A Mutation at Tyrosine 1062 in MEN2A-Ret and MEN2B-Ret Impairs Their Transforming Activity and Association with Shc Adaptor Proteins

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Germ line mutations of the ret proto-oncogene are associated with the development of three dominantly inherited neoplastic disorders, multiple endocrine neoplasia (MEN) 2A, MEN 2B, and familial medullary thyroid carcinoma. It has been demonstrated that the mutations result in constitutive activation of the Ret protein, leading to transformation of NIH 3T3 cells. In the present study we investigated the role of tyrosine residues present in the carboxyl-terminal sequence for the transforming activity of Ret with the MEN 2A or MEN 2B mutation (MEN2A-Ret or MEN2B-Ret). Substitution of phenylalanine for tyrosine at position 1062 (designated Y1062F) markedly impaired the transforming activity of both MEN2A-Ret and MEN2B-Ret, whereas substitution or deletion for four other tyrosines (codons 981, 1015, 1090, and 1096) did not affect their activity. The Shc adaptor proteins bound to the MEN2A-Ret and MEN2B-Ret proteins and were phosphorylated on tyrosine in the transfectants. The binding of Shc to the Y1062F mutant proteins was reduced by approximately 80% indicating that tyrosine 1062 is a major binding site for Shc. In addition, phosphopeptide analysis of MEN2A-Ret demonstrated that tyrosine 1062 represents an auto-phosphorylation site of the mutant Ret proteins.

The ret proto-oncogene encodes a receptor tyrosine kinase whose ligand has not been identified (1–3). It turned out that germ line mutations of ret are responsible for the development of four different neural crest disorders including multiple endocrine neoplasia (MEN) 2A, MEN 2B, familial medullary thyroid carcinoma, and Hirschsprung's disease. Among these, MEN2A, MEN2B, and FMTC are dominantly inherited neoplastic disorders, the former two of which share the clinical feature of medullary thyroid carcinoma and pheochromocytoma. FMTC is characterized by the development of medullary thyroid carcinoma alone. It is of note that the RET protein is mutated in these two disorders, whereas the MEN2A mutation results in inactivation of the Menin protein (4). The MEN2A mutations result in the activation of the RET protein, leading to its constitutive activation (5, 6).

Since the activation of MEN2A-Ret could mimic that of other receptor tyrosine kinases, it is of interest to determine the physiological significance of the RET sequence. To this end, we have initiated a study to identify the regulatory sequences of the RET protein.

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synthesized and used for amplification of c-ret sequences of approximately 100–150 base pairs. The corresponding sequences of the c-ret gene were replaced with the amplified fragments with the mutations.

**Transformation—**Each recombinant plasmid (0.05–0.2 μg) was transformed into NIH 3T3 cells (5 × 10^6 cells in a 60 mm-diameter dish) with 10 μg of NIH 3T3 DNA as described previously (1). Transformed cells were scored on day 12 after transformation. Then foci were picked up and grown into cell lines.

**Western Blotting—**Total cell lysates were prepared from NIH 3T3 cells and transfectants as described previously (26). The lysates were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes (Ni-hon Millipore Kogyo KK, Tokyo, Japan). After membranes were reacted with anti-Ret antibody (26, 27), anti-Shc antibody (Upstate Biotechnology Inc., Lake Placid, NY), or anti-phosphotyrosine antibody (Zymed Laboratories, Inc., South San Francisco, CA), the reaction was examined by the avidin-biotin complex immunoperoxidase method (26) or by the avidin-biotin complex immunoperoxidase method (26).

**Expression and Phosphorylation of Fusion Proteins—** A cDNA fragment with or without the Y1062F mutation comprising nucleotides 3075–3714 (numbered according to the published sequence (2)) was inserted between StuI and PstI sites of pMAL-c2 expression vector (New England BioLabs, Beverly, MA). The recombinant plasmids were transformed into Epicurian Coli TKX1 competent cells (Stratagene, La Jolla, CA) that contain a plasmid-encoded, inducible tyrosine kinase gene. To express the fusion proteins, transformed cells were grown in 2 × YTG broth (16 g of tryptone/liter, 10 g of NaCl/liter, and 10 g of yeast extract/liter) containing 2% (w/v) glucose, 50 μg/ml ampicillin and 12.5 μg/ml tetracycline, and the induction was performed with 0.3 μM isopropyl-β-D-thiogalactopyranoside (IPTG). The cultures reached an A600 = 1–2. The cells were spun down at 2,000 × g and resuspended in TK induction media (see manufacturer’s (Stratagene) protocol) to an A600 of 0.5. They were then grown for 2 h at 37°C to phosphorylate the fusion proteins. Phosphorylation of the fusion proteins was evaluated by Western blotting with anti-phosphotyrosine antibody.

**In Vitro Binding Assay—**Induced bacteria were lysed by sonication in lysis buffer (20 mM Tris-HCl pH 7.5, 50 mM NaCl, 2 mM EDTA, 0.1% Triton X-100). Lysates were clarified by centrifugation at 12,000 × g for 10 min, and the MBP-Ret fusion proteins were purified using the amylose resin. NIH 3T3 cells (approximately 1 × 10^6) were lysed in radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) containing 1 mM phenylmethylsulfon fluoride and 0.5 mM sodium orthovanadate, and clarified lysates were incubated with 1 μg of immobilized MBP-Ret fusion proteins at 4°C overnight. The protein complexes were washed four times with RIPA buffer and eluted in SDS-sample buffer (20 mM Tris-HCl, pH 6.8, 2 mM EDTA, 2% SDS, 10% sucrose, 20 μg/ml bromophen blue) by boiling for 3 min. Then protein complexes were subjected to Western blotting with anti-Shc antibody.

**Cell Labeling and Immunoprecipitation—**Cells were labeled for 4–5 h in phosphate-free RPMI medium containing [35S]methionine (1 mCi/ml; ICN) supplemented with 10% dialyzed fetal calf serum. After washing with phosphate-buffered saline, pH 7.2, cells were lysed in RIPA buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) containing 1 mM phenylmethylsulfon fluoride and 0.5 mM sodium orthovanadate. The lysates were clarified by centrifugation (15,000 × g) for 1 h, incubated with Sepharose beads conjugated with antibodies at 4°C overnight, and washed with RIPA buffer four times. The resulting antigen-antibody complex was suspended in SDS-sample buffer in the presence of 80 μM dithiothreitol and boiled for 3 min.

**C-termini Peptide Maps—**Immunoprecipitated phosphopeptides were resolved by SDS-8% polyacrylamide gel electrophoresis (28), and a 6-kDa phosphopeptide-related fragment was excised, digested with trypsin and separated in two dimensions. Then peptides were digested with trypsin and separated in two dimensions.

**RESULTS**

**Transforming Activity of Ret with Mutations of Tyrosine Residues—**We introduced the MEN 2A (Cys634-Arg, C634R) or MEN 2B (Met918-Thr, M918T) mutation into two Ret isoforms of 1114 amino acids (long isoform) and 1072 amino acids (short isoform) (Fig. 1) and investigated their transforming activity. The 51 carboxy-terminal amino acids of the long isoform were replaced by the 9 unrelated amino acids of the short isoform by alternative splicing in the 3′ region (30). The former sequence contains an additional two tyrosines (tyrosines 1090 and 1096) as compared with the latter sequence (Fig. 1). The mutant ret cDNAs were inserted into the expression vector containing the cytomegalovirus promoter and transfected into NIH 3T3 cells. As shown in Table I, the transforming activity of the long isoform with the MEN 2A mutation was 1.5-fold higher than that of its short isoform. On the other hand, the activity of the long isoform with the MEN 2B mutation was approximately 10-fold higher than that of the short isoform with the MEN 2B mutation and 2–3-fold higher than that of both isoforms with the MEN 2A mutation. Since the transfection efficiencies of each construct (5 × 10^6 G418-resistant colonies/μg of DNA) were comparable, these results suggested that the carboxy-terminal tail sequences of two isoforms differently regulate the activity of the mutant Ret proteins.

**To investigate whether the length of the carboxy-terminal sequence influences the activity of Ret with the MEN 2A or MEN 2B mutation (MEN2A-Ret or MEN2B-Ret), we truncated the long isoform after codon 1074 (designated DEL-1074). This truncation removed tyrosines 1090 and 1096 present in the long isoform. However, the truncation did not significantly affect the transforming activity of MEN2A-Ret and MEN2B-Ret (Table I). Since the length of the carboxy-terminal tail in the DEL-1074 mutant protein was comparable with that of the short isoform, the low transforming activity of the short isoform of MEN2B-Ret appears to be caused by its specific carboxy-terminal sequence. In addition, this result indicated that tyrosines 1090 and 1096 do not play a crucial role for the transforming activity of the long isoform of MEN2A-Ret and MEN2B-Ret.

**We next replaced tyrosines 1015 and 1062 in the carboxy-terminal tail of the long isoform as well as tyrosine 981 in the kinase domain with phenylalanine (Fig. 1; designated Y1015F, Y1062F, and Y981F, respectively). Although another tyrosine (codon 1029) was present in the carboxy-terminal sequence, substitution for this tyrosine has been unsuccessful in our
experiments. Among these, replacement of tyrosine 981 or tyrosine 1015 did not affect the transforming activity of MEN2A-Ret and MEN2B-Ret. In contrast, substitution for tyrosine 1062 severely impaired the activity of both of them (Table I), suggesting that tyrosine 1062 is one of major sites recognized by signaling molecules important for their transforming activity.

Establishment of the Cell Lines Expressing the Mutant Ret Proteins—In order to analyze the role of tyrosine residues in the intracellular signaling via the Ret protein, we established the cell lines expressing each mutant Ret protein at high levels (Fig. 2A). As expected, molecular mass of the short isoform and the DEL-1074 mutant protein (150 and 170 kDa) was approximately 5 kDa smaller than that of the long isoform (155 and 175 kDa). The cell lines expressing MEN2A-Ret with the Y1062F mutation or MEN2B-Ret with the Y1062F mutation (designated NIHret(C634R,Y1062F)L and NIHret(M918T,Y1062F)L cells) showed a partially transformed phenotype, whereas other cell lines expressing the Y981F, Y1015F, or DEL-1074 mutant proteins were spindle-shaped and highly refractile. The cells expressing the short isoform of MEN2B-Ret also showed a fully transformed phenotype. When these cells were injected subcutaneously into Scid mice, all of them formed solid tumors, although the latency of NIHret(C634R,Y1062F)L and NIHret(M918T,Y1062F)L cells was longer (15–21 days) than that of the other cell lines (6–7 days) (Table I).

Fig. 2B shows Western blot analysis with anti-phosphotyrosine antibody. The patterns of tyrosine phosphorylation are similar among the cell lines expressing each isoform of MEN2A-Ret or MEN2B-Ret or expressing the Y981F, Y1015F, or DEL-1074 mutant proteins. As we have already reported (11, 14), the level of tyrosine phosphorylation of the 170–175-kDa Ret proteins present on the cell surface was higher than that of the 150–155-kDa Ret proteins present in the endoplasmic reticulum. In addition, several other proteins including 74-, 58-, and 50-kDa proteins were phosphorylated on tyrosine at variable levels in each transfectant. On the other hand, the level of tyrosine phosphorylation somewhat decreased in NIH(C634R,Y1062F)L and NIH(M918T,Y1062F)L cells (Fig. 2B), although the Y1062F mutation did not influence the autokinase activity of MEN2A-Ret and MEN2B-Ret in vitro (data not shown).

Shc Adaptor Proteins Bind to Tyrosine 1062—Since Borrello et al. (31) reported that two forms of rearranged Ret (Ret/ptc1 and Ret/ptc2) found in human papillary thyroid carcinoma bound the Shc proteins in vivo, we investigated whether the MEN2A-Ret and MEN2B-Ret proteins also bind Shc. After the lysates from NIHret(C634R)L, NIHret(M918T)L, NIHret(C634R,Y1062F)L, and NIHret(M918T,Y1062F)L cells were immunoprecipitated with anti-Ret antibody, they were immunoblotted with the anti-Ret or anti-Shc antibody (Fig. 3A). As a result, it turned out that the 52- and 46-kDa Shc proteins were coprecipitated with the MEN2A-Ret or MEN2B-Ret protein. Interestingly, the degree of binding of Shc to MEN2A-Ret and MEN2B-Ret with the Y1062F mutation markedly decreased (~80%). Since the Y981F, Y1015F and DEL-1074 mutant proteins did not show a significant change of the binding ability for Shc as compared with that of the MEN2A-Ret and MEN2B-Ret proteins (data not shown), the results suggested that tyrosine 1062 of Ret represents a major binding site for Shc and that its binding is associated with the transforming activity of the mutant Ret proteins.

![Image](https://example.com/image.png)

**FIG. 2. Characterization of NIH 3T3 cells expressing the mutant Ret protein.** A, expression of the mutant Ret proteins in the transfecteds. Total cell lysates (20 μg of proteins) were prepared from the designated cell lines, separated on SDS-7.5% polyacrylamide gels under reducing conditions, and subjected to immunoblotting with anti-Ret antibody. The 150-, 155-, 170-, and 175-kDa Ret proteins are indicated. B, tyrosine phosphorylation in the transfecteds. The lysates were immunoblotted with anti-phosphotyrosine antibody. Several tyrosine phosphorylated bands detected in the transfecteds are indicated.

**TABLE I**

| DNA | Focus-forming activity (foci/μg of DNA) | No. of Scid mice with tumor formation/total number of Scid mice (3) | Latency (days) |
|-----|----------------------------------------|-------------------------------------------------|----------------|
| c-retS | <0.2 | 0/3 | - |
| c-ret | <0.2 | 0/3 | - |
| ret(C634R) | 50–80 | 3/3 | 6–7 |
| ret(C634R) | 70–120 | 3/3 | 6–7 |
| ret(M918T) | 150–300 | 3/3 | 6–7 |
| ret(C634R, DEL-1074) | 70–120 | 3/3 | 6–7 |
| ret(C634R, Y981F) | 70–120 | 3/3 | 6–7 |
| ret(C634R, Y1015F) | 70–120 | 3/3 | 6–7 |
| ret(C634R, Y1062F) | 70–120 | 3/3 | 6–7 |
| ret(M918T, DEL-1074) | 150–300 | 3/3 | 6–7 |
| ret(M918T, Y981F) | 150–300 | 3/3 | 6–7 |
| ret(M918T, Y1015F) | 150–300 | 3/3 | 6–7 |
| ret(M918T, Y1062F) | 10–20 | 3/3 | 15–21 |

a Transformed foci were counted on day 12 after transfection.

b NIH 3T3 cells (3×10⁴) expressing each construct were subcutaneously injected in female Scid mice. Mice were checked for tumor formation until 40 days after injection.

S, short isoform of ret.

d, long isoform of ret.
We next examined the level of tyrosine phosphorylation of the Shc proteins. The cell lysates were immunoprecipitated with anti-Shc antibody and immunoblotted with anti-Phosphotyrosine antibody. As shown in Fig. 3, the 52- and 46-kDa Shc proteins were phosphorylated on tyrosine in NIH ret(C634R) and NIH ret(C634R,Y1062F) cells, whereas the content of phosphotyrosine on the Shc proteins was significantly reduced in NIH ret(C634R,Y1062F) cells. This result suggested a role for the Y1062F mutation in impairing Shc binding to the mutant Ret protein.

DISCUSSION

In the present study, we first compared the transforming activity of two Ret isoforms that differ in their carboxyl-terminal sequence. The carboxyl-terminal 9 amino acids of the short isoform were replaced by the 51 amino acids of the long isoform that contains two additional tyrosine residues (Tyr1090 and Tyr1096) (1, 2, 30). Although the difference in the transforming activity between both isoforms of MEN2A-Ret was small, the activity of the short isoform of MEN2B-Ret was approximately 10-fold lower than that of its long isoform and 3–4-fold lower than that of both isoforms of MEN2A-Ret. These results were in agreement with the results reported by Borrello et al. (13) demonstrating the low transforming activity of the short isoform of MEN2B-Ret. Since most, but not all, transfectants that we isolated showed relatively low levels of expression of the short isoform of MEN2B-Ret in comparison with the expression levels of its long isoform (data not shown), the former protein may be more unstable than the latter. On the other hand, it is possible that the short isoform of MEN2A-Ret might be stabilized by its dimerization, resulting in higher transforming activity than that of the short isoform of MEN2B-Ret.

To examine whether the carboxyl-terminal sequence of the short isoform with tyrosines 1090 and 1096 plays a role in the transforming activity, we truncated its 41 carboxyl-terminal amino acids (DEL-1074). However, this truncation did not decrease the transforming activity of MEN2A-Ret and MEN2B-Ret. This result suggested that the low transforming activity of the short isoform of MEN2B-Ret was caused by its specific carboxyl-terminal sequence rather than by its short length.

Since deletion of tyrosines 1090 and 1096 did not affect the activity of the long isoform of MEN2A-Ret and MEN2B-Ret, we
Thus, it seems likely that the Shc proteins recognize phosphorylated tyrosine 1062 and transmit the signal of MEN2A-Ret and MEN2B-Ret. In this respect, it has recently been reported that substitution of phenylalanine for tyrosine 1062 abolished the mitogenic activity of Ret/ptc2, a rearranged form of Ret detected in human papillary thyroid carcinoma (32). On the other hand, replacement of tyrosine 1029, the biological role of which was not examined in our experiments, had no significant effect on the mitogenic activity. Furthermore, Borrello et al. (31) observed that two forms of rearranged Ret (Ret/ptc1 and Ret/ptc2) bound the Shc proteins in the transfectants. These also supported our results that tyrosine 1062 is a binding site for Shc that may play a crucial role in the transforming activity of MEN2A-Ret and MEN2B-Ret. Using the established cell lines expressing each mutant protein, we are currently investigating the signaling pathway via the Shc adaptor proteins responsible for cell transformation.

Shc contains two domains, SH2 domain and phosphotyrosine binding domain (33), both of which are known to recognize specific phosphotyrosine-containing sequences (34, 35). The binding specificity of Shc SH2 and phosphotyrosine binding domains is determined by residues carboxyl-terminal and amino-terminal to phosphotyrosine, respectively. The sequence (NK) amino-terminal to tyrosine 1062 of Ret matches the consensus sequence (NXXpY) for the binding of the Shc phosphotyrosine binding domain (34), whereas tyrosine 1062 is not embedded in the consensus sequence (pY(I/L/M)(C)(L/V/M)/) for the binding of the Shc SH2 domain (35). This finding suggested that tyrosine 1062 is the target to which the phosphotyrosine binding domain binds rather than the SH2 domain. In addition, since the sequence (GMS) carboxyl-terminal to this tyrosine does not match the consensus sequences for the SH2 domains of other signaling molecules including phospholipase C type γ, Grb2, phosphatidylinositol-3 kinase, Syp and Src family (23, 35), it seems unlikely that tyrosine 1062 is a major binding site for these molecules.

The fact that the Y1062F mutation did not completely abolish the transforming activity of MEN2A-Ret and MEN2B-Ret and their binding ability to Shc suggested that tyrosine residues other than tyrosine 1062 represent minor binding sites for Shc which may be necessary for low levels of their transforming activity. To elucidate other binding sites for Shc, further investigation including the introduction of double or triple mutations of the tyrosines present in the intracellular domain of Ret will be required.

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