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Increased *in Vivo* Phosphorylation of Ret Tyrosine 1062 Is a Potential Pathogenetic Mechanism of Multiple Endocrine Neoplasia Type 2B[1]

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ABSTRACT

Mutations of the Ret receptor tyrosine kinase are responsible for inheritance of multiple endocrine neoplasia (MEN2A and MEN2B) and familial medullary thyroid carcinoma syndromes. Although several familial medullary thyroid carcinoma and most MEN2A mutations involve substitutions of extracellular cysteine residues, in most MEN2B cases there is a methionine-to-threonine substitution at position 918 (M918T) of the Ret kinase domain. The mechanism by which the MEN2B mutation converts Ret into a potent oncogene is poorly understood. Both MEN2A and MEN2B oncoproteins exert constitutive activation of the kinase. However, the highly aggressive MEN2B phenotype is not supported by higher levels of Ret-MEN2B kinase activity compared with Ret-MEN2A. It has been proposed that Ret-MEN2B is more than just an activated Ret kinase and that the M918T mutation, by targeting the kinase domain of Ret, might alter Ret substrate specificity, thus affecting Ret autophosphorylation sites and the ability of Ret to phosphorylate intracellular substrates. We show that the Ret-MEN2B mutation causes specific potentiated phosphorylation of tyrosine 1062 (Y1062) compared with Ret-MEN2A. Phosphorylated Y1062 is part of a Ret multiple effector docking site that mediates recruitment of the Shc adapter and of phosphatidylinositol-3 kinase (PI3K). Accordingly, we show that Ret-MEN2B is more active than Ret-MEN2A in associating with Shc and in causing constitutive activation of the Ras/mitogen-activated protein kinase and PI3K/Akt cascades. We conclude that the MEN2B mutation specifically potentiates the ability of Ret to autophosphorylate Y1062 and consequently to couple to the Ras/mitogen-activated protein kinase and the PI3K/Akt pathways. The more efficient triggering of these pathways may account for the difference between MEN2A and MEN2B syndromes.

INTRODUCTION

Ret is the tyrosine kinase receptor for growth factors of the GDNF[3] family (GDNF, neurturin, persephin, and artemin), and it plays an important role in the development of the enteric nervous system and the kidney (1). Ligands of the GDNF family interact with glycosylphosphatidyl inositol-anchored membrane receptors, called GFRα1–4, that in turn induce dimerization and activation of Ret kinase (2).

Germ-line point mutations that cause oncogenic activation of Ret are responsible for the inheritance of MEN2A and MEN2B and FMTC. Each disease has a distinct phenotype: MEN2A is associated with MTC, pheochromocytoma, and parathyroid hyperplasia. The MEN2B phenotype is more severe, being characterized by an earlier occurrence of more aggressive MTC. Unlike MEN2A patients, MEN2B patients develop multiple mucosal neuromas and several skeletal abnormalities. Finally, FMTC consists only of an inherited predisposition to MTC (3). Involvement of different tissues corresponds to differences in the nature of the underlying *RET* gene mutation. In most MEN2A cases, *RET* mutations consist in the loss of one of six cysteines localized in the extracellular domain. This causes constitutive dimerization mediated by the formation of disulfide bonds and activation of Ret kinase (4, 5). The same mutations have been found in various FMTC patients (3). In >80% of patients, MEN2B is caused by substitution of methionine 918 with a threonine (M918T; Ref. 6). Interestingly, the same mutation at a somatic level has been identified in ~30% of sporadic MTC, whereas MEN2A-like mutations are very rare in sporadic tumors (7).

The MEN2B (M918T) mutation occurs in the "activation loop" of Ret kinase, a domain that is critical not only for kinase activation but also for substrate selection (6). The MEN2B mutation induces constitutive activation of Ret kinase; however, the intrinsic kinase activity of Ret-MEN2B is not more potent than that of Ret-MEN2A (4, 5, 8). It is believed that Ret-MEN2B is not simply an activated Ret kinase. Maps of phosphorylated Ret peptides and two-dimensional gel electrophoresis experiments have demonstrated that the MEN2B mutation alters Ret autophosphorylation sites as well as the pattern of intracellular proteins undergoing tyrosine phosphorylation with respect to wild-type ligand-triggered Ret and to Ret-MEN2A (4, 9). Thus, the shift of Ret autophosphorylation sites and of Ret intracellular substrates, rather than the simple potentiation of Ret kinase activity, may be crucial for the oncogenic activity of Ret-MEN2B alleles.

The biological effects of RTKs are exerted mainly through autophosphorylation of tyrosine residues. The substrates of the RTK protein are characterized by docking sites, *i.e.*, PTB or Src-homology 2 domains. By recognizing receptor phosphotyrosines, these docking sites provide a means whereby enzyme and substrate can physically interact to induce the phosphotransfer reaction and activation of intracellular signal transduction (10, 11). The cytoplasmic domain of Ret contains 14 tyrosine residues; a longer form (1114 residues long), which arises from alternative splicing, contains 16. Ret phosphorylated tyrosines 905 and 1015 are docking sites for the Grb7/Grb10 adapters (12, 13) and for phospholipase Cγ (14), respectively. Tyrosine 1062 (Y1062) is essential for Ret signaling. Mutation of Y1062 impairs neoplastic transformation mediated by Ret-derived oncogenes (Ret-MEN2A and Ret-MEN2B; Refs.15, 16) and Ret-mediated PC12 cell survival (17). Y1062 is necessary for the binding of Shc (15, 18, 19) and Enigma (20) to Ret. Shc is a docking protein involved in the coupling of several receptors to the Ras/MAPK pathway (21). Enigma is a PDZ- and LIM-domain containing protein whose function is relatively obscure; however, evidence suggests that Enigma is involved in recruitment and clustering of Ret protein products at mem-

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[3] The abbreviations used are: GDNF, glial cell line-derived neurotrophic factor; MEN2A and MEN2B, multiple endocrine neoplasia type 2A and type 2B; FMTC, familial medullary thyroid carcinoma; MTC, medullary thyroid carcinoma; RTK, receptor tyrosine kinase; PTB, phosphotyrosine binding; MAPK, mitogen-activated protein kinase; PI3K, phosphatidyl-inositol-3 kinase; ERK, extracellular signal-regulated kinase; GST, glutathione-*S*-transferase; RBD, Ras-binding domain; PTK, protein tyrosine kinase.

brane level (20). Tyrosine 1062 is essential for Ret-mediated activation of the Ras/MAPK cascade (17), for the binding of Ret to the p85 regulatory subunit of PI3K, and for activation of PI3K and of the serine-threonine kinase Akt(PKB), a well-known PI3K effector (16, 17).

Neither the signaling pathways triggered by the MEN2B mutation nor the specific Ret sites whose autophosphorylation is altered by the MEN2B mutation, are known. Phosphorylation of Y1096, a residue present only in the 1114-amino acid-long Ret isoform and involved in Grb2 binding to Ret, is reduced in the Ret MEN2B protein (9, 22). Furthermore, the M918T mutation modifies the pattern of tyrosine phosphorylated proteins that bind to the Nck and Crk adapters. However, the identities and roles of these molecules in Ret signal transduction have yet to be demonstrated (23).

We have used phosphorylated-Ret specific antibodies directed against Y1015 and Y1062 (24) to analyze the phosphorylation states of Ret-MEN2B and Ret-MEN2A. Here we show that Y1062 and Y1015 are constitutively phosphorylated in Ret-MEN2A and Ret-MEN2B; however, phosphorylation of Y1062, but not of Y1015, is greatly increased in Ret-MEN2B compared with Ret-MEN2A. *In vitro* assay confirmed the more potent autokinase activity toward Y1062 resulting from the MEN2B mutation. Consistent with the increased phosphorylation of Y1062, the Ret-MEN2B oncoprotein is more active than Ret-MEN2A in recruiting the Shc adapter and in inducing activation of the Ras/MAPK and PI3K/Akt cascades. We conclude that selective increase of Y1062 autophosphorylation and of coupling to the Shc/Ras/MAPK and PI3K/Akt pathways is part of the specific pathogenetic mechanism of the MEN2B syndrome.

MATERIALS AND METHODS

Reagents and Cell Lines. Rabbit anti-Ret *p*Y1015 and 1062 were produced in our laboratory and are described elsewhere (24). Mouse monoclonal anti-phospho-ERK and anti-Shc polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-Ras, anti-Akt, and anti-phospho-Akt (Ser473) antibodies were purchased from New England Biolabs (Beverly, MA). Rabbit polyclonal anti-Ret antibodies are described elsewhere (25), as are expression vectors LTR-Ret Cys634-Tyr (Ret-MEN2A) and LTR-Ret Met918-Thr (Ret-MEN2B; Ref. 4), and NIH-3T3 and PC12 cells expressing Ret-MEN2A and Ret-MEN2B (4, 26).

Cells and Transfection Experiment. PC12 cells were grown in RPMI medium 1640 supplemented with 10% horse serum and 5% FCS. NIH-3T3 and COS-7 cells were grown in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FCS and were transfected with 1 μg of epitope (AU5)-tagged Ras construct (V12) as a positive control of the Ras activation assay, using the calcium phosphate precipitation method as described previously (27).

Protein Studies and Calf Intestinal Phosphatase Treatment. Cells were lysed in a buffer containing 50 mM HEPES (pH 7.5), 1% (v/v) Triton X-100, 50 mM NaCl, 5 mM EGTA, 50 mM NaF, 20 mM sodium PP_i, 1 mM sodium vanadate, 2 mM phenylmethylsulfonyl fluoride, and 0.2 mg/ml each of aprotinin and leupeptin. Lysates were clarified by centrifugation at 10,000 $\times$ *g* for 15 min, and the supernatant was processed for immunoblotting or for immunoprecipitation. Protein concentrations were measured with a modified Bradford assay (Bio-Rad, Munich, Germany). Immunocomplexes were revealed with an enhanced chemiluminescence detection kit (ECL; Amersham, Bucks., England). The signal was quantified with scanner densitometry of the films.

For the phosphatase treatment, lysates were dialyzed twice against phosphatase reaction buffer [05 mM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, 150 mM NaCl] supplemented with 0.1% Triton X-100, 0.05% SDS, and 2 mM phenylmethylsulfonyl fluoride, suspended in 20 μl of phosphatase reaction buffer containing 1% SDS, 1% 2-mercaptoethanol, and 2 mM phenylmethylsulfonyl fluoride, and heated (60°C for 3 min). Samples were then divided in half (10 μl), diluted with 40 μl of phosphatase reaction buffer, and incubated for 3 h at 37°C with or without 3 units of molecular biology-grade calf intestinal alkaline phosphatase (Boehringer Mannheim, Mannheim, Germany). The reaction was terminated by the addition of sample buffer; samples were electrophoresed on 7.5% acrylamide SDS-PAGE and analyzed by immunoblotting.

Detection of Activated Ras. Gst-Raf(RBD) encodes amino acids 1–149 of c-Raf-1 fused to GST (28). Bacterial cultures were induced with 1 mM isopropyl β-D-thiogalactopyranoside for 3 h. The induced bacteria were lysed by sonication in PBS containing 10 μg/ml aprotinin, and GST-Raf(RBD) protein was purified using glutathione-Sepharose (Pharmacia). The beads were washed in a solution containing 20 mM HEPES (pH 7.5), 120 mM NaCl, 10% glycerol, 0.5% NP-40; 2 mM EDTA, and 10 μg/ml aprotinin and used within 2–3 days after preparation. Cells were lysed, and the clarified extract (1 mg) was incubated with 15 μg of immobilized GST-Raf(RBD) protein for 1 h at 4°C with rocking. Protein complexes were resolved by SDS-PAGE (10% acrylamide), transferred to polyvinylidene difluoride (Immobilon-P) membranes, and stained with anti-Ras antibodies.

Pull-Down Experiments Cell lysates were subjected to "pull-down" binding assays with purified GST-Shc(PTB) recombinant protein immobilized on agarose beads. The GST fusion protein of the PTB domain of Shc (29) was expressed in bacteria and purified with glutathione-conjugated Sepharose beads (Sigma, St. Louis, MO) by standard procedures. The protein complexes were washed several times with the cell lysis buffer, eluted, and resolved by SDS-PAGE. Western blotting with specific antibodies and enhanced chemiluminescence (ECL; Amersham) were used for immunodetection of proteins in the complex.

Kinase Assay. ERK activity was measured in PC12 cells that express Ret-MEN2A and Ret-MEN2B. Protein lysates (1 mg) were immunoprecipitated with anti-ERK; 20 μg of myelin basic protein was used as a substrate for the kinase reaction, as described previously (30). Ret kinase activity was assayed in PC12 Ret-MEN2A and Ret-MEN2B cells, essentially as described elsewhere (4). A GST fusion protein containing the COOH-terminal 72 residues of Ret, GST-Ret(C-tail), which include Y1015 and Y1062 cloned in the pGEX2T vector (Amersham Pharmacia Biotech), served as substrate. Protein lysates (1 mg) were immunoprecipitated with anti-Ret; 15 μg of GST-Ret(C-tail) was used as substrate for the kinase reaction.

RESULTS

Increased Phosphorylation of Y1062 in Ret-MEN2B with Respect to Ret-MEN2A. To analyze Ret-MEN2A and Ret-MEN2B (Fig. 1*A*) phosphorylation levels, we used antibodies that detect the phosphorylation of two critical autophosphorylation sites of human Ret (Ab-*p*Y1015 or Ab-*p*Y1062). Protein extracts were obtained from stably transfected NIH-3T3 and PC12 cells and subjected to immunoblotting with the anti-phospho-Ret antibodies. The oncoproteins of the two cell lines appeared as a doublet of 155 and 170 kDa and had similar Y1015 phosphorylation levels (Fig. 1*B*). In contrast, in both cell types, the phosphorylation levels of Y1062 were much higher (~5-fold) in the Ret-MEN2B protein than in the Ret-MEN2A protein (Fig. 1*B*).

To confirm the specificity of our antibodies, we analyzed by Western blot lysates from NIH-3T3/MEN2A either treated or untreated with calf intestinal phosphatase. As shown in Fig. 1*C*, the signal detected in NIH-2A was not present in the untransfected NIH-3T3 cells (Fig. 1*C*, *Lane C*) as well as in the phosphatase-treated NIH-2A (Fig. 1*C*, *Lane 2A-CIP*), indicating that the phosphorylated tyrosine residue is required for antibody recognition.

Ret oncoproteins bind the PTB domain of Shc through phosphotyrosine 1062 (15, 18, 19). Consequently, an independent way to determine the extent of Y1062 phosphorylation is to measure the Shc(PTB) interaction. Protein extracts from PC12-MEN2A and -MEN2B cells were subjected to a pull-down assay with a recombinant Shc(PTB) domain expressed in *Escherichia coli* as a GST fusion protein. Protein extracts from parental PC12 cells served as a control. Proteins interacting with GST-Shc(PTB) were separated by SDS-PAGE and immunoblotted with anti-Ret. As shown in Fig. 2*A*, higher amounts of Ret-MEN2B interacted with GST-Shc(PTB) than with Ret-MEN2A. This shows that Ret-MEN2B contains higher levels of

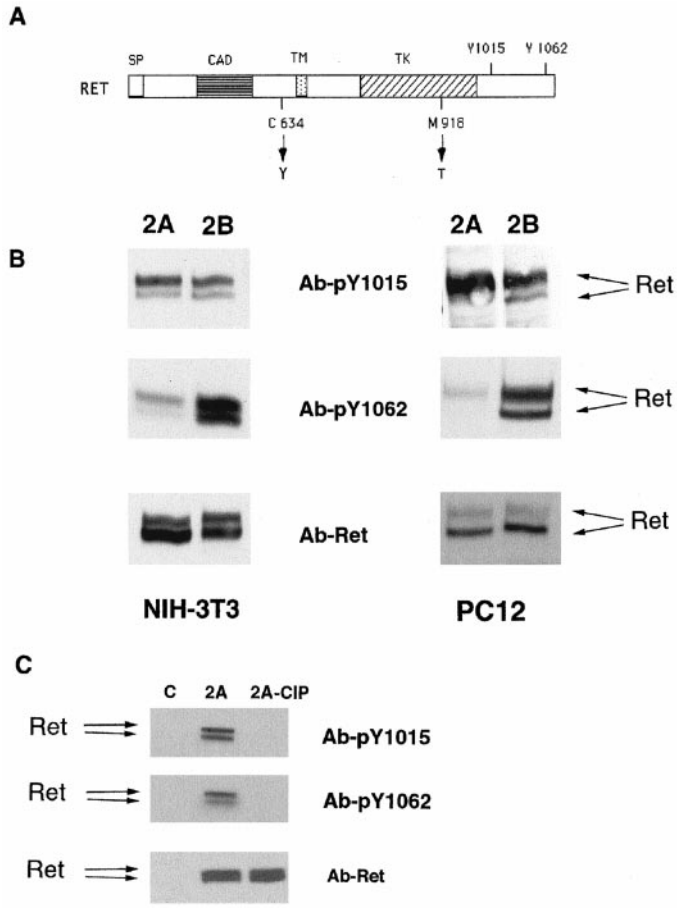

Fig. 1. *A*, schematic representation of the Ret protein. *SP*, signal peptide; *CAD*, cadherin homologous domain; *TM*, transmembrane domain; *TK*, tyrosine kinase domain. The mutations associated with MEN2A (C634Y) and MEN2B (M918T) analyzed in this study are shown. Y1015 and Y1062 Ret autophosphorylation sites are also indicated. *B*, different patterns of autophosphorylation of Ret-MEN2A (*Lane 2A*) and Ret-MEN2B (*Lane 2B*). Fifty μg of protein extract from PC12 or NIH-3T3 cells expressing Ret-MEN2A or Ret-MEN2B were used for Western blot as indicated (in the case of NIH-MEN2B, 100 μg of protein were used). Whereas the PC12-MEN2A and PC12-MEN2B cells expressed comparable levels of Ret oncoproteins, NIH-MEN2B expressed lower levels (4-fold) of oncogenic Ret with respect to NIH-MEN2A. *Ab*, antibody. *C*, phosphorylation of Y1015 and Y1062 is critical for antibody recognition. Protein extracts (50 μg) from NIH (*Lane C*) or NIH-MEN2A either treated (*Lane 2A-CIP*) or not treated (*Lane 2A*) with calf intestinal phosphatase were immunoblotted with the indicated phosphoantibodies (*Ab-pY1015* and *Ab-pY1062*) as well as with a polyclonal anti-Ret (*Ab-Ret*). Ret isoforms are indicated by *arrows*. In *B* and *C*, the 155–170 kDa doublet corresponding to Ret oncoproteins is indicated.

phosphotyrosine 1062 than Ret-MEN2A. An immunoblot stained with anti-Ret antibody demonstrated equal expression levels of the Ret mutants (Fig. 2*B*).

Ret MEN2B Has More Potent Kinase Activity than Ret-MEN2A toward Y1062 We next investigated whether the increased phosphorylation of Y1062 in Ret-MEN2B was attributable to an increased capacity of the Ret-MEN2B kinase to phosphorylate this residue. To this aim, we performed an *in vitro* kinase assay using as substrate a GST fusion protein containing the COOH-terminal 72 residues of Ret, *i.e.*, GST-Ret(C-tail), which include Y1015 and Y1062. Equal amounts of Ret proteins from PC12-MEN2A and PC12-MEN2B were immunoprecipitated with anti-Ret. Protein extracts from parental PC12 cells served as a control. The immunocomplexes were incubated with ATP and GST-Ret(C-tail). The reaction products were subjected to SDS-PAGE, and the phosphorylation of immunoprecipitated Ret oncoproteins and GST-Ret(C-tail) was measured by immunoblot with Ab-*p*Y1015 or Ab-*p*Y1062. As shown in Fig. 3*A* (*bottom*), Ret-MEN2B kinase was clearly more potent than Ret-MEN2A kinase in phosphorylating GST-Ret(C-tail) on Y1062. Consistently, the autophosphorylation of Ret-MEN2B on Y1062 was also clearly more potent than Ret-MEN2A (Fig. 3*A*, *top*). We know that most of the phosphorylation detected on the Ret oncoproteins occurred during the *in vitro* assay because cells were harvested in the absence of phosphatase inhibitors, thus minimizing the amount of phosphorylation occurred *in vivo* before collecting cells (data not shown). On the other hand, the two kinases phosphorylated GST-Ret(C-tail) on Y1015 to a similar extent. Immunoblotting demonstrated that equal amounts of Ret kinases were used for the assay (Fig. 3*B*).

Increased Phosphorylation of Y1062 in Ret-MEN2B Corresponds to Increased Activation of the Ras/MAPK and PI3K/Akt Cascades. Because Ret Y1062 is part of the docking site for Shc (15, 18, 19), we hypothesized that the increased phosphorylation of this tyrosine in Ret-MEN2B may correspond to greater recruitment of Shc

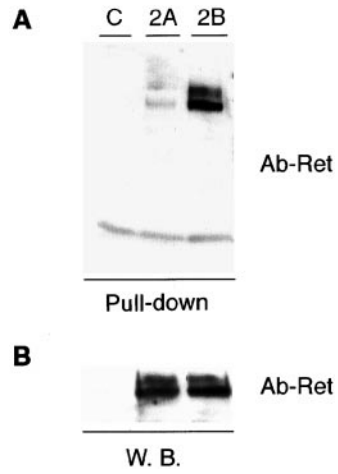

Fig. 2. Pull-down experiment of Ret-MEN2A and Ret-MEN2B with purified GST-Shc(PTB) recombinant protein. *A*, lysates (700 μg) from PC12-MEN2A (*Lane 2A*) and PC12-MEN2B (*Lane 2B*) cells were incubated with purified GST-Shc recombinant protein for 3 h at 4°C. The protein complexes were eluted and resolved by SDS-PAGE, and bound Ret levels were detected by immunoblotting with anti-Ret (*Ab-Ret*) as indicated. *Lane C*, control. *B*, 25 μg of lysates were analyzed by Western blotting (*W. B.*), as internal control for the experiment.

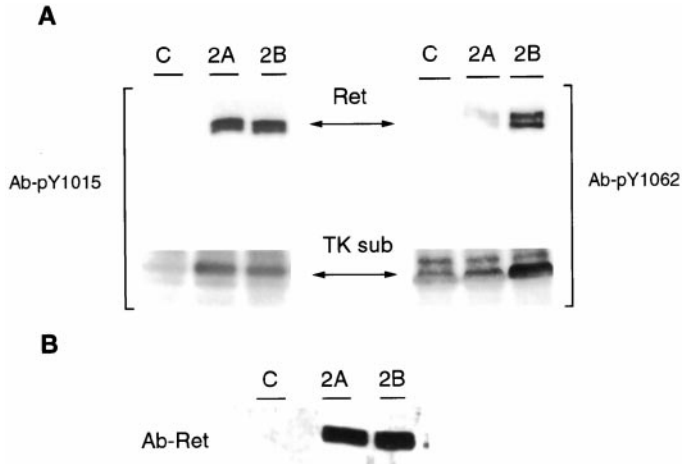

Fig. 3. Ret-MEN2B has a more potent kinase activity than Ret-MEN2A toward Y1062. *A*, protein lysates from PC12-MEN2A (*Lane 2A*) and PC12-MEN2B (*Lane 2B*) or untransfected PC12 (*Lane C*) were immunoprecipitated with anti-Ret. The immunocomplexes were washed three times with lysis buffer and incubated with ATP and GST-Ret(C-tail) (see "Material and Methods"). The reaction products were subjected to SDS-PAGE, and the phosphorylation of both immunoprecipitated Ret and GST-Ret(C-tail) was measured by immunoblot with Ab-*p*Y1015 or Ab-*p*Y1062. *B*, 25 μg of lysates were analyzed by Western blotting, as internal control for the experiment.

proteins and potentiated activation of the corresponding downstream pathways. To test this hypothesis, we examined the level of Shc phosphorylation in PC12 cells expressing Ret-MEN2A or Ret-MEN2B. Protein lysates from serum-starved cells were immunoprecipitated with anti-Shc antibodies and immunoblotted with antiphosphotyrosine antibodies. As shown in Fig. 4, the 66-, 52-, and 46-kDa Shc proteins were tyrosine-phosphorylated in PC12 cells expressing oncogenic Ret alleles with respect to parental cells. More importantly, Shc phosphorylation was at least 3-fold more pronounced in Ret-MEN2B- than in Ret-MEN2A-expressing cells (Fig. 4*A*). Immunoblot analysis with Ab-Shc, as a loading control, showed comparable levels of Shc proteins in the tested cell lines.

By recruiting Grb2/Sos complexes, Shc is involved in Ras activation by RTKs (10). Thus, we compared the extent of Ras activation in PC12-MEN2A and -MEN2B cells. To this aim, we exploited the interaction between Ras-GTP, the active Ras isoform, and the RBD of Raf-1 expressed as a recombinant GST fusion protein (28). Protein extracts from Ret-MEN2A and Ret-MEN2B cells were incubated with GST-Raf(RBD); protein complexes were separated by SDS-PAGE and stained with anti-Ras antibodies. As shown in Fig. 4*B*, Ras activation induced by Ret-MEN2B was ~2-fold stronger than that caused by Ret-MEN2A. Ras expression levels were comparable in MEN2A and MEN2B cells and were slightly elevated *versus* untransfected cells (Fig. 4*B*). The V12 oncogenic Ras mutant transiently expressed in COS-7 cells was used as a positive control for the assay.

Y1062 is also essential for recruitment of PI3K to the Ret receptor (16, 17). PI3K activation results in the triggering of multiple downstream effectors including the serine-threonine kinase Akt(PKB) (31). On the other hand, activation of the Ras cascade leads to a series of events culminating in activation of the MAPKs ERK1 and ERK2 and also contributes to the activation of PI3K/Akt pathways. Thus, we determined ERK and Akt activation as a measure of activation of the Ras and of the PI3K signaling pathways in cells expressing Ret-MEN2A or Ret-MEN2B.

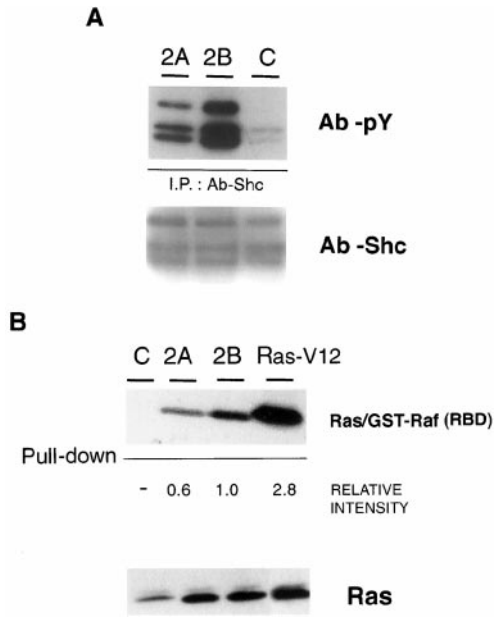

Fig. 4. Shc phosphorylation and ras activation in PC12 cells expressing Ret-MEN2A (*Lanes 2A*) or Ret-MEN2B (*Lanes 2B*). *A*, PC12 expressing Ret-MEN2A or Ret-MEN2B were serum starved. After 24 h, cells were harvested, and 1 mg of each protein extract was used for immunoprecipitation (*I.P.*) with anti-Shc and blotted with anti-Shc (*Ab-Shc*) or antiphosphotyrosine antibodies (*Ab-pY*) as indicated. *B*, 1 mg of cell lysate was incubated with 15 μg of immobilized GST-Raf(RBD) protein; recovered protein complexes were resolved by SDS-PAGE (10% acrylamide), transferred to polyvinylidene difluoride (Immobilon-P) membranes, and stained with anti-Ras antibodies. The same lysates (50 μg) were immunoblotted with anti-Ras antibody for normalization. Bands were analyzed using a Phospho Imager densitometer, and intensity is expressed as arbitrary units. *Lane C*, control; *Lane Ras-V12*, (AU5)-tagged Ras construct used as positive control.

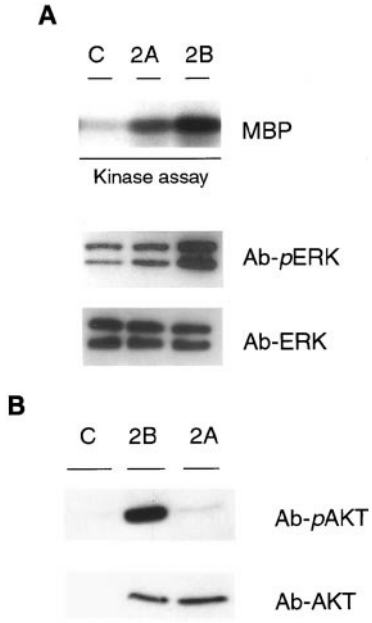

Fig. 5. Increased activation of ERK and pAKT by the Ret-MEN2B mutant. *A*, subconfluent PC12 cells expressing Ret-MEN2A (*Lane 2A*) or Ret-MEN2B (*Lane 2B*) were serum starved overnight. ERK proteins were immunoprecipitated from 1 mg of cell lysate, and the immunoprecipitates were incubated with 1 μCi of [γ-^{32}P]ATP and 20 μg of myelin basic protein (*MBP*). Parallel samples were immunoblotted with anti-phospho-ERK (*Ab-pERK*) or anti-ERK (*Ab-ERK*). *B*, subconfluent PC12 cells expressing Ret-MEN2A (*Lane 2A*) or Ret-MEN2B (*Lane 2B*) were serum starved overnight. Fifty μg were resolved by SDS-PAGE and blotted with anti-S473-phospho-AKT (*Ab-pAKT*) or anti-AKT (*Ab-AKT*) as indicated. *Lane C*, control.

We used two techniques to evaluate ERK activation. ERK2 protein was recovered by immunoprecipitation from PC12 cells expressing Ret-MEN2A or Ret-MEN2B. The immunoprecipitates were subjected to an *in vitro* kinase assay using myelin basic protein as substrate. Untransfected PC12 cells had very low MAPK activity, whereas Ret-MEN2A expression promptly induced MAPK activity (Fig. 5*A*). Activation of MAPK by Ret-MEN2B was ~2-fold higher than that caused by Ret-MEN2A. These findings were confirmed by an independent assay. Protein lysates from the same cells were analyzed by immunoblot with an antibody specific for the phosphorylated (*i.e.*, active), ERK proteins. Again, ERK phosphorylation levels in Ret-MEN2B cells was 2–3-fold higher than in Ret-MEN2A cells. An immunoblot with an anti-ERK antibody was performed for normalization (Fig. 5*A*).

Finally, we used immunoblotting to determine the levels of Akt phosphorylation on Ser473, a marker of Akt activation. Akt phosphorylation was detected in Ret-expressing but not in parental PC12 cells. The extent of Akt phosphorylation was much higher (~6-fold) in Ret-MEN2B- than in Ret-MEN2A-expressing cells.

DISCUSSION

MEN2B is the most severe variant of the MEN2 syndromes. It is characterized by aggressive MTC with a very early occurrence. Furthermore, the MEN2B phenotype is different from the other MEN2 syndromes, including mucosal neuromas, hyperganglionosis of the intestinal tract, marfanoid abitus, and ophthalmic abnormalities (3). At variance with MEN2A-associated mutations, the M918T MEN2B-type mutation is frequently found in sporadic MTCs (7). This also indicates that when the mutation occurs at somatic level, it can induce neoplastic transformation of thyroid C cells.

Oncogenic activation of RTKs is mediated by constitutive activation of their enzymatic activity. In most cases, this catalytic activation is caused by mutations that induce constitutive dimerization (32). This is the case of most of the Ret-MEN2A and Ret-FMTC mutants (4, 33). MEN2B is an exception to this paradigm. The M918T mutation does not cause constitutive dimerization, and the overall activation levels of the Ret kinase it induces can hardly account for its high oncogenic potential (4). Thus, Ret-MEN2B is probably more than simply an active Ret kinase, and qualitative changes in Ret kinase activity may be responsible for its specific neoplastic phenotype. M918 is highly conserved in RTKs, and it maps in a loop of the kinase domain that is predicted to interact with the protein substrate. A threonine is found at the equivalent position in cytosolic PTKs, and the two kinase classes (RTK and PTK) have different signaling specificities (34). Accordingly, the MEN2B mutation causes a shift of the optimal peptide substrate from that preferentially phosphorylated by RTKs to that selected by PTKs (34). The change in substrate specificity can affect Ret-mediated phosphorylation of intracellular proteins as well as the pattern of Ret autophosphorylation sites. Both possibilities have been experimentally proven. The pattern of phosphorylated intracellular proteins differs in Ret-MEN2B- and Ret-MEN2A-expressing cells (4). Moreover, phosphopeptide mapping has shown that Ret-MEN2B autophosphorylation sites differ dramatically from those of wild-type Ret and of Ret-MEN2A (4, 9).

We have produced phosphorylation-specific antibodies directed against Ret Y1015 and Y1062 (24). Here we demonstrate that although both residues are constitutively phosphorylated in Ret-MEN2A and Ret-MEN2B, Y1062 autophosphorylation is significantly increased by the MEN2B mutation with respect to the MEN2A mutation. In agreement with this finding, a MEN2B kinase phosphorylated Y1062 more efficiently than did a MEN2A kinase in an *in vitro* kinase assay, We believe that the increased phosphorylation of Y1062 depends on the altered specificity of the Ret-MEN2B kinase. Indeed, the Ret sequence that flanks Y1062 (Leu-Tyr-Gly-Arg-Ile) corresponds to the optimal substrate (Ile/Leu/Val-Tyr-Gly-X-hydrophobic) of PTK (to which Ret-MEN2B functionally belongs; see Ref. 35) and thus, Y1062 has the structural requisites for efficient phosphorylation by a Ret-MEN2B kinase.

Several lines of evidence suggest that the high phosphorylation of Y1062 has significant biological effects. Residue Y1062 is essential for oncogenic and survival Ret signaling and for activation of Ras/MAPK and PI3K/Akt. Parallel to the increased phosphorylation of Y1062, we show that the MEN2B mutation potentiates recruitment of Shc and activation of Ras/MAPK and of Akt with respect to Ret-MEN2A. Given the susceptibility of Ret-MEN2B to ligand triggering (36), the above-mentioned differences may be more pronounced *in vivo* in the presence of ligands.

In conclusion, we propose that the high level of Y1062 phosphorylation caused by the MEN2B mutation can be involved in determining the MEN2B phenotype. This suggests the intriguing possibility of using residue Y1062 as a target for therapeutic approaches to MEN2B as well as to sporadic MTC neoplasias.

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