Oncogenic RET Receptors Display Different Autophosphorylation Sites and Substrate Binding Specificities*

Communication

The c-ret proto-oncogene encodes a receptor tyrosine kinase which plays an important role in neural crest as well as kidney development. Genetic studies have demonstrated that germ line mutations in the ret oncogene are the direct cause of multiple endocrine neoplasia (MEN) 2A and 2B, familial medullary thyroid carcinoma (FMTC), and Hirschsprung's disease. However, despite the large body of genetic and biological evidence suggesting the importance of RET in development and neoplastic processes, the signal transduction mechanisms of RET remain unknown. To begin to understand the molecular mechanisms of the disease states caused by mutations in RET, the patterns of autophosphorylation of the wild-type RET and the MEN mutants were studied using site-directed mutagenesis and phosphopeptide mapping. Among the 6 autophosphorylation sites found in the wild-type RET receptor, the MEN2B mutant lacked phosphorylation at Tyr-1096, leading to decreased Grb2 binding, while simultaneously creating a new phosphorylation site. These changes in autophosphorylation suggest that the MEN2B mutation may result in the more aggressive MEN2B phenotype by altering the receptor's signaling capabilities.

EXPERIMENTAL PROCEDURES

Construction of the Full-length Recombinant RET—The full-length cDNA encoding RET was obtained by polymerase chain reaction amplification of total RNA isolated from a human medullary thyroid carcinoma and, after confirming the sequence of the polymerase chain reaction product, was cloned into the mammalian expression vector, pCDNA3 (Invitrogen). Three HA epitope sequences (YPYDVPDYA)(13) were inserted in-frame at the COOH terminus of RET prior to the stop codon. The resulting plasmid was named pRET-HA.

Site-directed Mutagenesis—Site-directed mutagenesis was performed on plasmid pRET-HA per the manufacturer's instructions (BioRad). The sequence of the DNA containing the mutated residues was confirmed by dyeoxy sequencing (14).

Transfection of COS Cells, in Vivo Labeling, and Immunoprecipitation—Subconfluent COS cells in 10-cm plates were transfected with 8 μg of the appropriate pRET-HA construct using LipofectAMINE reagent as described by the manufacturer (Life Technologies, Inc.). For the in vivo labeling experiments, 2.4 mCi [33P]orthophosphate were added 36 h post-transfection. Forty eight h after transfection, the cells were collected and lysed in 100 mM sodium phosphate, pH 7.4, 150 mM NaCl, and 1% Triton X-100 (PBS-T). The lysates were preclated with Protein A-Sepharose reagent (Calbiochem) and incubated with 2 μg of anti-HA antibodies (Boehringer Mannheim) followed by protein A-agarose beads (Life Technologies, Inc.). The immunoprecipitates were collected by centrifugation at 10,000 × g for 2 min, washed five times with 1 ml of PBS-T buffer, followed by resuspension in 40 μl of the same buffer. Typically, a 20-μl sample of the resuspended beads was analyzed by electrophoresis. The radioactive products were visualized by autoradiography.
Expression of RET in COS Cells—To express and immunoprecipitate the RET protein from mammalian cells, an expression vector containing three tandem HA sequences encoded at the COOH terminus of the RET cDNA was constructed. pRET-HA was then transfected into COS cells, and the recombinant protein was immunoprecipitated using a monoclonal antibody against the HA epitope tag. In Western blot analyses, the recombinant protein was detected in the transfected cells (Fig. 1 lane 1), but not in the mock-transfected cells (Fig. 1A, lane 2). Western blotting with antibodies directed against phoshothyrosine indicated that recombinant RET was capable of autophosphorylation in COS cells (Fig. 1A, lane 3).

In preparation for phosphopeptide mapping, immunoprecipitated RET was autophosphorylated in vitro with \( \gamma^{32} \text{P} \text{ATP} \). Consistent with the Western blotting experiment, a 170-kDa band was labeled strongly with \( ^{32} \text{P} \) (Fig. 1A, lane 4). To rule out the possibility that the observed phosphorylation of RET resulted from other kinases that co-immunoprecipitated with RET, a kinase-inactive form of the receptor was made by replacing lysine 758 with methionine (K758M). According to studies done on other receptor tyrosine kinases, replacement of this lysine residue is predicted to inactivate RET by disrupting the ATP binding site (18). The K758M mutant was expressed and immunoprecipitated and, as expected, no phosphorylated band was visible on the SDS-PAGE after the in vitro kinase assay (Fig. 1A, lane 5). An equivalent amount of RET receptor was produced in each experiment (data not shown). This result confirmed that the tyrosine phosphorylation of RET was due to autophosphorylation. To verify that RET was phosphorylated due to its tyrosine kinase activity, a phosphoamino acid analysis was performed on the labeled receptor. As shown in Fig. 1B, phosphorylated RET contained phosphotyrosine but no phosphoserine or phosphothreonine.

**RESULTS AND DISCUSSION**

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**The MEN2B Mutation in RET**—Results in an Altered Phosphopeptide Map—Mutations in the ret proto-oncogene which result in the manifestation of MEN2A are located in the cysteine-rich extracellular sequence adjacent to the transmembrane domain. It was previously demonstrated that MEN2A mutations result in the activation of the RET receptor by mechanisms involving inappropriate receptor dimerization leading to constitutive activation of the tyrosine kinase (9). In contrast, the MEN2B mutation, M918T, occurs in the tyrosine kinase domain and may alter the receptor’s substrate specificity with no apparent effects on dimerization (9). In order to determine if these two diseases could be differentiated at the molecular level, phosphopeptide maps of MEN2A (C634S) and MEN2B (M918T) mutations were generated and compared to the phosphopeptide map of the wild-type receptor. Both mutant receptors were readily phosphorylated in the in vitro kinase reaction, although the kinase activity of the MEN2B mutant was 3- to 5-fold lower than the MEN2A mutant or the wild-type receptor.

Analysis of the two-dimensional map for the MEN2A mutant receptor revealed 12 major \( ^{32} \text{P} \) labeled peptides (Fig. 2, A and C). The phosphopeptide map of the wild-type receptor did not show any significant difference compared to the MEN2A receptor, indicating that the MEN2A mutation does not affect the autophosphorylation state of RET. In addition, an NH\textsubscript{2}-terminal tagged receptor generated an identical phosphopeptide map demonstrating that the COOH-terminal HA peptide does not interfere with autophosphorylation (data not shown). In contrast, the phosphopeptide map of the MEN2B mutation
differed dramatically from the wild-type RET pattern (Fig. 2B). Notably, the MEN2B phosphopeptide map showed an absence of phosphopeptide 5, a diminished phosphopeptide 1, and the appearance of an unidentified phosphopeptide which runs very close to phosphopeptide 3. Phosphoamino acid analysis confirmed that the MEN2B receptor was still phosphorylated exclusively on tyrosine (data not shown).

To demonstrate that the phosphorylation obtained in vitro accurately represented phosphorylation in vivo, COS cells were transfected with the wild-type and K758M (kinase inactive) receptors and labeled with [32P]orthophosphate. The labeled wild-type receptor contained all the phosphopeptides obtained in the in vitro labeling experiments as well as additional labeled peptides (Fig. 2D). These additional peptides were also present in the kinase inactive receptor's phosphopeptide map indicating that they represent either background labeling or the phosphorylation of the RET receptor by associated cellular kinases (data not shown).

Two-dimensional Phosphopeptide Mapping of the Phosphorylation Sites in RET—To further dissect the signaling mechanism of the RET receptor, the autophosphorylated tyrosine residues in RET were identified by two-dimensional mapping using a selected set of tyrosine mutants. As described above, the two-dimensional phosphopeptide map of the wild-type receptor revealed 12 major 32P-labeled spots (Fig. 3A). We focused on the 8 tyrosine residues located in the juxtamembrane region, in the kinase insert, and in the COOH-terminal tail, as candidate autophosphorylation sites (Table I). These residues were selected because tyrosine residues in these regions were shown to be the major phosphorylation sites for the epidermal growth factor and platelet-derived growth factor receptors which are closely related to RET (19). Individual tyrosine residues in RET were replaced with phenylalanine by site-directed mutagenesis, and the two-dimensional peptide map of each mutant was compared with that of the wild-type protein.

Interestingly, mutation Y1096F resulted in the absence of phosphopeptide 5, thereby allowing us to assign one of the phosphopeptides absent in the MEN2B receptor (Fig. 3B). Although this phosphopeptide contains two potential Tyr phosphorylation sites, mutation of Tyr-1090 does not result in the reduction or disappearance of phosphopeptide 5 (data not shown), suggesting that the major phosphorylated residue of this peptide is Tyr-1096. This has major repercussions on RET receptor signaling as this phosphotyrosine and adjacent amino acids represent a potential Grb2 binding site (pYXNX) (20). Tyr-1096 is also not present in the short form of RET which is truncated near Tyr-1062 (1). It is possible that the disappearance of this putative Grb2 binding site in conjunction with the production of a new phosphopeptide and its alternative signal transduction pathway could result in the more aggressive MEN2B phenotype. Efforts are underway to determine the identity of this new phosphopeptide.

Mutations Y687F, Y826F, and Y1062F caused the absence of spots 1, 2, and 7, respectively (Table I, data not shown), indicating that these mutations replaced the phosphorylated tyrosines in the corresponding tryptic peptides. We therefore deduce that Tyr-687, Tyr-826, and Tyr-1062 are phosphorylated in the wild-type receptor. On the contrary, we did not observe any altered phosphopeptide pattern with mutants Y660F and Y1090F (as mentioned above), indicating that these two sites are probably not phosphorylated in the activated receptor. We also noticed that mutations Y1015F and Y1029F both caused partial disappearance of spot 10, consistent with the fact that these two tyrosines are located in the same tryptic peptide (Table I, data not shown).

Based on the phosphopeptide maps, we can identify six tyrosine phosphorylation sites in RET: Tyr-687, Tyr-826, Tyr-1062, Tyr-1096, Tyr-1015, and Tyr-1029. By comparing the peptide map of each mutant to the wild-type receptor, we can tentatively assign each tryptic peptide containing phosphoryl-

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**Table I**

Autophosphorylation sites in RET

| Residue | Location         | Peptide sequence   | Mutation | Phosphorylation | Assignment on two-dimension map |
|---------|------------------|--------------------|----------|----------------|--------------------------------|
| Tyr-660 | Juxtamembrane    | TIVIAAVLFS1VSVLLSADFCHCYHK | Y660F    | +              |                                |
| Tyr-687 | Juxtamembrane    | RPAQAFPV1SYSSSGAR  | Y687F    |                |                                |
| Tyr-752 | Kinase domain    | AGYTVAVKK          |          |                |                                |
| Tyr-791 | Kinase domain    | LYGACSDQGDPLILIVEYAK |          |                |                                |
| Tyr-806 |                 |                    |          |                |                                |
| Tyr-809 | Kinase domain    | YGSLR              |          |                |                                |
| Tyr-826 | Kinase insert    | VGPQYLSGSGSR       | Y826F    | +              |                                |
| Tyr-864 | Kinase domain    | ALTMGDLSFAWQI5GMOYLAEMK |          |                |                                |
| Tyr-900 | Kinase domain    | DVYEEDSYVK         |          |                |                                |
| Tyr-905 | Kinase domain    |                     |          |                |                                |
| Tyr-928 | Kinase domain    |                     |          |                |                                |
| Tyr-952 | Kinase domain    |                     |          |                |                                |
| Tyr-981 | Kinase domain    |                     |          |                |                                |
| Tyr-1015 | COOH-terminal tail | DYLDLAAPTS5DSL1YDDGLSEEET | Y1015F  | +              |                                |
| Tyr-1029 |                 |                     | Y1029F  | +              |                                |
| Tyr-1062 | COOH-terminal tail | LYGMSDPNPWPGESPVPLTR | Y1062F | +              |                                |
| Tyr-1090 | COOH-terminal tail | YPDSDVYANWMLSPSAAK | Y1090F  | +              |                                |
| Tyr-1096 | COOH-terminal tail |                         | Y1096F  | +              |                                |

*a* Sequences of the predicted tyrosine containing tryptic peptides.  
*b* + indicates tyrosine phosphorylation in the wild-type receptor.
To confirm one of the assignments made by site-directed mutagenesis, we synthesized a peptide with a sequence identical to the tryptic peptide containing the predicted phosphorylation site, Tyr-687, and tested the migration of the phosphorylated synthetic peptide on the two-dimensional map. The labeled synthetic peptide migrated to the same position as phosphopeptide 1, which was the assigned position for the tryptic peptide containing Tyr-687 (data not shown).

Durick et al. (21) recently determined several of the phosphotyrosines important for mitogenic activity of the RET/ptc2 oncogene. This version of the RET receptor contains the tyrosine kinase domain fused to the type I regulatory subunit of protein kinase A (22). In their assay, Tyr-826 and Tyr-900 were moderately important for mitogenic activity, while mutation of Tyr-826 or Tyr-900 completely abolished mitogenic activity. Our analysis confirms that Tyr-826 and Tyr-906 are phosphorylated and are contained within phosphopeptides 2 and 7, respectively, on our map.

As mentioned previously, phosphopeptide 5, which disappears in the MEN2B receptor, contains potential Grb2 binding sites specified by the consensus sequence pYXNX (20). In order to demonstrate Grb2 binding, we expressed wild-type, K758M (kinase inactive), Y1090F, Y1096F, the Y1090F/Y1096F double mutant, and M918T (MEN2B) in COS cells. The various receptors were immunoprecipitated to equivalent levels and allowed to interact with a GST fusion protein containing the SH2 domain of Grb2 (Fig. 4). As anticipated, wild-type RET bound the SH2 domain of Grb2 effectively. In contrast, the kinase inactive RET mutant (K758M) displayed no Grb2 binding, confirming that autophosphorylation of RET is required for Grb2 association. Analyses of the RET tyrosine mutants showed that although the Y1090F mutant bound to Grb2 in a manner similar to wild-type RET, the RET mutants lacking Y1096 and the MEN2B mutant all demonstrated weak association with Grb2. However, the Y1096 mutant was capable of binding to an SH2 domain of phospholipase C\alpha with the same affinity as the wild-type receptor demonstrating that the mutant receptor is capable of interacting with other SH2 domains through other phosphotyrosine residues (data not shown). It is important to note that Grb2 binding does not disappear completely, suggesting that other phosphotyrosine residues may have some affinity for Grb2 binding. While it is clear that the major binding site of Grb2 is Y1096 in vitro, confirmation of this result in vivo awaits isolation of the RET ligand.

Our analyses have resulted in the assignment of several autophosphorylation sites located in the juxtamembrane, kinase insert, and COOH-terminal tail of the RET receptor. In addition, we have determined a major difference in the phosphopeptide maps between the MEN2A and MEN2B mutants. This change in enzymatic activity results in the loss of a Grb2 binding site while simultaneously creating a new phosphotyrosine peptide with presumably new signaling capabilities. We are pursuing the identity of this new phosphopeptide and continuing our determination of the phosphorylation status of the remaining tyrosines in the RET kinase domain. The successful completion of our experiments will provide us with a more complete picture of RET signaling and will put us in a position to address the different mechanisms by which the RET mutants affect various second messenger cascades.

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