association between RET and Cbl was also observed in MN1 cells after GDNF stimulation and was accompanied by a concomitant increase in the tyrosine phosphorylation of Cbl (Fig. 4*B*). These results suggest that the association of Cbl with RET depends upon receptor activation and phosphorylation.

We then tested whether Cbl could influence the ubiquitylation of RET. When overexpressed together with MEN2A-RET51 in COS cells, Cbl did not enhance the ubiquitylation of the receptor (Fig. 5*A*), possibly because of saturating amounts of Cbl in these cells. However, a RING finger mutant of Cbl (C381A) that lacks ubiquitin ligase activity and has dominant negative effects (22) suppressed the ubiquitylation of MEN2A-RET51 (Fig. 5*A*). Moreover, the RING finger mutant of Cbl stabilized the levels of MEN2A-RET51 in transfected COS cells treated with CHX, making the turnover of the two isoforms indistinguishable in these cells (Fig. 5*B*). Together, these results demonstrate that Cbl mediates RET ubiquitylation. The preferential association of Cbl with RET51 could account for the stronger ubiquitylation and faster turnover of this receptor isoform.

RET Interacts with Cbl Indirectly—Because the recruitment of Cbl was dependent on the activation of RET, we set out to identify the RET autophosphorylation sites necessary for the interaction. Different phosphorylation site mutants of RET were compared for their ability to associate with Cbl by co-immunoprecipitation. Replacement of either Tyr-1062 or Tyr-1096 but not Tyr-1015 with Phe in RET51 reduced its association with Cbl, whereas the Y1062F/Y1096F double mutant failed to interact with Cbl (Fig. 6*A*). In the case of RET9, which lacks Tyr-1096, replacement of Tyr-1062 with Phe was sufficient to reduce its interaction with Cbl to background levels (Fig. 6*A*). Although Tyr-952 in RET is found within a sequence that conforms to the consensus binding motif of the tyrosine kinase binding (TKB) domain of Cbl (21), mutation of this residue into Phe had no effect on the interaction of RET with Cbl (data not shown). Together, these results identified a common residue present in both RET isoforms, namely Tyr-1062 and Tyr-1096 (which is only present in RET51), as important

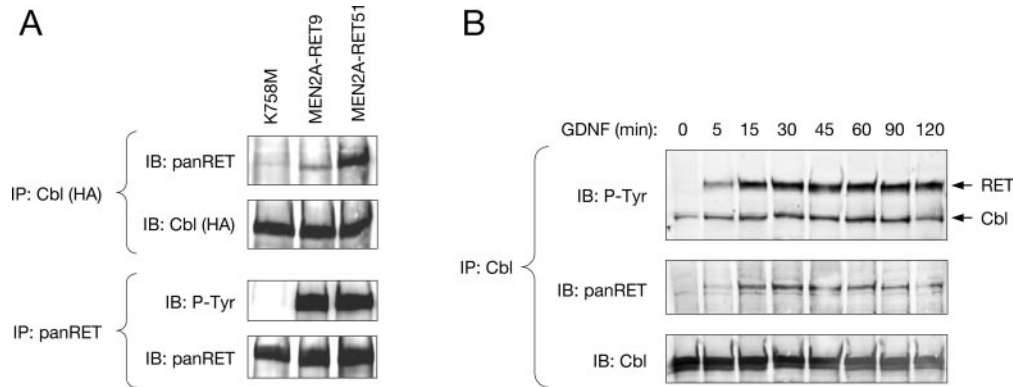


FIG. 4. Cbl is recruited to RET. *A*, Cbl associated with RET in a phosphotyrosine (*P-Tyr*)-dependent manner and interacted more strongly with RET51 than with RET9. Indicated RET constructs were co-expressed with HA-tagged Cbl in COS cells. RET-Cbl association was assessed by co-immunoprecipitation using an antibody directed against the HA tag of Cbl. *B*, endogenous Cbl associated with RET in MN1 cells in a ligand-dependent manner. MN1 cells were stimulated with GDNF for the indicated times. Cbl immunoprecipitates (*IP*) were then probed for *P-Tyr* and RET revealing a ligand-dependent phosphorylation of Cbl and association with RET. *IB*, immunoblot.

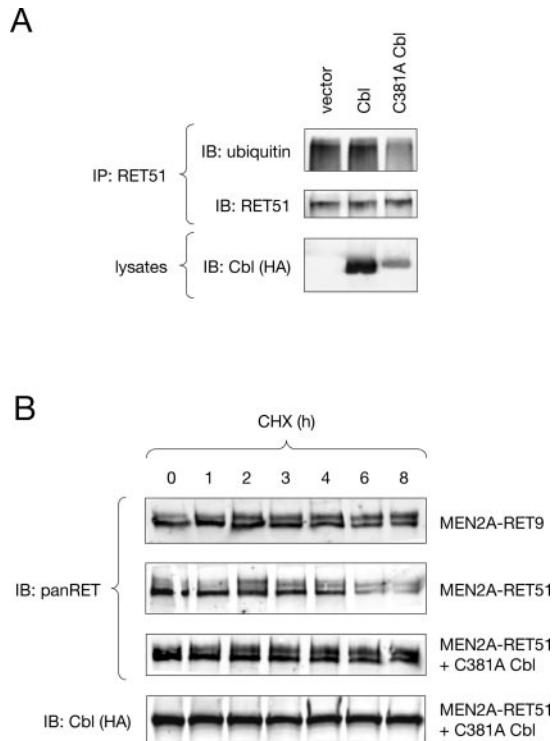


FIG. 5. Cbl mediates RET ubiquitylation. *A*, a RING finger mutant of Cbl suppressed RET ubiquitylation. Wild type and the RF mutant (C381A) of Cbl were co-expressed with MEN2A-RET51 in COS cells. MEN2A-RET51 co-expressed with the RF mutant was less ubiquitylated, as assessed by Western blotting (*IB*) for ubiquitin of RET51 immunoprecipitates (*IP: RET51*). *B*, co-expression of the RF mutant of Cbl led to sustained levels of RET even in the presence of CHX. Protein synthesis was inhibited at the indicated times, and the decay of MEN2A-RET51 and MEN2A-RET9 overexpressed in COS cells was assessed by Western blotting. Equal amounts of protein were loaded in the different lanes of each gel. The *two upper panels* show representative blots showing RET turnover when expressed alone. The *third panel* shows MEN2A-RET51 levels after co-expression of the RF mutant of Cbl, which has dominant negative effects. Reprobing for Cbl with anti-HA antibodies is shown in the *bottom panel*. When MEN2A-RET51 was co-expressed with the dominant negative Cbl mutant, the turnover of the two RET isoforms was indistinguishable.

determinants for the interaction of RET with Cbl. The presence of two alternative sites in RET51, compared with only one in RET9, was in line with the ability of the longer isoform to interact more strongly with Cbl. Intriguingly, neither Tyr-1062 nor Tyr-1096 are found within known consensus sequences for Cbl binding, which suggests that the interaction between RET

and Cbl was indirect. In agreement with this, recombinant full-length Cbl, produced as a fusion protein to GST, was unable to detect RET in Far Western overlay assays (data not shown).

To identify the regions of Cbl that are necessary for complex formation with RET, fusions between GST and different segments of Cbl were constructed and used in GST pull-down assays (Fig. 6*B*). The results of these experiments indicated that MEN2A-RET51 but not the kinase-dead receptor mutant K758M associated with the C-terminal region of Cbl (Fig. 6*B*, *Cbl-C*), spanning residues 350–906, which contains the RF domain that is important for its ubiquitin ligase activity and a proline-rich domain (Fig. 6*C*). The N-terminal region of Cbl (Fig. 6*B*, *Cbl-N*), containing the TKB domain, did not interact with MEN2A-RET51 (Fig. 6*C*), which is consistent with the lack of consensus binding sites for this domain around Tyr-1062 and Tyr-1096 in RET. Similar results were observed for MEN2A-RET9 (data not shown). In contrast, ligand-stimulated EGFR interacted with both the N- and C-terminal domains of Cbl (Fig. 6*D*) in agreement with previous results (22, 23). Together, these data indicated that intermediate molecules docking at phosphorylated Tyr-1062 and Tyr-1096 in RET mediate recruitment of Cbl to this receptor independently of the TKB domain of Cbl.

Grb2 and Shc Link Cbl to RET—The scaffold protein Shc and the adaptor Grb2 are known to bind to phosphorylated Tyr-1062 and Tyr-1096 of RET, respectively, via phosphotyrosine binding and SH2 domains (24–27). We therefore considered the possibility that Shc and Grb2 may mediate the interaction of Cbl with RET. In a pull-down assay using a GST fusion of Grb2, a complex between Cbl and Grb2 was observed to associate with MEN2A-RET51 produced in transfected COS cells (Fig. 7*A*, *third lane*). As expected, the inactive RET mutant K758M was not recovered in these conditions (Fig. 7*A*, *second lane*). Cbl was found associated with Grb2 even in cells that did not express RET (Fig. 7*A*), which was in agreement with previous observations indicating that Cbl and Grb2 are constitutively associated within cells (28, 29).

Further analysis of the domains in Grb2, required for its ability to bridge Cbl and RET, indicated that both SH3 domains were necessary for its interaction with Cbl. Mutation of the conserved Trp residues W36G and W193G within the N- and C-terminal SH3 domains of Grb2, respectively, affected its ability to associate with Cbl in pull-down assays but did not affect its interaction with RET (Fig. 7*A*). The fact that only a partial reduction was observed in the single mutants indicated that both SH3 domains of Grb2 are involved in its interaction

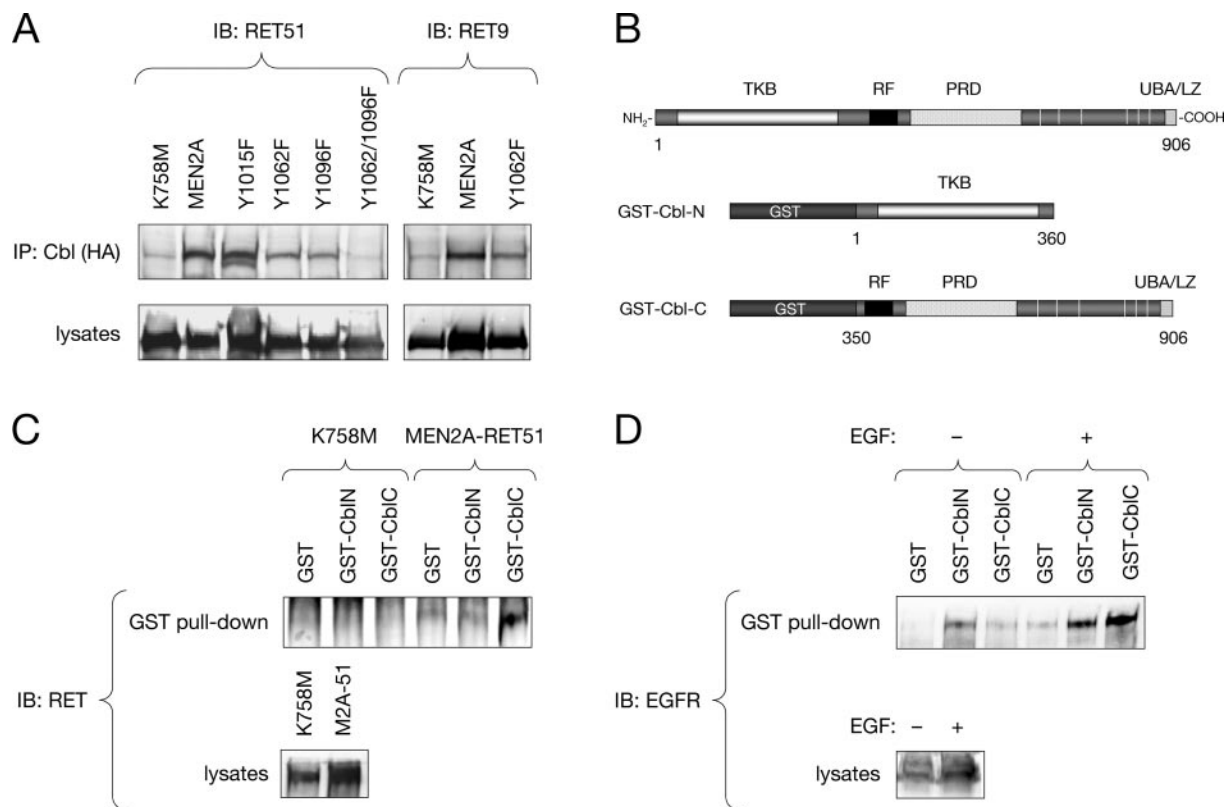


FIG. 6. Structural determinants of RET-Cbl association. *A*, RET autophosphorylation sites Tyr-1062 and Tyr-1096 were required for Cbl recruitment. MEN2A-RET51 and MEN2A-RET9 wild type and mutant constructs were overexpressed in COS cells together with HA-tagged Cbl. Cbl was immunoprecipitated by the tag (*IP*: Cbl (HA)), and co-immunoprecipitation of RET isoforms was analyzed using specific antibodies. *B*, protein fusions between GST and different segments of Cbl used for mapping regions necessary for RET association. NH₂-, N-terminal; COOH-, C-terminal; PRD, proline-rich domains; UBA/LZ, ubiquitin-associated/leucine zipper-like motif. *C*, the C-terminal region of Cbl but not its TKB domain was required for RET association. MEN2A-RET51 and the kinase-dead mutant K758M were transiently expressed in COS cells and subjected to a GST pull-down assay using GST-Cbl fusion constructs schematized in *B*. *D*, in contrast to RET, ligand-activated EGFR associated with Cbl via both the TKB and the C-terminal domains of Cbl. *IB*, Western blotting.

with Cbl. In co-immunoprecipitation assays in transfected COS cells, overexpression of wild type Grb2 but not the double mutant enhanced the interaction between RET51 and Cbl (Fig. 7B). Together, these findings indicate that Grb2 can bridge RET and Cbl via interaction of its two SH3 domains with the proline-rich C-terminal tail of Cbl and its SH2 domain with phosphorylated residues in RET, such as Tyr-1096, or in other adaptor proteins that are known to bind the receptor, such as Shc (see below).

To assess the role of Shc, we transfected a GST fusion construct of the p52 isoform of Shc together with Cbl and either RET9 or RET51 and subsequently precipitated GST-Shc with glutathione beads. Both RET isoforms could be co-precipitated with Cbl and Shc in these experiments (Fig. 7C). However, a GST-Shc construct carrying mutations in the three tyrosine residues that are known docking sites for Grb2 (30, 31) interfered with the recruitment of Cbl to either of the RET isoforms while still being able to interact with RET (Fig. 7C). Interestingly, the effect of the 3F-Shc mutant on the interaction between RET and Cbl was more pronounced in the case of RET9 than RET51 (Fig. 7C), in agreement with the existence of a secondary site for Cbl recruitment in the long isoform of the RET receptor. Together, these findings suggest that phosphorylated Shc recruits Cbl to RET through Grb2, a mechanism of Cbl recruitment that appears to be common to both RET9 and RET51 because of their shared ability to bind Shc via phosphorylated Tyr-1062. On the other hand, the enhanced association of RET51 with Cbl could be accounted for by the additional, direct recruitment of Grb2 to phosphorylated Tyr-1096, which is only present in the long isoform.

A Role for Shc as a Negative Regulator of RET Stability—One prediction of this model is the participation of Shc in the ubiquitylation of RET, a rather novel concept given the universally accepted role of Shc as a positive regulator of receptor tyrosine kinase signaling. In support of this notion, we found that overexpression of wild type Shc, but not its triple mutant, enhanced ubiquitylation of MEN2A-RET51 in transfected COS cells (Fig. 8A). Moreover, the 3F-Shc mutant, but not wild type Shc, was able to increase the half-life and thereby decrease the turnover of RET9 endogenously expressed in MN1 cells (Fig. 8B), indicating a novel function of Shc in the down-regulation of receptor tyrosine kinases.

DISCUSSION

The ubiquitylation, or covalent conjugation of ubiquitin to proteins, is an important posttranslational modification utilized in various cellular processes. Polyubiquitylation is an essential step in the targeting of proteins for proteasomal degradation (18, 32). Monoubiquitylation on the other hand provides recognition signals that assist in regulating endocytic trafficking, membrane transport, and transcriptional regulation (33). Ubiquitylation is a multistep event involving the sequential action of three different enzymes categorized as E1, E2, and E3 (18, 32). The first two steps involve the conjugation of ubiquitin to an E1 enzyme and the transfer of the ubiquitin moiety to an E2 enzyme. The final step catalyzed by an E3 enzyme is the transfer of the ubiquitin from the E2 enzyme to the final protein substrate. The E3 enzymes, in addition to their protein ligase activity, are primarily responsible for the selection and recruitment of target protein substrates. Cbl

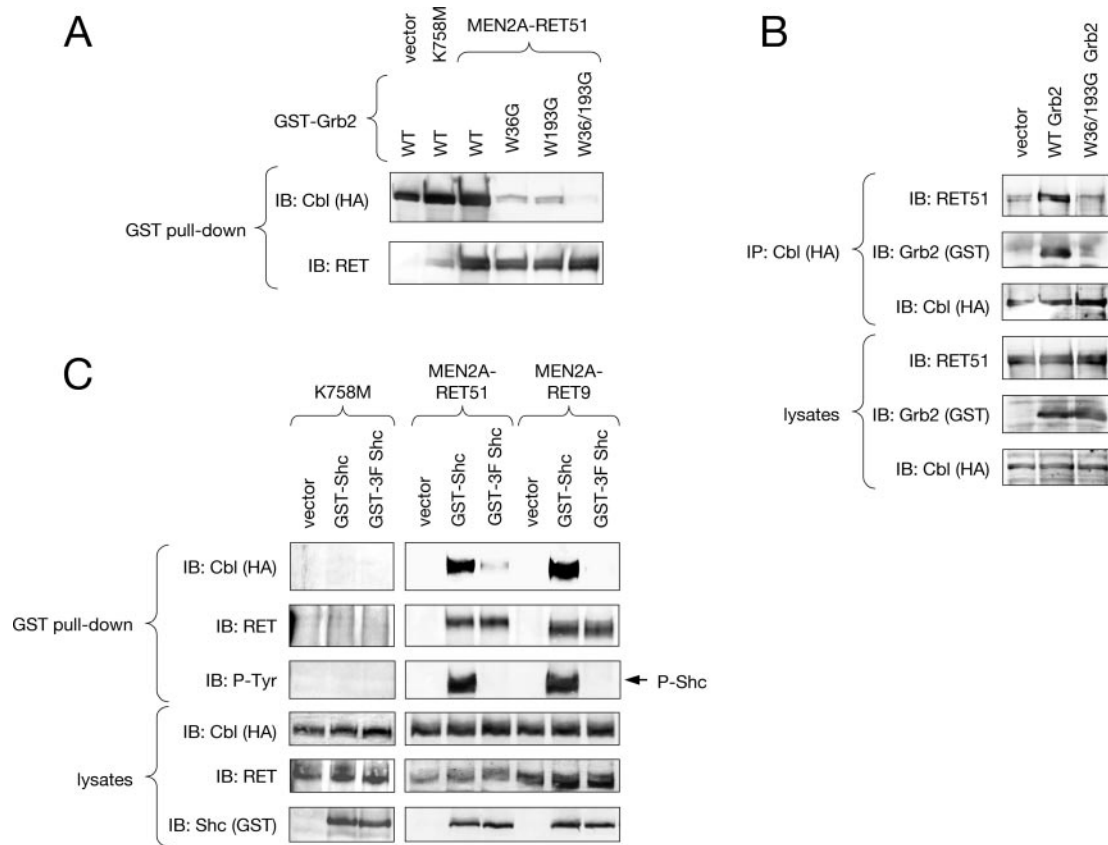


FIG. 7. Grb2 and Shc link Cbl to RET. A, Grb2 associated with Cbl via its two SH3 domains. Shown are immunoblots of GST-Grb2 pull-down assays using lysates from COS cells co-expressing HA-tagged Cbl and the indicated RET51 constructs. For pull-down assays, wild type (WT) or mutated GST-Grb2 fusion constructs were used as indicated. B, Grb2 mutated in both SH3 domains failed to co-immunoprecipitate with Cbl and reduced RET-Cbl interaction. GST-Grb2 (wild type and the SH3 double mutant W36G/W193G) were co-expressed together with MEN2A-RET51 and HA-Cbl in COS cells. Immunoprecipitates (IP) of Cbl (via its HA tag) were then probed for RET51 and Grb2 (via its GST tag). C, phosphorylated Shc (P-Shc) associates with Cbl. GST-tagged Shc (Shc (GST)) co-precipitated with HA-Cbl in transfected COS cells that also expressed constitutively active RET9 or RET51. Mutation of three tyrosine phosphorylation (P-Tyr) sites on Shc that are known docking sites for Grb2 interfered with the recruitment of Cbl to either of the RET isoforms without affecting the interaction of Shc with RET. The effect of the 3F-Shc mutant was more pronounced in the case of RET9 than RET51. IB, Western blotting.

belongs to a family of E3 ligases that require a RING finger domain for their catalytic activity (20, 21, 30). The role of Cbl proteins in the ubiquitylation and subsequent down-regulation of EGFR was the first demonstration of their role as ubiquitin ligases (34). Subsequently, other protein tyrosine kinases such as platelet-derived growth factor receptor, the receptor for colony stimulating factor-1, ErbB2, and Fyn are also reported to be ubiquitylated by Cbl (23, 35–37).

In this study, we found that RET activation was accompanied by ubiquitylation in agreement with a previous report using an oncogenic form of this receptor (38). Interestingly, the pattern of ubiquitylation was different between RET9 and RET51 isoforms, the latter being more ubiquitylated and consequently more efficiently down-regulated. RET ubiquitylation occurred as a consequence of the recruitment of Cbl. Consistent with the preferential ubiquitylation of the long isoform, Cbl interacted more strongly with RET51 than with RET9. The association of Cbl with RET9 was dependent on phosphorylation of Tyr-1062. However, Cbl could be additionally recruited through the phosphorylation of Tyr-1096 in RET51. Neither of the phosphorylation sites matched the canonical binding motif for the TKB domain of Cbl, and thus they do not mediate direct recruitment of this protein.

Unlike EGFR and platelet-derived growth factor receptor that recruit Cbl through direct interaction between the TKB domain of Cbl and phosphorylated residues on the active receptors (34, 36), RET recruited Cbl by an alternative route that

involved the adaptor molecules Shc and Grb2 (Fig. 8C). Our experiments demonstrated that upon interaction with RET9 and RET51 at phosphorylated Tyr-1062, Shc became phosphorylated and served as a platform that engaged Grb2 and Cbl. The interaction between Grb2 and Cbl appeared to be constitutive and involved the two SH3 domains of Grb2 and the proline-rich tail of Cbl. This was in agreement with our observation that deletion of the TKB domain of Cbl did not affect its association with either of the RET isoforms. The interaction of the SH2 domain of Grb2 with phosphorylated Tyr-1096 of RET51 provided an additional means to recruit Cbl for this isoform. Grb2 has also been shown to play a similar role in the recruitment of Cbl to the EGFR (39).

Our results implicate Shc in a previously undescribed role as a negative regulator of a receptor tyrosine kinase. Recently, the scaffold protein FRS2 has been reported to mediate the recruitment of Cbl via Grb2 to the fibroblast growth factor receptor (40). Because FRS2 can also dock at Tyr-1062 of RET (41, 42), it is likely that it shares functions with Shc in the down-regulation of this receptor. Perhaps the underlying mechanism in the hypomorphism of RET51 during embryogenesis lies in its more efficient down-regulation as a consequence of a tighter coupling to the ubiquitylation machinery. However, this interpretation does not preclude other not mutually exclusive possibilities, including differences in downstream signaling pathways activated by the two receptor isoforms.

Our inability to identify heterodimers of RET9 and RET51

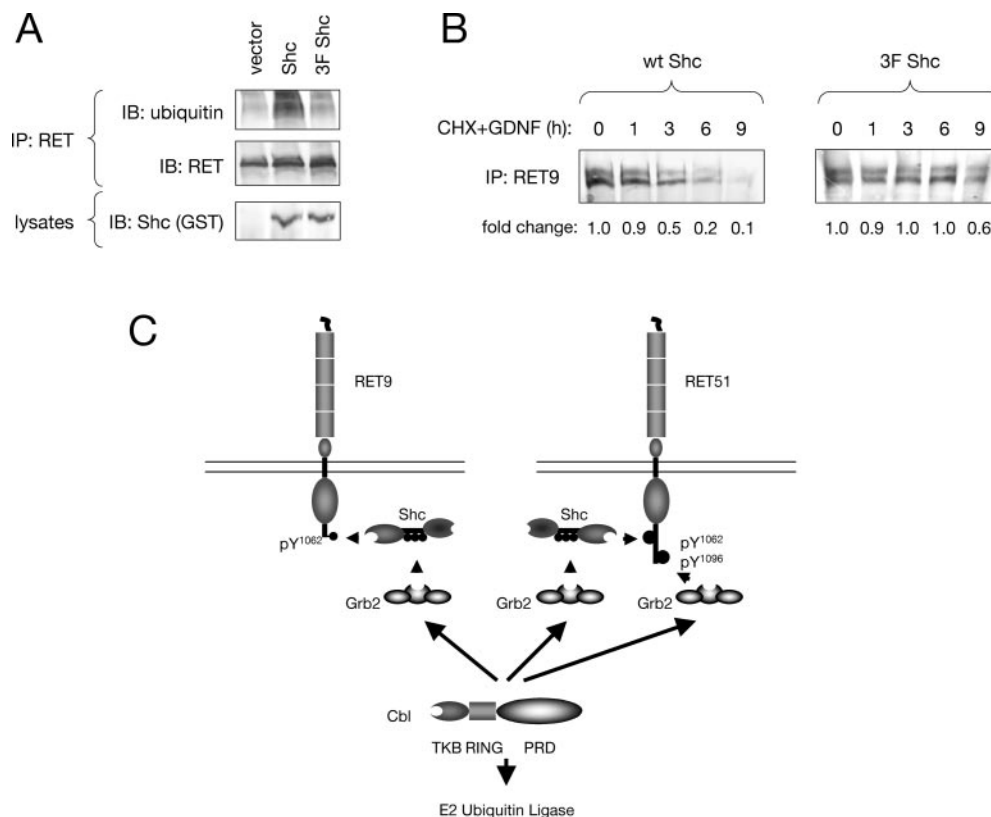


FIG. 8. A role for Shc as a negative regulator of RET stability. *A*, Shc potentiates RET ubiquitylation. Overexpression of wild type GST-Shc but not the GST-tagged triple mutant (*3F Shc*) increased the ubiquitylation of MEN2A-RET51 that was co-expressed in COS cells. *IB*, Western blotting. *B*, Shc negatively regulates RET turnover. MN1 cells were transfected with wild type (*wt Shc*) or mutant Shc as indicated and then treated with CHX in the presence or absence of GDNF for the indicated periods of time. RET9 was selectively immunoprecipitated (*IP*) from normalized cell lysates and receptor levels were assessed by Western blotting using a pan-RET antibody. *-fold change* relative to control levels (no CHX or GDNF) is indicated below the lanes. *C*, proposed model of Cbl-RET interactions via Shc and Grb2. Grb2 is pivotal for the association of Cbl with RET. A preformed complex of Cbl and Grb2 can be recruited to RET via Shc docked to phosphorylated Tyr-1062 (pY¹⁰⁶²), which is common to both of the RET isoforms. In the case of RET51, the presence of an additional phosphorylation site at Tyr-1096 (pY¹⁰⁹⁶) provides a second route for Cbl recruitment via direct binding of Grb2 to the receptor. *PRD*, proline-rich domain.

suggests that the signal transduction events mediated by each isoform are compartmentalized and independent of each other. Why several isoforms of RET are generated by alternative splicing can only be speculated about at this point. Expression of RET9 and RET43 precedes expression of RET51 during normal kidney development in humans (43), suggesting that RET51 may have functions more related to differentiation rather than induction of the ureteric bud. A more thorough analysis of isoform expression would be necessary to assert this hypothesis. The unexpectedly low number of genes found in the mammalian genome, compared with that of insects or nematodes, has been rationalized by a relatively higher incidence of alternative splicing in mammalian species that would presumably result in increased molecular diversity. Our analysis, revealing distinct mechanisms in the negative regulation of two alternatively spliced products of the *ret* gene, is in agreement with this notion and offers an explanation for some of the differences in the biological activities of these receptor isoforms.

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