Identification of RET Autophosphorylation Sites by Mass Spectrometry

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Summary

The catalytic and signaling activities of RET, a receptor-type tyrosine kinase, are regulated by the autophosphorylation of several tyrosine residues in the cytoplasmic region of RET. Some studies have revealed a few possible autophosphorylation sites of RET by $^32$P-phosphopeptide mapping or by using specific anti-phosphotyrosine antibodies. To ultimately identify these and other autophosphorylation sites of RET, we performed mass spectrometry analysis of an originally prepared RET recombinant protein. Both the autophosphorylation and kinase activity of myelin basic protein as an external substrate of the recombinant RET protein were substantially elevated in the presence of ATP without stimulation by a glial cell line-derived neurotrophic factor, a natural ligand for RET. Mass spectrometric analysis revealed that RET tyrosine 806 (Y806), Y809, Y900, Y905, Y981, Y1062, Y1090 and Y1096 were autophosphorylation sites. Levels of autophosphorylation and kinase activity of RET-MEN (multiple endocrine neoplasia) 2A, a constitutively active form of RET with substitution of Y900 by phenylalanine (Y900F), were comparable with those of original RET-MEN2A, whereas those of the mutant Y905F were greatly decreased. Interestingly, those of a double mutant, Y(900/905)F, were completely abolished. Both the kinase activity and transforming activity were impaired in the mutants Y806F and Y809F. These results provide convincing evidence for both previously suggested and new tyrosine
autophosphorylation sites of RET as well as for novel functions of Y806, Y809 and Y900 phosphorylation in both catalytic kinase activities and cell growth. The significance of the identified autophosphorylation sites in various PTKs registered in a database is discussed in this paper.
Introduction

Activation of receptor-type or nonreceptor-type protein tyrosine kinases (PTKs) plays a pivotal role in signal transduction in cells. The catalytic activity of PTKs is known to be regulated either positively or negatively by phosphorylation/dephosphorylation at specified tyrosine residues. RET is a proto-oncogene that encodes a receptor-type PTK with a cadherin-related motif and a cysteine-rich domain in the extracellular domain (1-3). It is known that rearrangement of RET is the major cause of papillary thyroid carcinoma (4) and that germ-line single-point mutations of RET cause multiple endocrine neoplasia (MEN) 2A, MEN2B, familial medullary thyroid carcinoma, and Hirschsprung’s disease (5-10). The glial cell line-derived neurotrophic factor (GDNF) family ligands (GFLs), including GDNF, neurturin (NRTN), artemin (ARTN) and persephin (PSPN), are involved in the survival and differentiation of neurons via activation of RET (11-14). Unlike most other receptor-type PTKs, RET is not activated via direct binding of GFLs but is activated by the formation of a multicomponent receptor complex that includes glycosylphosphatidylinositol (GPI)-anchored cell surface proteins called GFRαs, which bind GFLs directly. Four members of the family of GFRαs (GFRα1-4) have been identified, and it has been shown that they bind GFLs with high affinity. GDNF, NRTN, ARTN and PSPN use GFRα1, GFRα2, GFRα3 and GFRα4, respectively, as preferred ligand-binding receptors (11-14).
RET can activate various signaling pathways, including extracellular signal-regulated kinase (ERK), phosphatidylinositol 3-kinase (PI3K)/AKT, p38 MAPK and JNK pathways, responsible for cell survival or differentiation (15-18). Upon ligand binding, RET forms dimers and is phosphorylated at a specific tyrosine residue(s) in intracellular domains. RET has been shown to be alternatively spliced to produce three isoforms (short, middle and long isoforms) that differ in C-terminal residues (19, 20). RET short and middle isoforms contain 16 tyrosine residues in their intracellular domains, and RET long isoforms have two additional tyrosines in the C-terminal tail. Among these tyrosines, tyrosine 905 (Y905), Y1015, Y1062 and Y1096 are thought to be phosphorylated to become binding sites for GRB7/GRB10, phospholipase Cγ, SHC/SNT(FRS2)/Enigma and GRB2, respectively (21-26). For neuronal signal transduction, one model has suggested that GFRα functions to mediate localization of RET to a lipid raft, a sphingolipid and cholesterol-rich lipid micro-domain in the plasma membrane. GFRα stimulation via GDNF induces not only RET dimerization but also RET/Src association at the raft, and RET can be phosphorylated by activated Src kinase (27, 28).

In addition to endogenous ligands, oxidative agents such as H₂O₂, NO, HgCl₂, and ultraviolet rays are known to increase the levels of catalytic activity of various PTKs, including Src (29-32), Lck (33-35) and RET (36-38). Recently, we demonstrated that cysteine 987 (C987), which is in the kinase domain of RET, plays a critical role in the
upregulation of kinase activity upon stimulation by an oxidative agent (37, 38). It is known that some cysteine residues, including C987 of RET or its equivalents, are widely conserved among a number of receptor-type and nonreceptor-type PTKs and some serine/threonine kinases (39). By analyzing amino acid sequences of various PTKs registered in a database, we found that C987 exists in the motif sequence of Met-X-X-Cys-Trp (MXXCW), which is located in the C-end of α-helix H in the kinase domain (40), and that this motif initially turns on the Y905-dependent local switch of RET kinase for activation through oxidation of C987 in the MXXCW motif (40). Thus, after accumulation of RET proteins on the plasma membrane induced by ligand or potentially by oxidative stimulation, autophosphorylation that leads to kinase activation and downstream signaling will occur.

The tyrosine residues Y1015, Y1062 and Y1096 in activated RET were suggested to be autophosphorylated in earlier studies by phosphopeptide mapping (41) or by using a specific antibody recognizing phosphotyrosine (42). Results of studies using an anti-phosphotyrosine antibody also suggested that Y905 is an autophosphorylation site (43, 44). However, tyrosine residues in RET protein were not identified as autophosphorylation sites in earlier studies by reliable methods. In this study, we performed mass spectrometry analysis of purified recombinant RET proteins in order to determine RET autophosphorylation sites in the purified RET protein activated by ATP, and we found that Y806, Y809, Y900, Y905, Y981, Y1062, Y1090 and Y1096 are \textit{bona fide} autophosphorylation sites that are actually
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autophosphorylated. Phosphorylation of Y806, Y809, Y900, Y981 and Y1090 in RET is reported here for the first time. Furthermore, this study demonstrated for the first time that Y806, Y809 and Y900, which are relatively highly conserved in a number of PTKs registered in a database, play additional or supplemental roles to the role of Y905 in catalytic activity.
EXPERIMENTAL PROCEDURES

Cell culture

Murine NIH3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Insect *Spodoptera frugiperda* (Sf21) cells were grown in Grace’s insect medium supplemented with 10% fetal bovine serum.

Construction of recombinant baculoviruses

A cDNA clone containing the entire sequence of the human c-RET gene inserted into the Rc/CMV vector (Invitrogen) was digested by *Eco*RI and *Not*I ultimately to make glutathione S-transferase (GST)-fused plasmamembrane and extracellular regions-deleted RET, and then a 1.6-kb fragment was subcloned into the *Eco*RI and *Not*I sites of a baculovirus transfer vector, pAcGHLT™ (Pharmingen). The transfer vector was then co-transfected with linearized BaculGold™ (Pharmingen) DNA into Sf21 cells to generate a baculovirus that can produce a primary recombinant GST-fused deletion RET protein, designated GST-ΔRET. The GST-ΔRET baculovirus was then amplified and maintained in Grace’s insect medium at 4 °C until used for infection of Sf21 cells to generate large amounts of recombinant proteins.
Site-directed mutagenesis

The cDNA encoding human RET-MEN2A with substitution of cysteine 634 by arginine in c-RET was prepared as described previously (28). The RET-MEN2A gene was subcloned into the APtag-1 plasmid vector containing the moloney murine leukemia virus long terminal repeat. RET-MEN2A single mutants, tyrosine 806 to phenylalanine (Y806F), tyrosine 809 to phenylalanine (Y809F), tyrosine 900 to phenylalanine (Y900F) and tyrosine 905 to phenylalanine (Y905F), and double mutants, Y(806/809)F and Y(900/905)F, were prepared by using a QuickChange site-directed mutagenesis kit (Stratagene) according to the manufacturer's instructions. To confirm the introductions of mutations, the plasmids were sequenced by a DNA sequencer ABI prism (Applied Biosystems).

Purification and characterization of GST-ΔRET

We purified GST-ΔRET recombinant protein generated by the baculovirus system by using glutathione (GSH)-agarose beads. The kinase activity of the purified GST-ΔRET was measured by using an ELISA-based Tyrosine Kinase Activity Kit (Chemicon International) according to manufacturer’s protocol. Briefly, the purified GST-ΔRET bound to GSH-agarose beads was incubated in the absence or presence of ATP (1 mM) in kinase buffer [10 mM MgCl2, 5 mM Tris-HCl (pH 7.2), 30 mM NaCl] with a synthetic biotinylated peptide substrate [poly (Glu:Tyr), 4:1] at 30 °C for 20 min. The enzyme reaction was
terminated by adding 24 mM EDTA, and the peptide substrate samples in the supernatant were then transferred to streptavidin-coated wells, followed by incubation at 37 °C for 30 min. After blocking the wells with 3% BSA in PBS, an HRP-conjugated anti-phosphotyrosine antibody was added to the wells, and then tetramethylbenzidine as an HRP substrate was added. Finally, absorbance of sample wells was measured at OD450 nm.

The residual GST-ΔRET samples that bound to GSH-agarose beads after ATP reaction were mixed with 2 × sample buffer (125 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 0.2% blomophenol blue, pH 6.8, 10% β-mercaptoethanol) and subjected to SDS-PAGE. The levels of tyrosine phosphorylation were determined by immunoblotting as described below.

*Preparation of phospho-peptides of GST-ΔRET*

GST-ΔRET bound to GSH-agarose beads (20~30 µg) was washed with kinase buffer [10 mM Tris-HCl (pH 7.4), 5 mM MgCl2] four times and incubated in the absence or presence of ATP (1 mM) in kinase buffer for 30 min at 30 °C. The ATP-treated purified protein was washed three times with digestion buffer (50 mM NH₄HCO₃, pH 7.8) followed by overnight digestion at 24 °C in the presence of Glu-C endoproteinase (8 ng/µl) (Roche) or at 37 °C in the presence of sequencing-grade modified trypsin (8 ng/µl) (Promega). The collected phosphopeptides were then incubated in the presence or absence of phosphatases, LAR phosphatase [30 min at 30 °C, in reaction buffer; 25 mM Tris-HCl (pH 7.0), 50 mM NaCl, 2
mM Na₂EDTA, 5 mM dithiothreitol] or protein phosphatase1 [30 min at 30 °C, in reaction buffer; 50 mM Tris-HCl (pH 7.0), 0.1 mM Na₂EDTA, 5 mM dithiothreitol, 0.025% Tween 20, 1 mM MnCl₂] (New England Bio Labs).

Identification of tyrosine auto-phosphorylation sites by mass spectrometry

The sample peptides were desalted by using C18 Zip Tip (Millipore), eluted in α-cyano-4-hydroxycinnamic acid (α-CHCA) solution containing 50% acetonitrile and 3% folic acid, and then analyzed by using a Voyager Elite MALDI-TOF mass spectrometer (PerSeptive Biosystems) operated in the positive ion reflectron mode. For molecular mass determination of GST-ΔRET peptides, two-point external calibration based on angiotensin II (m/z 1046.54) and ACTH fragment peptide 18-39 (m/z 2465.20) was used. For ion-trap mass spectrometric analysis, a mixture of protease-digested peptides was desalted with C18 Zip Tip and eluted in 98% acetonitrile solution containing 0.1% formic acid. The eluate was mixed with the same volume of 2% acetonitrile solution containing 0.1% formic acid, and then the sample was directly infused at 1 µl/min into an ion-trap mass spectrometer, LCQ Advantage (Thermo Electron). The MS/MS spectra were analyzed by using the MS/MS ion searching program of MASCOT (Matrix Science, http://www.matrixscience.com) and BioworksBrowser (v.3.0) (Thermo Electron) to assign individual mass signals, and then amino acid sequences were determined.
Molecular modeling

Primary amino acid sequences of human PTK family proteins were obtained from the SWISS-PROT protein database. The tertiary structure of RET was simulated and determined using FAMS, a homology modeling software program, originally produced by H. Umeyama at Kitasato University, based on the tertiary structure of fibroblast growth factor (FGF) receptor 1 elucidated by X-ray diffraction. Subsequently, the structure was visualized with WebLab ViewerLite 3.20 software (Molecular Simulations Inc.).

Immunoblotting and antibodies

Western blotting was performed according to the method described previously (45). An anti-RET rabbit polyclonal antibody that recognizes both a 175-kDa form (a mature glycosylated form) and a 155-kDa form (an immature glycosylated form) of RET was produced as described previously (46). The GST-ΔRET samples or the lysates (20 µg/lane) of NIH3T3 cells were subjected to SDS-PAGE using 8% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was blocked with 5% non-fat milk for 1 hour at room temperature and then reacted with a first anti-phosphotyrosine or anti-RET antibody for 12 hours at 4 °C followed by reaction with a second anti-rabbit HRP conjugated antibody for 1 hour at room temperature. The levels of
the phosphotyrosine or the RET protein signal were detected by Western blot chemiluminescence reagent (PerkinElmer Life Sciences), and densitometry analysis was performed by using Scion Image software (Scion Corporation).

Immunoprecipitation and in vitro kinase assay

Immunoprecipitation and an in vitro RET kinase assay were performed as described previously (47). Briefly, RET proteins were immunoprecipitated with Protein-A beads (Pierce biotechnology) and washed three times with lysis buffer [30 mM Tris-HCl (pH 8.0), 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 0.5 mM Na$_3$VO$_4$]. The immunoprecipitated RET proteins were washed three times with kinase buffer (10 mM Tris-HCl, pH 7.4, 5 mM MgCl$_2$), suspended the kinase buffer with 2 µg of myelin basic protein (MBP) (Sigma) as an exogenous substrate, and radiolabeled with [$\gamma^{32}$P] ATP (370 kBq) (New England Nuclear). The kinase reaction was carried out for 20 min in a 30 °C water bath and was terminated by adding 2 × sample buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerin] with 2-mercaptoethanol (2ME). The immunoprecipitates were then boiled for 5 min and loaded on 12.5% SDS polyacrylamide gels. The gels were dried and exposed to Imaging Plate (Fuji photo film), and the radioactive signals were detected by using a Typhoon 8600 imaging system (Amersham Biosciences). Densitometry analysis of signal levels of phosphorylated MBP was performed by using Scion image software.
Transformation assay

NIH3T3 cells were transfected with RET-MEN2A and RET-MEN2A mutant cDNAs, and several G418-resistant cell clones were isolated. Trypsinized $1 \times 10^5$ cells were mixed with 2 ml of 0.36% soft agar in DMEM, poured onto slightly solid 0.72% hard agar in DMEM, and then cultured in a 5% CO$_2$ incubator for two weeks. The number of aggregated cell foci in a dish was counted, and colony-forming efficiency $\{ \% = \frac{\text{number of colonies}}{\text{number of seeded cells}} \times 100 \}$ was calculated.
RESULTS

Identification of tyrosine auto-phosphorylation sites

In order to investigate the auto-phosphorylation sites in the intracellular domain of RET, we made a deletion mutant RET gene in which the plasmamembrane and extracellular regions were deleted (designated as ΔRET). We then connected glutathione S-transferase (GST) with the N-terminus of ΔRET (Fig. 1) and constructed a baculovirus protein expression system. Silver staining was performed to evaluate the purity of GST-ΔRET. As shown in Fig. 2A, GST-ΔRET protein was highly purified but was contaminated by a small amount of endogenous GST, as shown by Western blotting using an anti-GST antibody (data not shown). By Western blotting using an anti-phosphotyrosine antibody, a tyrosine-phosphorylated protein that might be coprecipitated with GST-ΔRET was observed at about 40 kD. Since there in no known tyrosine kinase of less than 40 kD, we assumed that non-PTK phospho-proteins were phosphorylated by GST-ΔRET and that any phosphorylation that newly occurred \textit{in vitro} with purified GST-ΔRET resulted from autophosphorylation. Next, the levels of autophosphorylation of GST-ΔRET were examined by an \textit{in vitro} kinase assay. As shown in Fig. 2B, both levels of autophosphorylation of purified GST-ΔRET and tyrosine phosphorylation of the synthetic substrate peptide were greatly increased when ATP was added to the \textit{in vitro} kinase system, and these increases
were prevented by treating the sample with λ-PPase before addition of ATP. These results suggested that the recombinant RET has an enhanced activity in vitro for autophosphorylation in the absence of any ligands and can be used for analyzing tyrosine autophosphorylation sites.

We extracted phosphorylated peptides from in vitro autophosphorylation-promoted GST-ΔRET that had been treated with Glu-C endoprotease. The samples were then analyzed by MALDI-TOF mass spectrometry to identify the phosphorylated peptides derived from GST-ΔRET. Because of the negative charge of phosphate groups, phosphopeptides exhibit low response to mass spectrometry in the positive ion mode. Nevertheless, several substantial intensities of mass spectra corresponding to phosphorylated peptides containing tyrosine residue(s) from the Glu-C endoproteinase-treated GST-ΔRET were obtained: Y806+Y809 as 1718.53, Y900 as 2209.59, Y905 as 2343.69, Y981 as 3419.05 and Y1062 as 1687.41 (Fig. 3A, C). For confirmation of tyrosine phosphorylation of these peptides, mass spectrometry analysis was also performed with Glu-C endoproteinase-digested phosphopeptides from GST-ΔRET that had been treated with LAR protein tyrosine phosphatase (LAR). As shown in Fig. 3B and 3D, the masses of tyrosine-dephosphorylated peptides containing Y806+Y809, Y900, Y905, Y981 and Y1062 were shifted to 1559.97, 2130.07, 2263.08, 3339.74 and 1607.80, respectively, confirming that the tyrosines on these peptides were autophosphorylated. We also performed mass spectrometry analysis of
peptides digested with Glu-C that had been treated with protein serine/threonine phosphatase 1, which resulted in development of no significant dephosphorylated masses for the peptides containing Y806+Y809, Y900, Y905, Y981 and Y1062 (data not shown).

MS/MS spectrometry analysis was also performed for endoprotease Glu-C-digested GST-ΔRET peptides containing Y900 or Y905. Putative triply charged phospho-peptide signals containing Y900 and Y905 were observed as m/z 737.5 and m/z 782.5, respectively, by using the full-scan mode (m/z 150-2000). Subsequently, mass spectra of each parent ion were obtained, and those of amino acid sequences were determined using the MASCOT MS/MS ion searching program and BioworksBrowser software. The triply charged peptides were identified mainly by doubly charged b-type ion in the MS/MS spectra as GRKMKISDFGLSRDV(pY)EE (Y900) (Fig. 4A) and DS(pY)VKRSQGRIPVKWMAIE (Y905) (Fig. 4B).

We also performed MALDI-TOF mass spectrometry analysis of phospho-peptides that had been digested with another protease, trypsin (Fig. 3E-H). Corresponding to the Glu-C results, putative phosphorylated fragments containing Y900+Y905 (m/z 1406.77), Y981 (m/z 1739.04) and Y1062 (m/z 2195.32) were observed, although fragments containing Y806 or Y809 were not detected. In addition, a new putative phospho-peptide containing Y1090+Y1096 (m/z 2174.22) was observed. Tyrosine phosphorylation(s) within these phospho-peptides was confirmed by treatment with LAR (Fig. 3F, H).
Taken together, the results demonstrated that Y806, Y809, Y900, Y905, Y981, Y1062, Y1090 and Y1096 are autophosphorylation sites. The MALDI-TOF mass spectra are shown in Table I.

The RET protein has 18 tyrosines in the intracellular domain: 2 in the vicinity of the transmembrane, 11 in the intracellular kinase domain, including Y806, Y809, Y900, Y905 and Y981, and 5 in the carboxyl-terminal tail, including Y1062, Y1090 and Y1096. Y905, Y1015, Y1062 and Y1096 residues were previously suggested to be autophosphorylation sites by using phospho-specific antibodies after GDNF stimulation (42-44) and in part by phosphopeptide mapping (41). However, there have been no reports on the phosphorylation of Y806, Y809, Y900, Y981 and Y1090. For Y981, we previously reported that both the levels of autophosphorylation and kinase activity of RET-MEN2A Y981F mutant were significantly decreased (28).

Database-oriented evaluation of the significance of tyrosines determined as autophosphorylation sites

Since the tertiary structure of RET has not yet been determined, we performed in silico analysis by using FAMS computer simulation software to predict the structure. As shown in Fig. 5, the predicted RET structure was constructed by homology molecular modeling and visualized. The calculated tertiary structure of RET was found to be similar to that of c-Src
(48). RET carried an N-lobe consisting of 5 β-sheets and one α-helix and a C-lobe consisting of 2 β-sheets and 7 α-helices. The tyrosines that we identified as autophosphorylation sites were positioned in this tertiary structure model. As shown in this figure, Y806 and Y809 tyrosines existed in the contralateral side of the activation loop, in which Y900 and Y905 existed. Y981 was very close to the MXXCW motif (40), which is on an α-helix of the C-lobe. Y1062, Y1090 and Y1096 were not shown in the predicted RET structure because of less similarity between RET and FGF receptor 1 as the source of structural information.

To determine which tyrosine residues are conserved in the human PTK family, a search of the SWISS-PROT database was made and human PTKs were manually aligned according to previously described methods (49, 50). In the database, we found 101 proteins that are registered as human PTK family proteins. Twenty-one of those 101 PTKs have serine and/or threonine kinase activity in addition to tyrosine kinase activity; hence, these proteins were excluded for the comparison and 80 proteins were examined (Fig. 6). We previously reported that the motif sequence of MXXCW, which is located in the C-end of α-helix H in the kinase domain is highly conserved in all except two (BLK and FGR) of the 80 genes for human PTKs (40). Interestingly, 61 (76.25%) of the 80 human PTK genes encoding the MXXCW motif had a conserved tyrosine residue corresponding to Y905 of RET that is positioned 79 residues from the MXXCW motif and is located in the putative activation segment of the protein kinase domain. In addition, 41 genes (51.25%), 15 genes (18.75%),
21 genes (26.25%), and 58 genes (72.5%) had conserved tyrosine residues corresponding to Y806, Y809, Y900 and Y981, respectively. These observations suggest that all of the tyrosines as autophosphorylation sites play critical roles in the regulation of the kinase activation that are conserved evolutionarily.

In vitro kinase assays of RET-MEN2A mutants

We next focused on the functions of Y806, Y809, Y900 and Y905 and prepared phenylalanine mutants of them for kinase activity assays. The mutant plasmids were transiently expressed in NIH3T3 cells for performing in vitro kinase assays. As shown in Fig. 7A and B, the levels of both autophosphorylation and MBP phosphorylation of the Y806F mutant were decreased to about 65% of those of RET-MEN2A, which is a constitutively active form of RET. Those levels of the Y809F mutant were comparable to those of the Y806F mutant. For the Y(806/809)F double mutant, the levels of both autophosphorylation and MBP phosphorylation tended to be more decreased than those of the Y806F or Y809F single mutant, although the difference between the levels of the single mutant and double mutant was not significant. These findings suggest that Y806 and Y809 contribute to the kinase activity of RET.

Among the 80 PTKs, all human PTK genes except CSK had one or more tyrosine residue near the segment that contains a tyrosine(s) corresponding to RET Y900 or Y905,
suggesting that such tyrosine residues in the activation loop are essential for protein kinase activation as was previously reported (48, 49). In fact, autophosphorylation of Y416 of Src, which is equivalent to Y905 of RET, is likely to work as a local switch to induce a conformational change required for kinase activation (48). We previously showed that the RET-MEN2A-Y905F mutant had reduced but not completely abolished auto-phosphorylation and kinase activities. We therefore hypothesized that Y900 phosphorylation supplements the function of Y905 that works as an initial local switch. To test this hypothesis, we made not only Y900F and Y905F mutants but also a Y(900/905)F double mutant of RET-MEN2A construct, which were transiently expressed in NIH3T3 cells for performing in vitro kinase assays. Corresponding to our previous data, the levels of both autophosphorylation and phosphorylation of an exogenous substrate (MBP) of the Y905F mutant of RET-MEN2A were significantly reduced, but not completely abolished, compared with those of the original RET-MEN2A. The levels of RET autophosphorylation and MBP phosphorylation of the Y900F mutant were nearly equal to those of the original RET-MEN2A, but those of the Y(905/900)F double mutant were almost completely abolished (Fig. 7D). These results suggest that Y905 in RET-MEN2A plays a pivotal role in the activation of RET-MEN2A for both autophosphorylation and MBP phosphorylation and that phosphorylation of Y900 supplements the function of Y905 as a local switch for increasing catalytic activity, presumably by stabilizing the conformation of activated RET
protein.

Transforming activity of RET-MEN2A mutants

We previously reported that the RET-MEN2A-Y905F mutant dramatically reduced the transforming ability of NIH3T3 cells on soft-agar medium compared with the RET-MEN2A transfectant, whereas the RET-MEN2A-Y900F mutant did not. We performed transforming assays to examine the effect on downstream signaling in MEN2A-Y806F or MEN2A-Y809F mutants. As shown in Fig. 8, the colony-forming efficiency of stable transformants of both RET-MEN2A-Y806F and Y809F mutants was about 50% lower than that of the original RET-MEN2A. As expected, the efficiency of Y905F was drastically reduced. These results coincide with the kinase activities, suggesting that both Y806 and Y809 are needed for the kinase to exert maximum protein kinase activity that leads to excess cell growth.
DISCUSSION

In this study, we used recombinant RET protein without the plasmamembrane and extracellular regions, which contains the kinase domain, and MALDI-TOF and/or ion trap mass spectrometry for identification of RET autophosphorylation sites. We used this strategy because it has at least two advantages. First, the purified RET protein is suitable for defining “auto” tyrosine-phosphorylation sites \textit{in vitro}. Physiologically, RET protein is activated by stimulation with a ligand such as GDNF that prompts a monomeric RET protein to gain access close each other and to form a dimer. In this event, several other proteins, including Src family PTKs, would accumulate around RET, which should phosphorylate RET as a substrate. In fact, Tansey \textit{et al.} and other groups (27, 51, 52) demonstrated that GFRα1 recruits RET to a lipid raft, a detergent-insoluble cholesterol-rich lipid microdomain enriched with signaling proteins, which results in RET/Src association after GDNF stimulation. Recently, we also demonstrated that RET-MEN2A protein forms a complex with Src tyrosine kinase in a lipid raft, which phosphorylates Y905 in RET \textit{in trans} (28). In this study, GST-ΔRET protein was highly purified by glutathione (GSH) agarose beads, although there was slight contamination of intrinsic GST and coprecipitated proteins of less than 40 kD (see Fig. 2A). This ruled out the possibility of an effect of trans-acting Src or other PTKs on GST-ΔRET. We showed that the recombinant RET protein on GSH agarose
beads, which allowed protein-protein interaction in the absence of a ligand, had kinase activity and was autophosphorylated by itself (Fig. 2). This system was therefore considered to be suitable for analyzing autophosphorylation sites. Second, MALDI-TOF MS and MS/MS analyses directly demonstrate the existence and exact positions of phosphate groups in protease-digested peptides. For digestion of RET protein, we used two kinds of protease, trypsin and protease V8, to confirm the identification of phospho-peptides by MALDI-TOF MS. Then the mass identical to the phospho-peptide was analyzed by ion-trap MS/MS and defined the sequence of amino acid with phosphate group.

There have been several attempts to determine autophosphorylation sites of RET by $^{32}\text{P}$-phosphopeptide mapping (41) or by using phospho-tyrosine specific antibodies (42-44), and the results of those studies have suggested, but not proved, that Y905 (43), Y1015 (41,42), Y1062 (41,43) and Y1096 (41, 44) are phosphorylated. The former technique includes two-dimensional $^{32}\text{P}$-phosphopeptide mapping followed by peptide sequencing by Edman sequencing. These procedures are rather tedious, and Edman sequencing is methodologically problematic, especially for determining phosphotyrosine sites, because phosphotyrosine is almost insoluble in the conventional transfer solvents used in an Edman sequenator, yielding a gap in the sequence course (53). Actually, this technique failed to demonstrate any autophosphorylation sites inside the kinase domain. The latter technique, which is fully based on specificity of phosphotyrosine reactive antibodies, does not provide
direct evidence. Neither of these techniques is therefore suitable for identifying autophosphorylation sites directly and exactly. Our data obtained by mass spectrometry have provided for the first time solid evidence that Y806, Y809, Y900, Y905 and Y981 in the kinase domain and Y1062, Y1090 and Y1096 in the tail are autophosphorylated.

The tertiary structure of RET, which was simulated by FAMS software (Fig. 5), was found to be similar to that of Src (48) or insulin receptor tyrosine kinase (IRK) (54). The molecular mechanism of the kinase activation in relation to the function of tyrosine residues of RET might therefore be comparable to that of Src or IRK. Like Src, RET carries an N-lobe and a C-lobe with an activation loop that carries Y905. Y905 in RET, which was convincingly demonstrated to be an autophosphorylation site in this study, is equivalent in position to Y416 in Src, which is known as the major autophosphorylation site principally regulating autophosphorylation and catalytic activity of Src kinase. Phosphorylation of Y416 in Src is thought to induce an alteration in the three-dimensional structure of the activation segment of the kinase domain to make it suitable for transferring ATP from the ATP-binding site to tyrosine residue(s) on the substrate. In fact, it was previously shown that the Y905F mutant of RET-MEN2A has greatly reduced catalytic and cell-transforming activities (28). Taken together, the results suggest that Y905 is the major autophosphorylation site of RET in terms of position, function and ability to bind phosphorus.

In this study, we demonstrated that Y806, Y809, Y900, Y981 and Y1090 are new
autophosphorylation sites of RET. We first focused our functional study on Y900 as a newly identified autophosphorylation site in the activation loop in relation to Y905 as the major autophosphorylation site. There has been no report on the role of Y900 or phosphorylated Y900 of RET. Based on the fact that Y900 is close to Y905 in the activation loop and the fact that the mutant Y905F retains low levels of autophosphorylation and kinase activity (Fig. 7 and Ref. 28), we speculated that Y900 can partially replace the function of Y905 as a local switch for kinase activation. As expected, both the autophosphorylation and kinase activity were almost completely abolished in the Y(900/905)F double mutant of RET-MEN2A (Fig. 7D), although the Y900F mutant itself showed activities comparable to those of wild-type RET-MEN2A.

Y1158 of IRK, which is equivalent in position to Y900 of RET, was previously reported to be an autophosphorylation site on the basis of results of analysis by Edman sequencing (55), in addition to Y1162, Y1163 (Y1163 is equivalent to Y905 in RET.) as other autophosphorylation sites in the activation loop (55-57). Similarly, the TRK nerve growth factor receptor was suggested by results of analysis using the Edman method to be autophosphorylated at Y670 (corresponding to Y900 of RET), Y674 and Y675 (Y905 of RET) (58). Hubbard et al. (54, 59) demonstrated that autophosphorylation of tyrosine residues in the activation loop of IRK is the initial event leading to tyrosine kinase activation and induces a drastic conformational change in the activation loop. After
autophosphorylation of tyrosine in the activation loop, the DFG sequence, which is conserved in the beginning of the activation loop of the kinase for occupying the ATP binding site, shifts away from the ATP binding site and the loop is stabilized by the interaction of phospho-Y1163 (pY1163) with a side chain of Arginine 1155 and Glycine 1166 via hydrogen bonds. The phosphate group of pY1158, which corresponds to RET pY900, however, makes no protein contacts and is completely solvent-exposed. It should be noted that the mutant Y1158F in IRK in one case was reported to have no effect on in vitro kinase activity (60), whereas the same mutation was found in several other studies to reduce in vitro activity by more than 80% (61, 62). Cunningham et al. demonstrated that mutations of the activation loop of TRK yielded a hierarchical loss of nerve growth factor (NGF)-induced neurite outgrowth: wild type > Y670F >> Y675F ≫ Y(670/675)F >> Y(674/675)F (63). The function we demonstrated in this study with Y900 of RET, supplementing the role of Y905 as the major autophosphorylation site, thus differs from the reported functions of Y900 of RET-equivalent Y1158 of IRK and Y670 of TRK and is novel. Collectively, these findings and our results confirm a general role of phosphorylated Y905 of RET and its equivalents in other PTKs and some diverse additional roles of phosphorylated Y900 of RET and its equivalents in kinase activation and downstream signaling.

We also demonstrated that Y806 and Y809, both of which exist in the juxtamembrane of RET, were autophosphorylated. There have been very few studies in which the function of
Y806 or Y809 of RET was examined. According to results of amino acid alignment analysis of PTKs, Y806 and Y809 of RET corresponded to tyrosines of 41 and 15 PTKs registered in the SWISS-PROT database, respectively. In vitro kinase assays revealed that the kinase activity was impaired by substitution of phenylalanine for tyrosine 806 or 809. The transforming activities in both the Y806F and Y809F mutants were also impaired. This is also the first report that to present evidence of the existence of tyrosines as autophosphorylation sites in the kinase domain outside the activation loop that are involved in both kinase activation and downstream signaling. There has been no earlier report on functions of tyrosines equivalent to those of Y806 or Y809 in other PTKs. According to the tertiary structure model, both Y806 and Y809 exist in the contralateral side of the activation loop and in a very short region between the end of the C-terminal β-sheet of the N-lobe and the beginning of the N-terminal α-helix of the C-lobe. These tyrosines lie adjacent to the putative ATP-binding pocket, indicating that they can affect the efficiency of ATP binding (see Fig. 5). These facts suggest that Y806 and Y809, located in this unique position, play a novel supplemental role for the activation loop upon phosphorylation. Interestingly, a V804M/Y806C double mutation has been identified in a Japanese female MEN2B patient (64). This finding suggests that the Y806-dependent regulation of kinase activity is physiologically significant.

Phosphorylation of Y981 of RET was another novel finding in this study. This tyrosine
is also well conserved among PTKs [in 58 (72.5%) of the 80 PTKs]. Mohammadi et al. (65) reported that Y730, which is equivalent to RET Y981, is one of the autophosphorylation sites of the fibroblast growth factor receptor, though the biological function of Y730 was not determined. Our previous study showed that mutation of MEN2A-Y981F abolished the autophosphorylation and kinase activities of RET-MEN2A measured in vitro (28).

Interestingly, the defect in intrinsic kinase activity of the Y981F mutant was able to be repaired by Src kinase, which promoted Y905 phosphorylation in trans. Taken together, the results suggest that phosphorylation of Y981 is not obligatorily required for the catalytic activity but plays a supplementary role in initiating autophosphorylation of Y905, which brings about the overall kinase activity.

In this study, we obtained direct evidence of not only tyrosine autophosphorylation sites in the kinase domain of RET but also of intriguing functions of Y806, Y809 and Y900 by mass spectrometry and mutagenesis analysis. It is likely that tyrosine residues of other PTKs that correspond to the RET autophosphorylation sites revealed in this study are also autophosphorylation sites because these tyrosine residues are well conserved among both receptor-type and nonreceptor-type PTKs. It was, however, difficult to determine all of the RET autophosphorylation sites in our system because there are some methodological limitations in the use of mass spectrometry for analysis of phosphopeptides; for example, ionization efficiencies of some phosphopeptides under a MALDI or ion-trap condition are
sometimes too low to detect signals. Therefore, the characterization of all autophosphorylation sites requires the development of improved methods. Autophosphorylation and the downstream signaling effects of these phosphorylated tyrosines in cells under physiological conditions remain to be analyzed because we used an originally prepared recombinant GST-ΔRET protein without any ligand stimulation and we did not use cells that normally express RET. However, mass spectrometry proved to be a useful technique for directly identifying sites of post-translational phospho-modification of RET, and the data we obtained by using this technique have contributed to an understanding of the mechanisms of activation of not only RET but also any other PTK proteins and to an understanding of downstream signaling under the condition of activated PTKs.
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The abbreviations used are:

GDNF, glial cell line-derived neurotrophic factor; RET-MEN2A, RET with multiple endocrine neoplasia type 2A mutation; MBP, myelin basic protein; PTK, protein tyrosine kinase; GST, glutathione S-transferase; λ-PPase, lambda protein phosphatase; LAR, leukocyte antigen related; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; FAMS, full automatic modeling system; FGF, fibroblast growth factor; PDB, protein data bank.
FIGURE LEGENDS

**Fig. 1. Preparation of GST-fusioned recombinant RET and MEN2A mutants.**

A c-RET gene fragment for the cytosolic region obtained by digestion with *Eco*RI and *Not*I was subcloned into the baculovirus expression vector pAcGHLT in which a RET insert was fused with the GST gene (designated GST-ΔRET). The RET-MEN2A gene was constructed by replacement of cysteine 634 with arginine of c-RET. Mutant RET-MEN2A cDNAs in which tyrosine at codon 806, 809, 900 or 905 was replaced by phenylalanine were then constructed. TK1, tyrosine kinase domain 1; TK2, tyrosine kinase domain 2.

**Fig. 2. Autophosphorylation of purified recombinant RET protein.**

A, GST-ΔRET was purified by using glutathione (GSH)-agarose beads and incubated for 30 min at 30 °C with or without ATP (1 mM) in kinase buffer containing MgCl₂. These samples were then subjected to SDS-PAGE followed by silver staining for the detection of overall proteins and by Western blotting for the detection of tyrosine-phosphorylated proteins. B, *In vitro* kinase assay was performed with ATP-treated or untreated purified GST-ΔRET for determination of the levels of PTK activity to phosphorylate a synthetic substrate (B-1) and of autophosphorylation (B-2) according to the method described in EXPERIMENTAL PROCEDURES. A portion of the sample (right end lane) was treated
with λ-PPase (100 units) for 30 min at 37 °C. Sodium orthovanadate (0.5 mM) was added to
the λ-PPase-treated sample to inhibit the phosphatase activity prior to the addition of ATP.
Quantitation of the tyrosine-phosphorylation level of GST-ΔRET was performed by using
Scion Image software. Data represent means ± S.D. of three independent experiments.

Fig. 3. Identification of RET phospho-peptides by MALDI-TOF mass spectrometry.

ATP-treated GST-ΔRET was digested with V8 protease (a Glu-C endoproteinase) (A, B,
C, D) or trypsin (E, F, G, H), and phosphorylated peptides in the samples were determined
by MALDI-TOF mass spectrometry. To confirm tyrosine phosphorylation, portions of
peptide samples were treated with tyrosine phosphatase (LAR) (B, D, F, H) at 30 °C for 30
min as described in EXPERIMENTAL PROCEDURES. Results shown are representative of
more than three independent experiments.

Fig. 4. Analysis of phosphopeptides by ion-trap mass spectrometry.

Phosphopeptide samples containing Y900 or Y905 were obtained from V8
protease-digested products from GST-ΔRET as described in EXPERIMENTAL
PROCEDURES. MS/MS spectra from candidates of phosphopeptides were acquired by
using an ion-trap mass spectrometer, and amino acid sequences were determined by using
the MS/MS ion search program of MASCOT and BioworksBrowser software. A partial
fragment ion spectrum of a triply charged precursor ion of phosphopeptides at m/z 737.5 was obtained (A) and identified as a tyrosine-phosphorylated peptide at Y900 from MS/MS signal analysis. MS/MS spectra of phosphopeptides at m/z 782.5 were analyzed in a similar manner (B), resulting in identification of tyrosine phosphorylation at Y905. The results show some characteristic y-type and b-type ion products. \((bn)^{+2}\) denotes a doubly charged b-ion series product. The peaks denoted by \(bn^0\) and \(bn^*\) are the results of water (−18 Da) and ammonia (−17 Da) losses from the corresponding ion, respectively.

**Fig. 5. Tertiary structure of RET protein simulated by FAMS computer software.**

Since a structural homologue with the extracellular and transmembrane region of RET does not exist in a protein structure database, PDB (Protein Data Bank), intracellular region of RET was simulated by FAMS. This tertiary structure was constructed by homology modeling based on FGF receptor 1 (PDB ID code, 1FGI), which shares the highest amino acid homology with RET. The stereo view of this figure was made with WebLab ViewerLite 3.20 software. The β-sheet and α-helix are colored light blue and red, respectively. Positions of identified autophosphorylation sites are shown in the picture. The putative ATP-binding site is also indicated.

**Fig. 6. Comparison of PTKs sequences.**
Eighty human PTKs were found in the SWISS-PROT database, and the positions of the tyrosines in these PTKs equivalent to Y806, Y809, Y900, Y905 and Y981 in RET from the MXXCW motif are displayed. The sequences were compared with a part of RET (G798-L1005). Gaps, represented by (--), were introduced into the sequences to optimize the alignment. The putative activation loop domain of PTKs is displayed in a red-colored box around the center of sequences. The MXXCW motif is shown as a red-colored box on the right side of the sequence. The alphanumeric characters on the left side of individual sequences represent human protein names registered in the database. The tyrosine residues equivalent to RET Y806, Y809, Y900, Y905 and Y981 are colored red.

**Fig. 7. Levels of kinase activity of RET-MEN2A and its mutants measured by in vitro kinase assays.**

*RET-MEN2A* or *RET-MEN2A* mutant cDNA was cloned into a mammalian expression vector, Rc/CMV vector, and RET protein was transiently expressed in NIH3T3 cells. Cell lysates from non-*RET*-transfected NIH3T3 cells or mutant *RET*-transfected NIH3T3 cells were subjected to immunoprecipitation with an anti-RET antibody, and the samples were analyzed by both *in vitro* kinase assays (*A*) and immunoblotting with the anti-RET antibody (*B*). 175-kD and 155-kD isoforms of RET were detected, and measurements of these bands were integrated for the determination of overall phosphorylation level. Quantitation of
phospho-RET (pRET) levels and phospho-MBP (pMBP) levels were performed by using Scion Image software. The calculated values for pRET and pMBP of an NIH3T3 sample were used as background, and the levels of experimental samples over the background were measured and then divided by those for pRET \((C)\) and pMBP \((D)\) of parental RET-MEN2A, respectively. Bars represent means ± S.D. of three independent experiments. The value for each of the tyrosine mutants \((\dagger)\) is significantly \((P<0.05)\) different from that for RET-MEN2A. The value for the Y(900/905)F mutant \((\ddagger)\) is significantly \((P<0.05)\) different from that for the Y905F mutant.

**Fig. 8. Transforming activities of the RET-MEN2A and RET-MEN2A mutant genes with mutations of tyrosine residues.**

RET-MEN2A and its mutant genes were transfected to NIH3T3 cells and transforming assays were performed as described in EXPERIMENTAL PROCEDURES. Bars represent means ± S.D. of four independent experiments. The values for each of the tyrosine mutants \((\ddagger)\) is significantly \((P <0.001)\) different from that for RET-MEN2A.
### Table I. Summary of tyrosine-phosphorylated peptides in RET analyzed by MALDI TOF mass spectrometry.

GST-ΔRET treated with ATP was digested with V8 protease or trypsin, and portions of peptide samples were treated with tyrosine phosphatase (LAR). The peptide samples were analyzed by MALDI TOF mass spectrometry as described in EXPERIMENTAL PROCEDURES. The mass values obtained were compared with the theoretical values calculated from known amino acid sequences.

<table>
<thead>
<tr>
<th>protease digested products</th>
<th>position</th>
<th>Mass of MH+ (Da)</th>
<th>dephosphorylated (LAR)</th>
<th>phosphorylated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Observed</td>
<td>Calculated</td>
<td>Observed</td>
</tr>
<tr>
<td>YAKYGSLRGFLRE</td>
<td>Y806+Y809</td>
<td>1559.97</td>
<td>1559.83</td>
<td>1718.53</td>
</tr>
<tr>
<td>GRKMKIDSFGLSRDVYEE</td>
<td>Y900</td>
<td>2130.07</td>
<td>2130.06</td>
<td>2209.59</td>
</tr>
<tr>
<td>DSYVKRSQGRIPVKWMAIE</td>
<td>Y905</td>
<td>2263.08</td>
<td>2263.20</td>
<td>2343.69</td>
</tr>
<tr>
<td>MRYLMLQCWKQEPDKRVPFAKDDKLE</td>
<td>Y981</td>
<td>3339.74</td>
<td>3339.67</td>
<td>3419.05</td>
</tr>
<tr>
<td>NKLYGMSDPNWPG</td>
<td>Y1062</td>
<td>1607.80</td>
<td>1607.72</td>
<td>1687.41</td>
</tr>
</tbody>
</table>

| DVYEDSYVK                  | Y900+Y905| 1246.68          | 1246.54                | 1406.77        | 1406.48        |
| MERPDNCSEEMYR              | Y981     | 1659.77          | 1659.66                | 1739.04        | 1739.64        |
| LYGMSDPNWPGESPVLTR         | Y1062    | 2116.16          | 2116.01                | 2195.32        | 2195.98        |
| YPNDSYYANWMLSPSAAK         | Y1090+Y1096| 2014.04        | 2013.94                | 2174.22        | 2173.90        |
Fig. 1

Transmembrane
Extracellular
Intracellular

ATG

C634R
=RET-MEN2A

TAA

c-RET

N terminus

TK1

EcoRI

TK2

C terminus

NotI

GST

GST-ΔRET

Y900
Y905
Y806-Y809
Y981
Y1015
Y1096
Y1090
Y826
Y864
Y928
Y952
Y1062
Y1029
Y752
Y791
Y687
Y660
Y46
Y46
Fig. 2

A

Silver staining

IB: α-phosphotyrosine

kD

GST-ΔRET

p-GST-ΔRET

GST

B-1

PTK activity (Fold)

- + ATP

* p<0.001

B-2

Tyr-autophosphorylation (Fold)

- + ATP

λ-PPase

* p<0.001
Fig. 3

Tyrosine phosphatase

% Intensity

Mass (m/z)

A

B

C

D

E

F

G

H

Tyrosine phosphatase

Tyrosine phosphatase

Tyrosine phosphatase

Tyrosine phosphatase
Fig. 4 B
Fig. 5
Fig. 8
Identification of RET autophosphorylation sites by mass spectrometry
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