Repair by Src Kinase of Function-impaired RET with Multiple Endocrine Neoplasia Type 2A Mutation with Substitutions of Tyrosines in the COOH-Terminal Kinase Domain for Phenylalanine

Masashi Kato, Kozue Takeda, Yoshiyuki Kawamoto, Toshihide Iwashita, Anwarul A. Akhand, Takeshi Senga, Masahiko Yamamoto, Gen Sobue, Michinari Hamaguchi, Masahide Takahashi, and Izumi Nakashima

ABSTRACT

An oncogenic mutant of c-RET as a receptor-type tyrosine kinase, termed RET-MEN2A, displays both cell-transforming activity in vivo and strong catalytic activity in vitro. In this study, we compared the activities of mutant RET-MEN2A with substitutions of nine tyrosines for phenylalanine (Y1062F, Y1015F, Y981F, Y952F, Y928F, Y905F, Y900F, Y864F, and Y826F), which had been transfected into NIH 3T3 cells. In RET-MEN2A with the Y905F mutation, the cell-transforming activity was drastically reduced with a great reduction in the in vitro catalytic activity. Unexpectedly, we found that in vitro kinase activity was severely impaired in RET-MEN2A with Y981F, Y952F, or Y928F mutation, which displayed near-normal cell-transforming activity and only a partially impaired tyrosine phosphorylation level in vivo. Phosphoamino acid analysis actually demonstrated some increase in phosphotyrosine in the Y905F mutant but no or barely detectable increase in the Y981F, Y952F, or Y928F mutant after incubation for in vitro kinase assay. This suggested a crucial role of the Y981F/Y952F/Y928F-linked structural integrity of the COOH end of the catalytic domain of RET in starting Y905 autophosphorylation. Interestingly, the apparent defect in intrinsic kinase activity in vitro in the Y981F, Y952F, or Y928F mutant, but not the reduction in activity in the Y905F mutant, could be partially repaired or restored by v-Src or, more extensively, by v-Src, which promoted Y905 phosphorylation in trans. A complex was shown to be formed between v-Src and RET-MEN2A through association of both with a cholesterol-rich membrane microdomain known as “raft,” possibly for efficient contact of submembranous domains of Src and RET to promote phosphorylation of Y905 of the latter. Finally, endogenous c-Src was shown to promote Y905 phosphorylation of the Y981F mutant in vivo. These results reveal a novel Src kinase-mediated repair mechanism of otherwise function-impaired mutant RET kinases.

INTRODUCTION

The c-RET proto-oncogene encodes a receptor-PTK, that is an essential component of a signaling pathway required for renal organogenesis and enteric neurogenesis (1, 2). Germ-line single-point mutations of the c-RET proto-oncogene are associated with MEN2A and MEN2B, familial medullary thyroid carcinoma, and Hirschsprung disease (3–8). The catalytic activities of many PTKs are regulated through control of the phosphorylation/dephosphorylation of specified tyrosine residues (Y) in kinase molecules. The RET protein has 16 tyrosines in the intracellular domain, including six tyrosines (Y864, Y900, Y905, Y928, Y952, and Y981) in its kinase domain 2, and Y905 has been suggested to play a crucial role in its catalytic and cell-transforming activity (9). Y1062 on the COOH-terminal tail of the RET protein has been reported to be the binding site of Shc and to thereby be involved also in cell-transforming activity (9, 10). Y1062 and Y1015, which was identified as the binding site for phospholipase Cy (11), on the COOH-terminal tail and Y826 on the kinase insert in the RET protein have also been reported to be autophosphorylation sites of RET (12). It is unknown, however, whether any tyrosines downstream of Y905 in the kinase domain are indispensably involved in the structural integrity needed for the catalytic activity of RET kinase.

From a comparison of the amino acid sequence of c-RET (13, 14) with those of c-Src and other PTKs such as Lck, Hck, IRK, FgfRK, and cAPK (15), it is evident that all of these PTKs have a kinase domain with a conserved basic structure scattered with a number of highly conserved amino acids, including Y905 of c-RET (corresponding to Y416 of c-Src) in the activation segment (13–15) and two specified cysteines (C) with an interval of 10 amino acids (C976 of c-RET, corresponding to C487 of c-Src; C987 of c-RET, corresponding to C498 of c-Src) near the COOH end of the catalytic domain (15–18). Y928, Y952, and Y981 of c-RET, which are located downstream of the Y905-containing activation segment-like sequence of the kinase domain, are also relatively conserved in a number of PTKs (Y981 in Lck, Hck, and FgfRK; Y952 in Src, Lck, Hck, IRK, FgfRK, and cAPR; and Y928 in FgfRK and cAPR). Xu et al. (15), who determined the three-dimensional structure of c-Src, suggested that both the “C” helix of the kinase NH3 lobe and activation segment with a major autophosphorylation site of the kinase COOH lobe form a local switch for activation of the kinase, which is under global regulation linked to the change in conformation of the whole kinase molecule. A well-known global regulation of c-Src kinase is linked to change in molecular conformation attributable to association or dissociation of the regulatory tyrosine (Y527)-containing COOH-terminal tail of the kinase domain with the SH2 domain of the kinase (15, 19). The potential role of the COOH-terminal kinase domain upstream of the tail and downstream of the activation segment in the global regulation of the catalytic activity has not been investigated.

In this study, we established mutants of RET-MEN2A in which each of nine tyrosines was replaced by phenylalanine (F), and we compared their kinase activities. The results show that whereas Y905 is needed for both cell-transforming activity and full in vitro kinase activity, Y981-, Y952-, and Y928-linked structural integrity of the COOH-terminal catalytic domain, downstream of the Y905-bearing activation segment, is essential for the tyrosine 905-dependent catalytic activity of RET-MEN2A to start in vitro, suggesting an involvement of this part of the molecule in the global regulation of the Y905-dependent local switch for kinase activation. Interestingly, the impaired in vitro catalytic activity of RET-MEN2A with the Y981F, Y952F, or Y928F mutation was partially restored by Src kinase.

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2 To whom requests for reprints should be addressed, at Department of Immunology, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan.

3 The abbreviations used are: PTK, protein tyrosine kinase; MBP, myelin basic protein; RET-MEN2A, RET with multiple endocrine neoplasia type 2A mutation; GDNF, glial cell-derived neurotrophic factor; PP1, 4-amino-5-(4-methylphenyl)-7-([4-butylypyrazolo-[3,4-d]pyrimidin; EGFR, epidermal growth factor receptor.
which is anchored to a detergent-insoluble, cholesterol-rich membrane microdomain known as “raft” (20, 21) in association with RET-MEN2A and phosphorylates Y905 in trans either in vitro or in vivo, suggesting the presence of a new Src-mediated repair mechanism for the kinase compensating for the Y928, Y952, or Y981 mutation-oriented defect in global regulation to start Y905 autophosphorylation.

MATERIALS AND METHODS

Preparation of the Mutant cDNAs of RET and Src and Their Transfectants. A cDNA clone containing the entire sequence (for 1114 amino acids) of the human c-RET gene was inserted into the vector containing the Moloney murine leukemia virus long terminal repeat as described previously (9, 10, 17). Each mutation was introduced by PCR. The c-RET cDNA with the MEN2A (cysteine 634—arginine; C634R) mutation, with or without the substitution of each of nine positions of tyrosine for phenylalanine (Y1062F, Y1015F, Y981F, Y952F, Y928F, Y905F, Y900F, Y864F, and Y826F) was prepared and inserted into the expression vector and transfected into NIH3T3 cells as described previously (9). Cells were cultured in DMEM (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 8% bovine calf serum (HyClone Laboratories, Inc., Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C in a humidified 5% CO2 atmosphere. As shown in Fig. 1, Y1062 and Y1015 are localized between the tyrosine kinase domain 2 and COOH terminal; Y981, Y952, Y928, Y905, Y900, and Y864 are in the tyrosine kinase domain 2; and Y826 is localized between tyrosine kinase domains 1 and 2 (kinase insert).

The method for preparing NIH3T3 cells transfected with v-src or v-src with the substitution of tyrosine 416 for phenylalanine (Y416F) was described previously (22). Cell lysates of normal and v-src-transfected NIH3T3 cells, which were used for incubation in vitro with immunoprecipitated RET for immunoblot and kinase assay, were prepared by lysing 5 × 106 cells in 500 μl of kinase buffer (see below) by sonication. Baculovirus containing full-length v-Src tagged with the histidine was prepared using pHBlueBacHis2 kit (Invitrogen, Groningen, the Netherlands), according to the manufacturer’s directions for use. After SP insect cells were infected with the baculovirus, purified v-Src preparation (concentration, 1 ng/μl) was prepared by passing the cell lysate through nickel-agarose beads.

For some experiments, SYF cells and their control cells, obtained from American Type Culture Collection (Manassas, VA), were transfected with mutant RET. SYF cells were originally generated from mouse embryos carrying functional null mutations in both alleles of the Src family protein tyrosine kinases, Src, Yes, and Fyn and immortalized by infection with a retroviral vector transducing the SV40 large T antigen (23). Src++ cells, which were derived from a control mouse embryo and express endogenous wild-type c-Src but not Yes and Fyn, were used as littermate controls for SYF cells. SYF + c-Src cells, which had been prepared by reintroducing c-Src into SYF cells, were used as another control. The recombiant plasmid carrying mutant RET (6–8 μg of DNA), was transiently introduced into SYF, Src++, or SYF + c-Src cells (5 × 105 cells in a 60-mm diameter dish) by using GenePORTER Transfection Reagent (Gene Therapy Systems, Inc., San Diego, CA). Cells were cultured in DMEM supplemented with 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO).

Immunoblot and Antibodies. Western blotting was performed according to the method described previously (24). An anti-RET rabbit polyclonal antibody was produced as described previously (25). Anti-phosphotyrosine polyclonal antibody was purchased from Transduction Laboratories (Lexington, Ky). Anti-Src monoclonal antibody (mAb327) was kindly donated by Dr. J. S. Brugge (State University of New York, Albany, NY; Ref. 26). Polyclonal antibodies for detection of phosphorylated Y1062 (anti-Y1062-P) and Y905 (anti-Y905-P) in RET protein were prepared by immunization of rabbits with 1-E-N-K-L-(Y)-G-M-S-D-P (Y1062-P) and V-Y-E-E-D-S-(Y)-V-K-R-S-Q (Y905-P) peptides, respectively. These antibodies were purified by two steps of affinity chromatography: (a) to eliminate nonphosphorylated peptide-binding antibodies; and (b) to collect tyrosine-phosphorylated peptide-binding antibodies. The specificities of these antibodies were examined by Western blot after immunoprecipitation of RET proteins from c-RET and c-RET with the Y1062F or Y905F mutation-transfected 5K-MC cells (primitive neuroectodermal cells) that had been incubated in the presence or absence of 200 μM/ml of its ligand GDNF. As expected, the anti-Y905-P antibody and anti-Y1062-P antibody demonstrated a clear protein band on the membrane for c-RET-transfectants, which was increased by incubation in the presence of GDNF (ref. 27; and data not shown). The anti-Y905-P antibody and anti-Y1062-P antibody, however, barely produced a detectable protein band from the c-RET with the Y1062F or Y905F mutation-transfected 5K-MC cell lysates, even after incubation in the presence of GDNF (Ref. 27; see also Fig. 7). The specificities of these antibodies have also been confirmed by immunohistochemistry of the same set of MC cells (28).

Immunoprecipitation, Kinase Assay, and Phosphoamino Acid Analysis. Immunoprecipitation was performed as described previously (29). The RET proteins immunoprecipitated from lysates of cells in lysis buffer [30 mM Tris-HCl (pH 8.0), 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, and 0.3 mM Na3VO4] were subjected to either immunoblot or kinase assay. In vitro RET kinase assay was performed as described previously (30). Briefly, the immunoprecipitated RET proteins were suspended in a kinase buffer [10 mM Tris-HCl (pH 7.4), 5 mM MgCl2 with 2.0 μg of MBP (Sigma Chemical Co.) as an exogenous substrate and radiolabeled [γ-32P]ATP (370 kBq; DuPont NEN, Wilmington, DE)]. The kinase reaction was carried out for 20 min in a 30°C water bath and was terminated by adding SDS sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, and 10% glycerin] with 2-mercaptoethanol. Phosphoamino acid analysis was carried out according to the method we described previously (31).

RESULTS

Cell-transforming Activity and Protein Phosphorylation Levels in Cells Carrying Y→F Mutants of RET-MEN2A. Table 1 shows a summary of the cell-transforming activities of the nine Y→F mutants of RET-MEN2A for NIH3T3 cells, a part of which was reported previously (9, 10). With more comprehensive data than before, we confirmed that there is a dramatic loss of cell-transforming activity in RET-MEN2A with the Y905F mutation and a considerable loss in RET-MEN2A with the Y1062F mutation but no loss of activity

<table>
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<tr>
<th>DNA</th>
<th>Focus-forming activity (foci/μg of DNA)</th>
<th>Cell morphology</th>
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<tr>
<td>RET-MEN2A (C634R)</td>
<td>70–120</td>
<td>Spindle</td>
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<td>RET-MEN2A (C634R, Y1062F)</td>
<td>10–20</td>
<td>Spindle</td>
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<tr>
<td>RET-MEN2A (C634R, Y1015F)</td>
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<td>RET-MEN2A (C634R, Y928F)</td>
<td>&lt;0.2</td>
<td>Flat</td>
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<td>RET-MEN2A (C634R, Y900F)</td>
<td>70–120</td>
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<td>RET-MEN2A (C634R, Y864F)</td>
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<td>RET-MEN2A (C634R, Y826F)</td>
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<td>NIH 3T3</td>
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*Transformed foci were counted on day 12 after RET transfection to NIH 3T3 cells.
in any of the other seven mutants. Corresponding to these findings, tyrosine 905 in the kinase domain of RET-MEN2A was shown to be necessary for maintaining high levels of tyrosine phosphorylation of cellular proteins, possibly including RET proteins in NIH 3T3 cells, by analysis of simple Western blotting with anti-phosphotyrosine and anti-RET antibodies (Fig. 2, A and B). Mutation of each of the other eight tyrosines of RET-MEN2A had smaller effects on the levels of protein tyrosine phosphorylation in the cells, although the Y981F, Y952F, and Y928F mutations reduced the protein tyrosine phosphorylation levels (Fig. 2A and data not shown). Correspondingly, Western blot analysis with anti-phosphotyrosine antibody on immunoprecipitated RET proteins showed that Y905F mutation caused a marked reduction of tyrosine phosphorylation of RET proteins, but that Y981F, Y952F, and Y928F mutations only partially impaired it (Fig. 2C).

Catalytic Activity in Vitro of Y→F Mutants of RET-MEN2A. Further study confirmed that the catalytic activity of the Y905F mutant of RET-MEN2A for both autophosphorylation and phosphorylation of an exogenous substrate (MBP), which was measured by in vitro kinase assay, was greatly reduced compared with that of the original RET-MEN2A (Fig. 3). To our surprise, however, the levels of catalytic activity measured in vitro of the Y981F, Y952F, and Y928F mutants of RET-MEN2A, which displayed near-normal (Table 1) or almost exclusively consisted of phosphotyrosine, even after a long film exposure. These results suggest that RET-MEN2A with the Y981F, Y952F, or Y928F mutation, which displayed obvious cell-transforming activity in vivo, is defective in in situ catalytic activity that could be demonstrated by in vitro kinase assay. It was noted that Y981, Y952, and Y928, of which exchange to phenylalanine causes impairment of the catalytic activity, are all located on the COOH-terminal catalytic domain downstream of Y905, suggesting a crucial role of the Y981-, Y952-, and Y928-linked structural integrity of this part of the molecule in the catalytic activity of RET-MEN2A, a role that is possibly distinct from that of Y905.

NIH3T3 Cell Lysate Partially Repairs the Impaired Catalytic Activity in Vitro of Mutant RET-MEN2A. Regarding the reason for dissociation between in vivo and in vitro actions of RET-MEN2A with the Y981F, Y952F, or Y928F mutation, we tested a hypothetical view that some intermolecular interaction between RET and other elements or endogenous kinase proteins in cells, which persistently operates in vivo but is absent for in vitro isolated RET proteins, might maintain the activity of the otherwise catalytic activity-defective mutant RET proteins, possibly continuously counteracting the negative regulation by protein tyrosine phosphatases. The immunoprecipitated RET-MEN2A with the Y981F mutation, which was supposed to be separated from cellular elements that potentially maintain the otherwise-defective catalytic activity in vivo, was mixed with the lysate of parental cells (NIH 3T3 cells). Because the cell lysates contained phosphatases as known potent negative protein kinase regulators that might prevent detection of the potential positive regulatory elements, Na3VO4, as an inhibitor of phosphatases, was added to this mixture. The mixture was then incubated so that the once-isolated (immunoprecipitated) function-impaired mutant RET-MEN2A could again interact with potential positive regulatory elements in the lysate in vitro. An in vitro kinase assay and Western blotting with anti-phosphotyrosine and anti-RET antibodies were performed with these in vitro incubated RET-MEN2A proteins. The results are shown in Fig. 5. The levels of phosphorylation of the mutant RET-MEN2A and MBP in the in vitro kinase assay (Fig. 5B) as well as the level of tyrosine phosphorylation (Fig. 5E) of the mutant RET-MEN2A were clearly elevated (equal loading of RET protein is shown in Fig. 5H) by...
incubation for a short time with the cell lysate in vitro, as compared with those for the mutant RET-MEN2A incubated without the cell lysate (control). Because the cell lysate alone did not induce any detectable level of phosphorylation of MBP (Fig. 5A), the function-restored mutant RET-MEN2A was thought to be responsible for the promoted MBP phosphorylation in Fig. 5B. This in turn suggests that a portion of the increase in phosphorylation of the mutant RET-MEN2A was attributable to autophosphorylation by the function-restored mutant RET-MEN2A, although some of the increased phosphorylation of the mutant RET-MEN2A might be attributable to phosphorylation in trans by the endogenous kinase in the lysate as the potential repair mechanism. Interestingly, the cell lysate did not act to
increase the in vitro kinase activity and tyrosine phosphorylation level of the Y905F-mutant RET-MEN2A, which were less extensively impaired than those of the Y981F-mutant RET-MEN2A (Fig. 5, C and F). This result further supported the view that promotion of phosphorylation of the Y981F-mutant RET-MEN2A and MBP shown in Fig. 5, B and E, was not primarily a result of direct phosphorylation by endogenous enzyme in the cell lysate.

We examined whether the elements in the cell lysate that cooperate with RET-MEN2A with the Y981F, Y952F, or Y928F mutation actually include a third-party endogenous PTK(s). One candidate for such a PTK is c-Src, and this possibility was tested by the addition of PP1 as a specific Src kinase inhibitor (32) to the cell lysate. As shown in Fig. 5J, the addition of PP1 into the normal NIH 3T3 cell lysate before incubation abolished the activity of the lysate to restore the kinase activity of RET-MEN2A with the Y981 mutation for phosphorylation of the kinase and MBP.

**v-Src Repairs the Impaired Catalytic Activity of Mutant RET-MEN2A.** We next demonstrated that cell lysate containing v-Src as a constitutively activated Src kinase caused more extensive phosphorylation of RET-MEN2A with the Y981F, Y952F, or Y928F mutation and MBP than did normal NIH 3T3 cell lysate (Fig. 6). Extensive phosphorylation of MBP was not a result of direct phosphorylation by v-Src as shown in the second lane from the left end in Fig. 6A but developed through cooperation of v-Src and the function-impaired mutant RET-MEN2A, of which the catalytic activity was thought to be repaired by coexisting v-Src. The lysate containing v-Src with the Y416F mutation, of which the catalytic activity was shown previously impaired than those of the Y981F-mutant RET-MEN2A (Fig. 5, C and F). This result further supported the view that promotion of phosphorylation of the Y981F-mutant RET-MEN2A and MBP shown in Fig. 5, B and E, was not primarily a result of direct phosphorylation by endogenous enzyme in the cell lysate.

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**Src Kinase Repairs the Kinase Activity of the Function-impaired Mutant RET-MEN2A, Possibly through Promotion of Y905 Phosphorylation.** We next conducted experiments to examine whether phosphorylation of RET-MEN2A with the Y981F mutation, which was promoted through cooperation with v-Src in vitro, involved phosphorylation of Y905 as the suggested major autophosphorylation site of RET kinase that plays a crucial role in activation of the RET kinase. We first examined the specificities of the anti-Y905-P and anti-Y1062-P antibodies we prepared recently. The anti-Y905-P antibody reacted well with the original RET-MEN2A (Fig. 7B, Lane 1) but not with RET-MEN2A with the Y905F mutation (Lane 4), whereas the anti-Y1062-P antibody, which failed to react with RET-MEN2A with the Y1062 mutation (Fig. 7C, Lane 2), reacted well with the original RET-MEN2A (Lane 1) and less extensively with RET-MEN2A with the Y905F mutation (Lane 3). These results confirmed the specificities of the two antibodies for detection of either phosphorylated Y905 or phosphorylated Y1062 and supported the view that Y905 is a phosphorylation site in the kinase domain of the RET kinase.

Using these antibodies, we compared the background Y905 phosphorylation levels in the cells on the original RET-MEN2A and RET-MEN2A with the Y981F mutation that were isolated by immunoprecipitation. As shown in Fig. 7A, the level of phosphorylation of Y905 on RET-MEN2A with the Y981F mutation (Lane 1) was much lower than that on the original RET-MEN2A (Lane 3). This did not correspond well with the results shown in Fig. 2C showing that the total phosphotyrosine level of the former was only partially reduced as compared with that of the latter. This suggested that the Y905 phosphorylation-linked intrinsic kinase activity of Y981F-mutant RET-MEN2A is more extensively impaired than expected from the level of total tyrosine phosphorylation of the kinase (Fig. 2C) and probably works by continuous collaboration with Src kinase for inducing cell transformation (Table 1), only a part of which can be reconstructed in the in vitro system by supplementing Src kinase-containing cell lysate to the mutant RET-MEN2A isolated by immunoprecipitation. We next attempted to set up a modified in vitro kinase assay system by incubating the immunoprecipitated RET-MEN2A proteins in the presence of cold ATP, instead of radiolabeled ATP, and by measuring the Y905 phosphorylation level by Western blotting, instead of radioautography for measurement of the total protein phosphorylation level. Fig. 7A also shows how this new assay system works. Incubation of the immunoprecipitated original RET-MEN2A in kinase buffer with cold ATP only in vitro for a short time clearly promoted the Y905 phosphorylation (Lane 4, compared with Lane 3), corresponding to the results of the conventional in vitro kinase assay shown in Fig. 3A. In contrast, no increase in Y905 phosphorylation was observed even after in vitro incubation of the Y981F-mutant RET-MEN2A (Lane 2), again corresponding to the results of the conventional in vitro kinase assay (see Fig. 3A).

Using this modified in vitro kinase assay system, we investigated whether the impaired kinase activity of mutant RET-MEN2A, assessed by the level of possibly catalytic activity-linked Y905 phosphorylation, can be repaired by Src kinase. In agreement with the results of measurement of total autophosphorylation levels (Fig. 6), incubation of the immunoprecipitated kinase activity-impaired
REPAIR OF FUNCTION-IMPAIRED RET-MEN2A

Y981F-mutant RET-MEN2A with cell lysate containing v-Src in vitro clearly promoted phosphorylation of Y905 (Fig. 7B, Lane 3, compared with Lane 2 as a control incubated without v-Src and with Lane 5 as a specificity control for Y905). Interestingly, incubation of the Y981F-mutant RET-MEN2A with v-Src-containing cell lysate also induced an increase in Y1062 phosphorylation (Fig. 7C, Lane 5). This contrasted with the finding that incubation with v-Src-containing cell lysate never increased (but rather decreased because of an as-yet-unclarified mechanism) the level of phosphorylation of Y1062- or Y905-defective mutant of RET-MEN2A (Lane 7 compared with Lane 6 as a control incubated without v-Src). This observation suggested that v-Src does not have catalytic activity to directly promote Y1062 phosphorylation and therefore that the observed promotion of Y1062 phosphorylation in the Y981F-mutant of RET-MEN2A is through autophosphorylation by the mutant kinase, of which the catalytic activity has been repaired through induction of Y905 phosphorylation by v-Src.

Promotion of Y905 phosphorylation of Y981F-mutant RET-MEN2A was also observed when the RET kinase was incubated with purified v-Src protein prepared from Sf9 insect cell lysate (Fig. 7D, Lane 2, compared with Lane 1 as a control incubated without purified v-Src), whereas incubation with purified v-Src protein did not promote Y905 phosphorylation of the original MEN2A (Lane 4, compared with Lane 3). This result ruled out the possibility that v-Src-associated metabolites in cell lysate rather than v-Src itself were responsible for the promotion of Y905 phosphorylation of RET-MEN2A with the Y981A mutation. All of these results suggested that the Y981F-mutant RET-MEN2A lacks the capability to start phosphorylation of Y905 as the major autophosphorylation site but that Y905 phosphorylation of the Y981F-mutant RET-MEN2A, which is crucial for its own catalytic activity to work for both autophosphorylation of Y905 and Y1062 and MBP phosphorylation, could be initially induced by Src kinase in trans, and the catalytic activity of the mutant RET-MEN2A is thereby repaired for further autophosphorylation and MBP phosphorylation.

Formation of a Complex of RET and Src Kinases in Association with Membrane Rafts. It is possible that molecular cooperation occurred through formation of a complex of the two molecules. As shown in Fig. 8A, incubation of immunoprecipitated RET-MEN2A with purified v-Src proteins induced formation of a complex of the two molecules, which was demonstrated by Western blot analysis of both RET-MEN2A and Src that had bound to RET-MEN2A-carrying immunobeads during incubation. Interestingly, a complex was also formed between Y981F- or Y905F-mutant RET-MEN2A and v-Src. This suggested that neither Y981 nor Y905 was essential for the mechanism of complex formation. To further test whether complex formation was RET phosphorylation-dependent as suggested previously for the formation of a complex of c-RET and c-Src (33), we compared the levels of formation of a complex of RET-MEN2A and c-Src after incubation in the presence and absence of ATP. As shown in Fig. 8B, the level of formation of a complex of RET-MEN2A and c-Src was not changed detectably by the presence or absence of ATP during incubation, suggesting that the complex formation was not exclusively dependent on the RET phosphorylation event. The results shown in Fig. 8, A and B, suggest that an alternative or additional mechanism prevails over the phosphorylation-dependent one for formation of a complex of RET-MEN2A and v-Src. It has been reported recently that a portion of RET proteins, upon stimulation with a ligand (GNDF) in combination with glycosylphosphatidyl inositol-anchored coreceptors, resides in the membrane raft (17, 34). On the other hand, Src family kinases are known to be anchored to the detergent (Triton)-insoluble, cholesterol-rich membrane raft with myristylated glycine and palmitoylated cysteine(s) at their NH2 termini (20, 35, 36). We therefore speculated that RET-MEN2A, a large portion of which is constitutively dimerized by the S-S bond attributable to C634R mutation (37), somehow associates with the raft to be colocalized with Src on the membrane. To test this possibility, cells carrying MEN2A or MEN2A with the Y981 or Y905 mutation were treated with β-cyclodextrin, which eliminates cholesterol from the cell membrane and disrupts the cholesterol-rich raft structure, before lysis for immunoprecipitation of RET proteins. As shown in Fig. 8C, this treatment severely inhibited complex formation between RET and Src proteins. This result suggests that formation of a complex of RET-MEN2A and Src requires the cholesterol-rich membrane rafts, which may be carried over by RET-MEN2A during immunoprecipitation from cell lysates in vitro and associate with externally added v-Src. Such
results of three experiments with consistent results are shown. MEN2A, with the Y905F mutation; carrying RET-MEN2A with the Y981F mutation; Y905F, C are for immunoglobulin heavy chains used for immunoprecipitation. 

B, then carried out in the presence of cold ATP. Lower bands below the Src band in min at 37°C before lysis for immunoprecipitation with anti-RET antibody. Incubation was carried out in the presence or absence of cold ATP. B, incubation was carried out in the presence of cold ATP (100 μM). Western blot analysis with anti-RET antibody for the same sample confirmed equal protein loading. B, incubation was carried out in the presence or absence of cold ATP. C, cells were treated or not treated with 10 mM β-cyclodextrin (Sigma Chemical Co.) for 15 min at 37°C before lysis for immunoprecipitation with anti-RET antibody. Incubation was then carried out in the presence of cold ATP. Lower bands below the Src band in B and C are for immunoglobulin heavy chains used for immunoprecipitation. Y981F, beads carrying RET-MEN2A with the Y981F mutation; Y905F, beads carrying RET-MEN2A with the Y905F mutation; MEN2A, beads carrying original RET-MEN2A. Representative results of three experiments with consistent results are shown. 

DISCUSSION

Here we first provided evidence for distinct roles of Y905 and a group of tyrosines (Y981, Y952, and Y928)-linked structural integrity of the COOH-terminal kinase domain in preparing the kinase activity of RET-MEN2A for cell transformation. In agreement with earlier results (9), Y905 was shown to be essential for both inducing cell transformation and elevated autophosphorylation of the kinase in vivo and for in vitro catalytic activity that induces extensive autophosphorylation and phosphorylation of an exogenous substrate MBP (Table 1; Figs. 2–4). Y905 of RET kinase, of which the homologue is highly conserved in the activation segment of the catalytic domain of various PTKs and corresponds to Y416 of c-Src and v-Src as the central autophosphorylation site essential for kinase activity (9, 13–15), is suggested to be the major autophosphorylation site of RET, which plays a key role in the catalytic activity of the kinase. Supporting this view, we confirmed in this study, by using a newly prepared antibody, that Y905 is actually a phosphorylation site (Fig. 7B). Mutation of either of the three tyrosines Y981, Y952, and Y928, which are located downstream of Y905, to phenylalanine, which barely (Table 1) or only partially (Fig. 2) impaired in vivo RET activity for inducing cell transformation (Table 1) and for being autophosphorylated (Fig. 2), was found to profoundly reduce the intrinsic catalytic activity of the mutant RET-MEN2A that was measured by the levels of promotion of autophosphorylation and phosphorylation of MBP as an exogenous substrate in vitro (Figs. 3 and 4). Further study revealed that the level of phosphorylation of Y905 of the Y981F mutant of RET-MEN2A in the cell was much lower than that of the original RET-MEN2A (Fig. 7A). It was hypothesized from these observations that a function of the Y981-, Y952-, and Y928-linked COOH-terminal kinase domain is needed for the RET-MEN2A to display catalytic activity for inducing Y905 autophosphorylation and MBP phosphorylation, and that the Y981F, Y952F, or Y928F mutant RET-MEN2A, which is missing the original function, only works in live cells in which the mutant RET-MEN2A maintains membrane raft-mediated molecular association at the first step might subsequently cause interaction of submembranous domains of RET and Src at the second step for acceleration of phosphorylation of mutant RET-MEN2A by Src.

c-Src Promotes Y905 Phosphorylation of Function-impaired Mutant RET-MEN2A in Vivo. To determine whether c-Src promotes Y905 phosphorylation of function-impaired mutant RET-MEN2A in vivo, we transfected Src family protein tyrosine kinase-defective SYF cells and their control cells (23) with RET-MEN2A with the Y981F mutation, and we compared the levels of Y905 phosphorylation of the mutant RET-MEN2A in those cells. As shown in Fig. 9, the level of Y905 phosphorylation of the mutant RET-MEN2A in SYF cells was much lower than that in control Src++ cells carrying endogenous c-Src, although both types of cells equally expressed RET proteins. The difference in the levels of phosphorylation was particularly evident with the mature form of the M, 175,000 RET protein (upper band of the doublet). In SYF + c-Src cells carrying exogenous c-Src, the level of phosphorylation at Y905 of the mutant RET-MEN2A was almost restored, although the RET protein expression level was lower than that in SYF cells. These results show that either endogenous (in Src++ cells) or exogenous (in SYF + c-Src cells) c-Src works to promote the Y905 phosphorylation in vivo.
continuous interaction with cellular elements that repair the function-impaired kinase molecule.

The molecular mechanism by which Y981, Y952, and Y928 maintain the basic function of the COOH-terminal catalytic domain to start the machinery leading to Y905 autophosphorylation and MBP phosphorylation remains unknown. Evidence that Y981, Y952, and Y928 are themselves phosphorylation sites has not been obtained; our recent efforts to obtain a potentially phosphorylated Y952-specific antibody were unsuccessful, even when we used the same protocol as that used for obtaining phosphorylated Y905-specific and phosphorylated Y1062-specific antibodies. Because their homologues are relatively conserved in various PTKs (see “Introduction”), these three tyrosines are thought to be involved as key amino acids in the basic structure (conformation) of the COOH-terminal kinase domain, which is needed for initial kinase activation. Recently, mutations at some other amino acids close to Y981, Y952, and Y928, such as M980T, R972G, and E921K, have been shown to impair the RET kinase activity as a basis of Hirschsprung disease (38). This also supports the above-described hypothetical view.

We conducted experiments to determine whether a homologue of the in vivo environment for rescuing otherwise function-impaired mutant RET-MEN2A could be reconstructed in vitro by incubating the mutant kinase with cell lysates that might contain function-repairing molecules. Three types of experiments using normal cell lysates, cell lysates containing v-Src, and purified v-Src proteins as potentially function-repairing agents revealed that the otherwise function-impaired Y981F, Y952F, or Y928F mutant RET-MEN2A was partially repaired by co-precipitation with one of the three above-mentioned agents for promoting Y905 phosphorylation (Fig. 7) of the mutant RET-MEN2A as well as its catalytic activity for MBP phosphorylation. It could be argued that mutations of Y981, Y952, or Y928 to phenylalanines result in nonspecific destabilization of the whole protein structure in vitro, which might be stabilized in vivo by chaperone activity or simply by high protein concentration in the cell. We, however, demonstrated that the action to restore the impaired catalytic activity of the mutant RET-MEN2A by normal cell lysates was inhibited by PP1 as a specific inhibitor of Src kinase and that v-Src kinase was definitely more active than that by function-deteriorated v-Src with Y416F mutation or normal cell lysates. Therefore, phosphorylation in trans by Src kinase rather than nonspecific protein protection is probably the main mechanism by which impaired catalytic activity of mutant RET-MEN2A is repaired. We further demonstrated that v-Src promoted phosphorylation of both Y905 and Y1062 of the Y981F mutant but neither Y905 nor Y1062 of the Y905F-mutant RET-MEN2A (Fig. 7). This supported the view that phosphorylation of Y905 in trans by Src kinase is the basic mechanism for the repair of the catalytic activity-impaired Y981F-mutant RET-MEN2A and that once-repaired mutant kinase induces not only phosphorylation of MBP as an exogenous substrate but also autophosphorylation of Y1062 and, probably, Y905 itself of other RET molecules.

There are some precedents for cooperation between Src family kinases and receptor-type tyrosine kinases. For example, Tice et al. (39) reported that overexpression of c-Src and EGFR occurs in many of the same human tumors and may functionally interact and contribute to the progression of cancer. They actually demonstrated that overexpression of c-Src in murine fibroblasts potentiated the mitogenic and tumorigenic capacity of the overexpressed EGFR and that the potentiation correlated with the ability of c-Src to physically associate with the activated EGFR attributable to an unclariﬁed mechanism. A cooperative role of Src family kinases in the stem cell factor

We conclude that the mechanism for starting phosphorylation of tyrosine 905 in the activation segment, which is crucial for turning on the local switch to activate the kinase (15), normally requires the Y981, Y952, and Y928-linked structural integrity of the COOH-terminal catalytic domain, and that this requirement could be partially replaced by Y905 phosphorylation by Src kinase in trans. The question arose as to how the Src kinase interacts with the catalytic function-impairment of the mutant RET-MEN2A for repair. We demonstrated that RET-MEN2A proteins form a complex with Src proteins in vitro (Fig. 8A). However, we also obtained evidence that contradicts the view that a complex is exclusively formed by protein (SH2 domain of Src)-phosphoprotein (phosphorylated RET) interaction as suggested previously in the mechanism of the support of c-Ret-mediated mitogenesis by c-Src (33): (a) complex formation occurred for purified v-Src proteins no less extensively with poorly phosphorylated Y981F- or Y905-mutant RET-MEN2A than with highly phosphorylated parental RET-MEN2A (Fig. 8A); (b) the absence or presence of ATP in the medium for incubation of the mixture of RET-MEN2A and v-Src did not affect the level of complex formation (Fig. 8B).

We then searched for an alternative or additional, although not mutually exclusive, mechanism of complex formation between RET-MEN2A and v-Src. We demonstrated that treatment of RET-MEN2A-carrying cells with β-cyclodextrin for sequestration of cholesterol before cell lysis to immunoprecipitate RET proteins severely inhibited complex formation between the immunoprecipitated RET proteins and v-Src proteins by incubation in vitro. This result indicated that the cholesterol-rich, detergent-insoluble membrane microdomain (raft) structure of the cell as the source of RET-MEN2A is needed for full complex formation of the immunoprecipitated RET-MEN2A with externally added v-Src. We speculated from this result that to the raft communoprecipitated with RET-MEN2A, which could associate with and carry over the raft through an as-yet-undetermined molecular mechanism, the externally added Src proteins were anchored by their NH2-terminal myristoylated or palmitoylated amino acids for complex formation. In cells, such cholesterol-rich membrane raft-mediated association of RET-MEN2A and Src may subsequently promote protein–protein interaction between intracellular kinase domains of RET and Src beneath the plasma membrane. The molecular mechanisms of association of RET-MEN2A with rafts and protein–protein interaction between RET and Src after raft-mediated molecular association, however, remain to be studied. The Src kinase-mediated repair mechanism we demonstrated here did not work for the mutant RET that had been isolated by immunoprecipitation, although it was thought to be bound to some pieces of rafts carrying endogenous c-Src. It is therefore likely that efficiency of this repair mechanism decreased to an undetectable level when cells were lysed by detergent and thereafter subjected to immunoprecipitation, which would, however, be reinforced by addition of either cell lysate with excess raft and raft-associating Src or of purified v-Src proteins.

Apart from the precise molecular action mechanism, we obtained evidence that either endogenous or exogenous c-Src actually works in vivo for inducing otherwise-defective phosphorylation of Y905 as the major autophosphorylation site in mutant RET-MEN2A using Src family kinase-defective SYF cells and appropriate control cells as the

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acceptor of the mutant RET-MEN2A. In general, tyrosine kinases may undergo a number of mutations during cellular ontogeny and onco-
genesis, some of which may promote or inhibit the intrinsic kinase activity. The newly proposed repair mechanism for the catalytic activity-impairing kinase may play physiologically or pathologically significant roles in preventing the occurrence of diseases with loss of function, such as Hirschsprung’s disease, on the one hand and in maintaining oncogene-mediated tumorigenesis on the other hand (in the case reported here) by counteracting emerging function-impair-
mutations. We should consider such a repair mechanism in situ, which sometimes operates in association with a specific molecular property of oncogene products, such as the property to bind to rafts (this study), to understand the exact molecular basis of oncogenic activity of individual elements for oncogenicity and for establishing a strategy for molecular engineering to attenuate oncogenic activities.

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REFERENCES


Repair by Src Kinase of Function-impaired RET with Multiple Endocrine Neoplasia Type 2A Mutation with Substitutions of Tyrosines in the COOH-Terminal Kinase Domain for Phenylalanine

Masashi Kato, Kozue Takeda, Yoshiyuki Kawamoto, et al.


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