Structure and Chemical Inhibition of the RET Tyrosine Kinase Domain*

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The RET proto-oncogene encodes a receptor tyrosine kinase for the glial cell line-derived neurotrophic factor family of ligands. Loss-of-function mutations in RET are implicated in Hirschsprung disease, whereas activating mutations in RET are found in human cancers, including familial medullary thyroid carcinoma and multiple endocrine neoplasias 2A and 2B. We report here the biochemical characterization of the human RET tyrosine kinase domain and the structure determination of the non-phosphorylated and phosphorylated forms. Both structures adopt the same active kinase conformation competent to bind ATP and substrate and have a pre-organized activation loop conformation that is independent of phosphorylation status. In agreement with the structural data, enzyme kinetic data show that autophosphorylation produces only a modest increase in activity. Longer forms of RET containing the juxtamembrane domain and C-terminal tail exhibited similar kinetic behavior, implying that there is no cis-inhibitory mechanism within the RET intracellular domain. Our results suggest the existence of alternative inhibitory mechanisms, possibly in trans, for the autoregulation of RET kinase activity. We also present the structures of the RET tyrosine kinase domain bound to two inhibitors, the pyrazolopyrimidine PP1 and the clinically relevant 4-anilinoquinazoline ZD6474. These structures explain why certain multiple endocrine neoplasia 2-associated RET mutants found in patients are resistant to inhibition and form the basis for design of more effective inhibitors.

The RET (rearranged during transfection) gene was originally isolated as an oncogenic fusion protein in cell transformation assays (1). The RET proto-oncogene (2), on human chromosome 10q11.2, encodes a receptor tyrosine kinase (RTK) (3–5) activated by members of the glial cell line-derived neurotrophic factor (GDNF) ligand family (GDNF, neurturin, artemin, and persephin) (6) in conjunction with a ligand-specific coreceptor (GFRα1–4) (7). RET signaling is essential for development, survival, and regeneration of many neuronal populations such as those in the enteric and sympathetic nervous systems (6) and the kidney (8, 9). The domain organization of RET is shown in Fig. 1A; orthologs exist from Drosophila to human and share a high degree of sequence similarity (90% in vertebrates) throughout the cytoplasmic domain and, to a lesser extent, within the extracellular region.

GDNF family ligands do not interact directly with RET; instead, signaling via RET depends on formation of a tripartite complex of RET, a GDNF family ligand, and its cognate glycosylphosphatidylinositol-linked GFRαs (10, 11). In addition, ligand binding requires Ca2+ ions chelated to the RET extracellular domain (12, 13). According to the classical RTK paradigm, formation of the complex promotes RET dimerization, leading to trans-autophosphorylation within the RET intracellular domain (RET-ICD). There are two tyrosine residues ( Tyr900 and Tyr1065) in the RET tyrosine kinase domain (RET-KD) activation loop (A-loop), both of which have been shown to be autophosphorylation sites by mass spectrometry (14); Tyr1065 has been shown to be required for RET downstream signaling (15). Phosphorylation at Tyr900, Tyr1062, and Tyr1096 has been shown to be of major importance for further signal propagation (16), and each forms a binding site for one or more downstream adaptor molecules (see Arighi et al. (11) for a recent review). RET has attracted considerable clinical interest.

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The atomic coordinates and structure factors (codes 2IVS, 2IVT, 2IVU, and 2IVV) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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4 The abbreviations used are: RTK, receptor tyrosine kinase; GDNF, glial cell line-derived neurotrophic factor; RET-ICD, RET intracellular domain; RET-KD, RET tyrosine kinase domain; A-loop, activation loop; MTC, medullary thyroid carcinoma; MEN2, multiple endocrine neoplasia 2; PTC, papillary thyroid carcinoma; STAT3, signal transducer and activator of transcription 3; VEGFR, vascular endothelial growth factor receptor; EGFR, epidermal growth factor receptor; RET-JM-KD, RET juxtamembrane domain and tyrosine kinase domain; GST, glutathione S-transferase; RET-KD-P, phosphorylated RET tyrosine kinase domain; RET-KD-0P, non-phosphorylated RET tyrosine kinase domain; r.m.s.d., root mean square deviation; IRK, insulin receptor kinase; GFRα, GDNF receptor α.
Structure and Inhibition of the RET Tyrosine Kinase Domain

because of the range of mutations found in diverse conditions that include Hirschsprung disease and a variety of cancers involving the thyroid gland. RET is the gene most commonly mutated in patients with familial or sporadic Hirschsprung disease, which is characterized by deficiencies in the enteric nervous system and intestine malfunction (17, 18). RET-related cancers with thyroid involvement include sporadic and familial medullary thyroid carcinoma (MTC; OMIM #155240), multiple endocrine neoplasia 2 (MEN2; OMIM #171400) syndromes MEN2A and MEN2B, and papillary thyroid carcinoma (PTC).

To date, >100 mutations in the RET gene have been described, and with the exception of a few dual-phenotype mutations (19), they can be generally partitioned into loss- or gain-of-function groups. RET mutations in Hirschsprung disease are characterized by loss of function and can be broadly classified into three groups: extracellular residues that are essential for protein folding, the alteration of which leads to immature non-folded RET being retained in the endoplasmic reticulum (20); residues in RET-KD that adversely affect protein stability or activity (21, 22); and docking residues required for downstream signaling (23). RET-related cancers generally involve gain-of-function mutations in RET that can be divided into two groups: those affecting cysteine residues in the cysteine-rich domain and those altering residues in RET-KD. In the first group, the most frequently mutated residue found in MEN2A patients is Cys634, where removal of one-half of an intramolecular disulfide bond allows formation of an intermolecular disulfide bond with a second mutant molecule, thus leading to constitutive receptor dimerization and aberrant signaling. Activating mutations within RET-KD are more varied in mechanism and not known to directly lead to constitutive dimer formation. A range of mutations such as L790F, Y791F, S891A, and R844L produce transforming RET, but with the relatively mild MTC and MEN2A phenotypes. In contrast, the M918T mutation has a very high transforming ability and is found in 95% of MEN2B patients (24). The MEN2B phenotype involves not only the thyroid and adrenal glands, but also a selection of mucosal, ocular, and skeletal abnormalities. M918T RET does not need Tyr905 for activation, in marked contrast to the MEN2A dimerizing mutations, where Tyr905 is required for oncogenesis (14, 24–27). This suggests that different underlying mechanisms subvert normal control of RET activation in MEN2A and MEN2B (28). In addition, M918T RET targets unique substrates such as STAT3 that may contribute to cell transformation (29, 30). As well as transforming mutations within intact RET, chromosome translocations can produce oncogenic fusions including the RET kinase domain (RET/PTC oncogenes) that give rise to PTC. RET/PTC fusion proteins are cytosolic and contain RET-KD from exon 12 (starting at Gln715) through to the C terminus (31). In many cases, the N-terminal domain of RET/PTC is a dimerization domain from the fusion partner. Notably, mutation of the residue in RET/PTC corresponding to Tyr905 in wild-type RET leads to reduced transforming ability (32).

Because RET point mutations in familial MTC, MEN2A, and MEN2B and the RET/PTC fusions all result in inappropriate kinase activation, RET-KD presents a unique therapeutic target for treatment of the associated cancers (33, 34). Evidence for its suitability as a target comes from suppression of oncogenic RET in a thyroid carcinoma model system by small molecule inhibition (35) as well as from the beneficial effect of a dominant-negative RET mutant in MTC cells blocking tumor growth (36). Small molecule inhibitors of protein kinases have already been proven clinically useful for a variety of human diseases (37, 38). Potent inhibitors of wild-type RET and oncogenic forms have been described, including the pyrazolopyrimidines PP1 and PP2 and the 4-anilinoquinazoline ZD6474 (ZacitumTM) (34). PP1 was first recognized as a Src family inhibitor, but also inhibits a number of RTKs (39), including RET (40). ZD6474 targets RET, vascular epidermal growth factor receptor (VEGFR), and epidermal growth factor receptor (EGFR) tyrosine kinases and is being tested in advanced trials against lung cancer as well as against locally advanced or metastatic hereditary MTC (41). The most oncogenic MEN2-associated RET-KD mutants are highly susceptible to PP1 and ZD6474 inhibition, with the exception of MEN2-associated V804L and V804M, which are resistant (42). A similar mutation of the analogous kinase “gatekeeper” residue of the BCR-ABL onco- gene develops in imatinib-treated leukemia patients, thus requiring strategies to overcome inhibitor resistance (43).

In this study, we have expressed, purified, and biochemically characterized RET-KD and determined its structure in non-phosphorylated and phosphorylated forms. Both show the same interlobe orientation characteristic of active tyrosine kinases and have essentially identical A-loop conformations. We demonstrate that no major cis-inhibition mechanism operates within RET-ICD. We also describe the structural basis for inhibition of RET by PP1 and ZD6474 and how drug-resistant RET mutations ablate interaction with these inhibitors.

EXPERIMENTAL PROCEDURES

Recombinant Baculovirus Preparation and Protein Production—The cDNAs encoding RET-KD (residues 705–1013), RET-KD with its juxtamembrane domain (RET-JM-KD; residues 666–1013), and the entire RET-ICD (residues 666–1114) were PCR-amplified and subcloned into the HindIII and XhoI restriction sites of a modified pBacPak-His3 vector (Clontech). The modified vector contains a glutathione S-transferase (GST) coding region upstream of a 3C protease cleavage site. The RET-KD mutation Y905F was introduced by QuikChange mutagenesis (Stratagene) according to standard procedures. Generation of recombinant baculovirus was carried out according to the manufacturer’s protocols (Clontech). The viruses were used to infect Sf9 cells with a multiplicity of infection of 5; cells were grown in shaker flasks in Grace’s medium supplemented with 10% fetal bovine serum, 0.1% PLEURONIC F-68, and 10 μg/ml gentamycin. The cells were harvested 72 h post-infection and lysed by sonication. RET-KD was purified by incubation with glutathione-Sepharose 4B beads (Amersham Biosciences), followed by removal of the GST affinity tag using GST-linked 3C protease (PreScission protease, Amersham Biosciences). To produce phosphorylated RET-KD (RET-KD-P), soluble RET-KD was treated with 1 unit of biotinylated alkaline phosphatase.
Biophysical Characterization of RET-KD—Sucrose density gradient centrifugation of RET-KD was carried out on a 4–15% sucrose gradient in an SW40 rotor at 40,000 rpm for 16 h at 4 °C. RET-KD (0.5 mg/ml) was mixed with appropriate molecular mass standards and loaded onto the gradient solution. The gradient solutions were subsequently fractionated and analyzed by SDS-PAGE. Static light scattering analysis with concentrations of RET-KD between 0.5 and 1.0 mg/ml using a Zetasizer Nano (Malvern Instruments) was used to calculate a Debye plot, from which the molecular mass and second virial coefficient were derived according to the manufacturer’s instructions.

Kinetic Parameters for RET-KD—Initial rates of phosphorylation by RET-KD were determined using a continuous ADP-coupled kinase assay with the random polymer poly(E4Y) peptide as a substrate (44). The final assay mixture contained 100 mM Tris (pH 8.0), 10 mM MgSO4, 2 mM phosphoenolpyruvate, 1 mM NADH, 20 μg/ml pyruvate kinase/lactate dehydrogenase (Roche Applied Science), and 25 μg/ml RET-KD in Buffer A. No ATP turnover was detectable without the addition of the poly(E4Y) substrate, indicating that no RET-KD trans-phosphorylation or uncoupled ATPase activity occurred under these conditions. The \( K_m \) for the poly(E4Y) peptide was determined using peptide concentrations of 0–16 mg/ml and a fixed ATP concentration of 500 μM. The \( K_m \) for ATP was determined using ATP concentrations of 0–600 μM and 4 mg/ml poly(E4Y). The \( K_i \) for ZD6474 was determined by measuring the apparent \( K_m \) for ATP using the previous conditions in the presence of 300 nM ZD6474. The experiments were carried out in 96-well plates, and the NADH consumption was continuously read at 340 nm. Kinetic constants were determined by fitting data points to the Michaelis-Menten equation using the GraFit program (Erichth Software). Each assay was performed at least three times with independent RET-KD preparations.

Western Blot Analysis—RET-KD-0P and RET-KD-P proteins were each resolved by SDS-PAGE and electroblotted onto polyvinylidene difluoride membranes. The blocked membranes were probed with anti-phosphotyrosine antibodies, and the Western blot was finally developed with the Western Blot Detection System kit (Sigma). Proteins were concentrated in Buffer A (20 mM Tris-HCl (pH 8.0), 100 mM NaCl, and 1 mM dithiothreitol).

Data Collection and Structure Solution—Data were collected (see Table 1) and processed with the program MOSFLM (48). The RET-KD-P structure was solved by molecular replacement using the program MOLREP (49) as implemented in the CCP4 Program Suite (50) using the fibroblast growth factor receptor 2 structure (Protein Data Bank code 1GJO) as a search model. After positioning the molecule, RESOLVE (51) was used in prime-and-switch mode to reduce phase bias. The structure was refined using REFMAC5 (52) and rebuilt using O (53) and Coot (54). Ligand libraries were generated with PRODRG (55). RET-KD-0P and RET-KD-P complexes with PP1 and ZD6474 were solved and refined using a similar protocol, except that a partially refined model of RET-KD-P with the flexible loops deleted was used as the search model. Refinement statistics are given in Table 1, and coordinates have been deposited in the Protein Data Bank with codes 2IVS (RET-KD-0P), 2IVT (RET-KD-P), 2IVU (RET-KD-P-ZD6474), and 2IVV (RET-KD-P-PP1).

The RET-KD-P structures have continuous electron density from Gly700 (including five N-terminal vector-derived residues) to Ile33579 and from Pro715 to Arg1012, omitting the kinase insert domain residues 823–843, which are assumed to be disordered, and the molecules formed dimers related by a crystallographic 2-fold axis. RET-KD-0P has two molecules (related to as A and B) related by a non-crystallographic 2-fold axis in the asymmetric unit, resulting in an AB dimer with an overall arrangement that is essentially the same as that found in the RET-KD-P.
structure (root mean square deviation (r.m.s.d.) of 0.5 Å for molecule A or B with RET-KD-P). Molecules A and B each have the same relative orientation of the N- and C-lobes (r.m.s.d. of 0.39 Å for 251 C-α atoms). Differences between molecules A and B are that, in molecule A, the N-terminal helix (helix αN) is only very poorly defined and is not included in the model, whereas the main chain for the A-loop is continuous with the N) is present and clearly defined, but the A-loop residues 900–909 are disordered. Molecule A has been used in the comparison of phosphorylated RET. Two very clear formate ions are common to all the structures. One bridges the NH1 and NH2 atoms of Arg973 and the main chain nitrogen at residue 912, where it forms part of the surface of the ligand-binding pocket; and the other is at the interface between helix αN and the His926 side chain of an adjacent protomer. Structural figures were made with PyMOL (DeLano Scientific, South San Francisco, CA).

RESULTS

Characterization of Recombinant RET-KD—As produced in S9 cells, purified RET-KD is partially phosphorylated, and we therefore treated these preparations with alkaline phosphatase to produce RET-KD-0P (Fig. 1B). To generate RET-KD-P and to ensure full phosphorylation, we incubated purified RET-KD with Mg2⁺-ATP (Fig. 1B). Mass spectrometric analysis indicated that Tyr900 and Tyr905 were phosphorylated in vitro, as were Tyr752, Tyr726, Tyr928, and Tyr981, consistent with previous reports (14). Biophysical characterization of RET-KD by sucrose density gradient centrifugation and by gel filtration indicated an approximate molecular mass of 30 kDa, indicating that the protein was monomeric in solution (calculated molecular mass of 35.9 kDa); this was independently confirmed by static light scattering, where RET-KD had an apparent mass of 29 kDa.

We measured the enzyme kinetic constants for RET-KD-0P and RET-KD-P using a continuous coupled spectrophotometric assay to detect ADP generation (44). For these experiments, we used the synthetic peptide poly(E4Y) as substrate and found that it could be efficiently phosphorylated with good saturation kinetics. We found a reproducible 3–4-fold increase in kcat for the poly(E,Y) substrate between RET-KD-0P and RET-KD-P (Table 2); the Km value (and therefore, the affinity for poly(E,Y)) was approximately the same in both cases, resulting in a 3–4-fold increase in overall catalytic efficiency (kcat/Km) upon phosphorylation of RET-KD. This is a markedly smaller difference than the 10–200-fold changes in catalytic efficiency found upon phosphorylation in other tyrosine kinases, e.g. the increase for insulin receptor kinase (IRK) with peptide substrate is 32-fold (56); it is comparable with the kcat/Km increase due to phosphorylation of VEGFR2 kinase using the poly(E,Y) substrate (11-fold) (57), but without the parallel change in Km. To confirm that the small kinetic differences in RET-KD were due to phosphorylation-dependent events, we performed similar measurements using the Y905F RET-KD mutant and found no change in the kcat or Km values following phosphorylation.

<table>
<thead>
<tr>
<th>Data collection</th>
<th>Protein</th>
<th>RET-KD-P</th>
<th>RET-KD-P</th>
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<th>RET-KD-0P</th>
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<tr>
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<td>PP1</td>
<td>C2</td>
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<tr>
<td>Space group</td>
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<td>C2</td>
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<tr>
<td>Unit cell dimensions (Å)</td>
<td>a = 72.13, b = 70.85, c = 78.92; β = 101.13°</td>
<td>a = 72.13, b = 71.42, c = 78.83; β = 101.23°</td>
<td>a = 72.13, b = 70.85, c = 78.92; β = 101.79°</td>
<td>a = 50.40, b = 80.22, c = 79.68; β = 100.09°</td>
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<td>Resolution (Å)</td>
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<td>2.5 (2.64-2.5)</td>
<td>2.25 (2.37-2.25)</td>
<td>2.0 (2.07-2.0)</td>
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<tr>
<td>Beamline</td>
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<td>In-house</td>
<td>ESRF ID14-4</td>
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<tr>
<td>Wavelength (Å)</td>
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<td>1.5418</td>
<td>1.5418</td>
<td>0.939</td>
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<td>13,506</td>
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<td>41,522</td>
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<td>Average redundancy</td>
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<td>3.7 (3.7)</td>
<td>3.9 (3.9)</td>
<td>4.0 (2.0)</td>
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<td>Completeness</td>
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<td>Rmerge (%)</td>
<td>4.9 (13.4)</td>
<td>6.4 (16.5)</td>
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<td>I/σ(I) (%)</td>
<td>12.4 (5.1)</td>
<td>9.6 (4.6)</td>
<td>10.6 (3.9)</td>
<td>16 (10)</td>
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<td>Wilson B</td>
<td>21.5</td>
<td>39.0</td>
<td>32.6</td>
<td>20.7</td>
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</tbody>
</table>

TABLE 1
Data processing and refinement statistics

DL, Daresbury Laboratory; ESRF, European Synchrotron Radiation Facility; FMT, formate.

<table>
<thead>
<tr>
<th>Protein</th>
<th>RET-KD-P</th>
<th>RET-KD-P</th>
<th>RET-KD-P</th>
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<td>PP1, 21; FMT, 6;</td>
<td>C2, 2; -cAMP, 44; FMT, 6;</td>
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<td>HOH, 122</td>
<td>HOH, 262</td>
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<tr>
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<td>30, 31, 26</td>
<td>41, 36, 44</td>
<td>19 (Å)/23 (B), 23, 27</td>
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<td>Bonds (Å)</td>
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<td>92.2</td>
</tr>
<tr>
<td>Generously allowed</td>
<td>92.8</td>
<td>6.0</td>
<td>7.4</td>
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<tr>
<td>Disallowed</td>
<td>0.4</td>
<td>0.8</td>
<td>0.0</td>
<td>0.4</td>
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We concluded that Tyr905 is not required for RET catalytic activity in vitro and that only a small catalytic enhancement occurs upon RET-KD autophosphorylation.

To investigate whether an additional regulatory mechanism exists outside the kinase domain, we prepared two additional baculoviruses and recombinant proteins: RET-JM-KD (residues 666–1013), including the juxtamembrane domain, and RET-ICD (residues 666–1114), encompassing the entire intracellular portion of RET. Again, we assayed these proteins in the phosphorylated and non-phosphorylated states and found the same 3–4-fold increase in $k_{\text{cat}}$ and no significant lowering of the $K_m$ upon phosphorylation. Thus, the in vitro activity of soluble RET-ICD by only modestly enhanced by phosphorylation, and no cis-inhibitory mechanism was evident even within the entire soluble intracellular domain, unlike the kinases present in other receptors (58).

To attempt to distinguish between the roles of Tyr900 and Tyr905 in activation and substrate recognition, we prepared peptide arrays based on the RET A-loop. These synthetic RET substrates were attached to cellulose membranes by SPOT synthesis (59). The peptides SRDVY$_{900}$EEDSF$_{905}$VKRSQG (peptide 2) was efficiently phosphorylated only when residue 905 was Tyr or Val, but this effect could not be mimicked by an acidic residue.

We concluded that Tyr$^{905}$ is not required for RET catalytic activity in vitro and that only a small catalytic enhancement occurs upon RET-KD autophosphorylation.

To investigate whether an additional regulatory mechanism exists outside the kinase domain, we prepared two additional baculoviruses and recombinant proteins: RET-JM-KD (residues 666–1013), including the juxtamembrane domain, and RET-ICD (residues 666–1114), encompassing the entire intracellular portion of RET. Again, we assayed these proteins in the phosphorylated and non-phosphorylated states and found the same 3–4-fold increase in $k_{\text{cat}}$ and no significant lowering of the $K_m$ upon phosphorylation. Thus, the in vitro activity of soluble RET-ICD by only modestly enhanced by phosphorylation, and no cis-inhibitory mechanism was evident even within the entire soluble intracellular domain, unlike the kinases present in other receptors (58).
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Structure of RET-KD—RET-KD adopts a characteristic protein kinase fold consisting of a smaller N-lobe (residues 713–805) and a larger C-lobe (residues 812–1013) connected by a hinge/linker (residues 806–811). The relative orientation of the N- and C-lobes is essentially the same in the phosphorylated and non-phosphorylated RET forms, with an r.m.s.d. for 268 C-α atoms of 0.49 Å (Fig. 2A), despite their having adopted two different crystal lattices. Structural alignment using the Secondary Structure Matching server (www.ebi.ac.uk/msd-srv/ssm/) showed the closest similarity to active kinase forms, including fibroblast growth factor receptor 2 (Protein Data Bank code 1OEC; r.m.s.d. of 0.87 Å), Kit (Protein Data Bank code 1PKG; r.m.s.d. of 1.07 Å), ACK1 (Protein Data Bank code 1U46; r.m.s.d. of 0.81 Å), and IRK (Protein Data Bank code 1IR3; r.m.s.d. of 1.22 Å) (Fig. 2B). A notable feature of the RET-KD structure is the presence of residues 705–711 of the RET juxtamembrane domain. In RET-KD-P, these residues form part of an N-terminal helix (helix αN) that connects to the β1 strand of the kinase domain via loop 712–714, for which no electron density was observed. Helix αN packs against and tethers the functionally important helix αC through apolar contacts around Phe709 and Phe776. A similar N-terminal helix is found in the autoinhibited c-Met kinase structure (60).

In both RET-KD-0P and RET-KD-P structures, the A-loop is ordered. In the crystals of RET-KD-P, Tyr905 is clearly phosphorylated, and Tyr900 is not; in RET-KD-0P, Tyr905 is hydrogen-bonded to one water molecule, and the side chain of Tyr900 is disordered. Because mass spectrometric results showed that Tyr900 is phosphorylated, phospho-Tyr900 may be labile to water-mediated hydrolysis, which would be favored by the high solvent accessibility of Tyr900 and the absence of a binding pocket for this residue. A similar situation was observed in the VEGFR2 kinase domain structure, where Tyr1054, the structural equivalent of Tyr900, is phosphorylated in solution, but not in crystals (61). In contrast, phosho-Tyr905 is protected through interaction with a group of basic side chains; three residues, Arg770, Arg997, and Lys907, contact phospho-Tyr905, in contrast to the single tether found in other RTKs, e.g. Arg1155 in the activated IRK structure (which is structurally equivalent to Arg997 in RET). The position of the Arg897 side chain is the same in the RET-KD-P and RET-KD-0P
structures and is stabilized by interaction with the carbonyl oxygen at position 894, but in RET-KD-0P, the side chains of Arg775 and Lys907 are disordered. The observed $k_{cat}$ enhancement could be explained by phospho-Tyr905 organizing this cluster of side chains. The conserved salt bridge between Lys758 and Glu775 from helix αC, equivalent to that found in most activated RTKs, is formed in both structures, although the torsion angles within the two side chains are slightly different. The relative position of the N- and C-lobes is further stabilized by a salt bridge between Arg912 and Asp771, which is not generally present in RTKs. The global RET-KD conformation is thus independent of the phosphorylation state of the A-loop and suggests that the A-loop exhibits no major autoinhibitory effect on binding of either ATP or substrate, consistent with our kinetic data. RET-KD is basic (calculated pl 8.6), but the majority of the accessible acidic residues are clustered within the A-loop. Positive charge found in the vicinity of the substrate-binding site should favor the acidic A-loop as a substrate and may contribute to the efficiency with which RET phosphorylates poly(E,Y) peptides.

A Possible trans-Inhibited RET-KD Dimer—Although apparently monomeric in solution, as shown by sucrose density gradient ultracentrifugation and static light scattering, RET-KD adopts two independent crystal forms that each contain an identical head-to-tail dimer generated by either non-crystallographic (RET-KD-0P, P2₁) or crystallographic (RET-KD-P, C2) symmetry, with ~1090 Å² of buried surface/monomer (Fig. 2, C and D). We also observed the same dimer in the preliminary crystal structure of RET-JM-KD, which includes both the juxtamembrane and kinase domains, suggesting a strong preference for this particular arrangement. The first contact area within the dimer involves N762 ASP767, prior to helix C interacting with the P+3 pocket and specifically Met918. Residues 763–767 are in an extended conformation that masks the substrate-binding site of the second molecule in a manner reminiscent of both the peptide-bound form of triphosphorylated IRK (Protein Data Bank code 1IR3) and the interaction of the juxtamembrane domain of c-Kit in the product complex (Protein Data Bank code 1PKG) with a second molecule. In both triphosphorylated IRK and c-Kit, the main chain nitrogen and oxygen of the P+1 residue form hydrogen bonds with main chain atoms in the C-terminal segment of the A-loop (Fig. 2E). There are no directly equivalent hydrogen bonds in RET, although there are main chain contacts between O-764 and N-915 and between N-766 and O-911 and a central van der Waals contact from Pro760 of one protomer to Met918 within an apolar cleft formed by the P+1 loop of the other protomer (discussed below) (Fig. 2F). A second, adjacent contact area in the dimer involves helix αN interacting with the side chains of Phe794 and Phe851 and (via a formate ion) and with Gln910 and His926. This dimer structure represents a trans-inhibited state despite being formed by molecules in an active conformation.

Small Molecule Ligands—We have determined the structures of RET-KD-P with AMP, PP1, and ZD6474 in the ATP pocket and of RET-KD-0P with 2',3'-cAMP. AMP binds in a manner similar to ADP/ATP observed in other RTKs. Although we might have expected a product complex to contain ADP, there is no electron density for a β-phosphate, and there does not appear to be space to accommodate it.

Co-crystallization of RET-KD-P with ZD6474 and PP1 led to well ordered ligands in the ATP-binding site (Fig. 3, A and B). Compared with the AMP complex, the site accommodated the larger PP1 and ZD6474 by adjusting the nucleotide-binding loop and a bulging of the linker strand 804–808. This is most obvious when RET-KD-P-ZD6474 is compared with RET-KD-P-AMP; the α-carbon atoms of residue 734 in the two structures are 1.57 Å apart, and the side chain of Phe735 is not seen in the ZD6474 complex, whereas in the AMP complex, it packs against the aliphatic part of the Lys758 side chain. Small movements of residues 804–806 produced a different conformation for the side chain of Glu805, where it cannot form a hydrogen bond with Lys889. PP1 forms the two canonical nucleotide hydrogen bonds with the linker region (Fig. 3D), whereas the quinazoline moiety of ZD6474 can mimic only one, the equivalent of N1–807N in the AMP complex (Fig. 3C). Other contacts between RET-KD and the inhibitors are shown in Fig. 3 (C and D). The methylphenyl group of PP1 and the bromofluorophenyl group of ZD6474 each occupy the small hydrophobic cavity at the back of the ATP site, which has Val804 in the gatekeeper (62, 63) position (Fig. 3, E–G). In RET-KD-P-ZD6474, this cavity also encloses one water molecule that bridges the side chain of Glu775 and the main chain nitrogen atom of Asp892.

DISCUSSION

In the majority of previously characterized RTK-KD structures, A-loop phosphorylation stimulates kinase activity by causing structural changes that relieve cis-autoinhibition. The non-phosphorylated basal states of many RTKs have low catalytic activity because of a suboptimal A-loop conformation that interferes with either the ATP- or substrate-binding sites (reviewed, for example, by Schlessinger (64)). In contrast, we have found that the A-loop phosphorylation state of RET-KD made no substantial difference to the three-dimensional structure and only modestly affected the level of its catalytic activity. Other tyrosine kinases with active A-loop conformations in their non-phosphorylated states include EGFR kinase (65) and the non-receptor tyrosine kinase ACK1 (66). For EGFR, ligand-dependent allosteric changes are required for receptor activation (67). Similar to ACK1, phospho-Tyr905 of RET-KD stabilizes a surrounding constellation of basic residues, rather than the single one found in most tyrosine kinases. A mechanism similar to that proposed for ACK1, in which a favorable electrostatic environment promotes an increased rate of phosphoryl transfer, may explain the increased catalytic efficiency of RET-KD-P.

It is not yet clear whether the essential role of Tyr905 in constitutive (MEN2A and PTC) and transient (GDNF-dependent) RET dimers can be explained simply by the modest increase in catalytic rates of RET-KD. We have shown that, in vitro, Tyr905 and Tyr900 (or a phosphomimetic at position 900) are required for efficient phosphorylation of the RET-KD A-loop peptide, but it is possible that further functions of phospho-Tyr905 remain to be found, such as promoting an active RET dimer or
prolonging RET activation to allow other phosphotyrosine sites to engage with adaptor molecules. In contrast, highly transforming MEN2B mutants do not require Tyr905 phosphorylation, possibly because cell transformation can occur in the absence of detectable RET dimerization. However, M918T RET can be further activated by an additional MEN2A mutation (C634R) and stimulation by GDNF (68) or PTC fusion (69), suggesting that dimerization is important for maximal activation.

Our results show no cis-inhibitory mechanism within the
would both be expected to destabilize the structure. Mutations of essential residues, such as R873Q in the pink for E768D/A919P and
the red part of the RET dimer interface. It is conceivable that, with-

mutagenesis in a full-length RET context. This model is remi-

tation with ligand and co-receptor and subsequent Tyr905 auto-

phosphorylation. This suggests that RET kinase activity must
be regulated in a manner that is not apparent from studying
RET-KD in solution and does not involve the juxtamembrane
domain, in contrast to the autoinhibition of c-Kit (70). We

phosphorylation. Asp771 and Arg912 form a salt bridge that
links the N- and C-lobes, stabilizing the active conformation. E734K at the tip of the conformationally sensitive
nucleotide-binding loop mutation may perturb its interaction with Arg912, although this is not structurally
critical, as Arg912 is disordered in RET-KD-P. Both S765P and S767R, in positions preceding helix C, may affect
helix C orientation. Other mutations, including E762Q and R982C, are solvent-accessible and have no obvious
detrimental impact on the RET-KD structure. B, activating mutation sites in RET-KD identified in MTC, MEN2A,
and MEN2B. Carbon atoms of the side chains are colored magenta for M918T, the predominant MEN2B mutant;
pink for E768D/A919P and white for V804M/V806C, two paired mutations where there is synergy; and cyan for
L790F, Y791F, S891A, and R844L.

FIGURE 4. Mapping of disease-linked mutations on the RET kinase structure. The backbone schematic of
RET-KD is shown in green, with the link to the N-terminal helix and the kinase insert domain indicated by
dashed lines. The side chains of wild-type RET-KD are shown, and the bound nucleotide is indicated in stick form. A, inactivating mutation sites in RET-KD found in familial and/or sporadic Hirschsprung disease (18). Carbon
atoms of the side chains are colored orange to mark mutation sites. The effects of some of these mutations can be
rationalized from the structure, e.g. W942C in the conserved kinase core and F961L in a hydrophobic region
would both be expected to destabilize the structure. Mutations of essential residues, such as R873Q in the
kinase RD motif and F893L and G894S in the invariant DFG motif, will lower or abolish catalytic activity. Muta-
tions of basic side chains that coordinate phospho-Tyr905, including R897Q and K907E, will perturb the optimal
active kinase conformation and lower affinity for phosphorytrosine. Asp771 and Arg912 form a salt bridge that
links the N- and C-lobes, stabilizing the active conformation. E734K at the tip of the conformationally sensitive
nucleotide-binding loop mutation may perturb its interaction with Arg912, although this is not structurally
critical, as Arg912 is disordered in RET-KD-P. Both S765P and S767R, in positions preceding helix C, may affect
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L790F, Y791F, S891A, and R844L.

is isolated RET-ICD and only a low activation threshold, which
together could lead to uncontrolled intracellular phosphoryla-
tion events. However, in cells, RET signaling requires interac-
tion with ligand and co-receptor and subsequent Tyr905 auto-

phosphorylation. This suggests that RET kinase activity must
be regulated in a manner that is not apparent from studying
RET-KD in solution and does not involve the juxtamembrane
domain, in contrast to the autoinhibition of c-Kit (70). We
therefore considered whether RET might be trans-inhibited and
whether there is an allosteric component to RET activation.
The crystallographic RET dimer described here has mutually
occurred substrate-binding sites (Fig. 2D) and persists in both
RET-KD crystal forms and in RET-JM-KD; it may therefore
represent a biologically relevant trans-inhibited state of RET.
Several transforming mutations map to positions close to the
dimer contacts, including M918T, P766S, and E768D/A919P
(see below), and may destabilize this dimer. If this dimer were
to define an inhibited form of RET, a large conformational
change would be required to relieve the trans-inhibition, and it may be
driven by interaction with GDNF/GFRα1. RET/PTC dimers are
constitutively active and therefore must have accessible
substrate-binding pockets. These molecules lack the jux-
tamembrane domain including helix αN, which forms an integ-
ral part of the RET dimer interface. It is conceivable that, with-
out the juxtamembrane domain, RET/PTC cannot form the
trans-inhibited dimer, but is still capable of forming an active
RET dimer. Further investigation of the biological relevance of
the trans-inhibited dimer is merited, through directed
mutagenesis in a full-length RET context. This model is remi-
lated types of inhibitor (73). In BCR-ABL, the equivalent resi-
due (Thr315) forms a hydrogen bond with Gleevec and is small
enough to allow access to the pocket, but mutations of larger
side chains lead to Gleevec-resistant chronic myeloid leukemia
(74). Although Val804 of RET cannot form hydrogen bonds with
the inhibitors, the size of the side chain at this position controls
access to the pocket, explaining why Val804 mutants with the
bulker leucine or methionine side chains that are found in
MTC and MEN2 cases are not inhibited by ZD6474 (42, 75). As
with other kinases (73), mutation of Val804 to alanine or glycine
renders RET even more sensitive to inhibition by ZD6474 and
PP1 (42), possibly because of greater plasticity in the pocket;
however, such small side chains are not generally found as the
gatekeeper in wild-type kinases (63). The identity of the gate-
keeper shows why ZD6474 is able to selectively inhibit EGFR,
VEGFR2, and RET, but not IRK (41), in which the larger methi-
onine side chain occupies the gatekeeper position. Other resi-
dues in contact with ZD6474 (Fig. 3C) are conserved in
VEGFR2 and EGFR. These residues are also conserved within
fibroblast growth factor receptor 1, which is less potently inhib-
ited by ZD6474 (IC50 > 1 μM) (41). Here, variations in residues
further removed from the ligand may lower the nucleotide-
binding loop flexibility required to accommodate the inhibitor;
in particular, the equivalents to RET-KD Thr729 and Glu734 are
proline and cysteine in fibroblast growth factor receptor 1.

Familial and sporadic missense mutations in RET-KD resulting
in Hirschsprung disease are generally inactivating, and in struc-
tural terms, they fall roughly into three categories: those that
directly affect essential kinase residues, those that lead to charge

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reversals or loss of charged residues, and those that remove or introduce bulky hydrophobic residues (Fig. 4A). Several germ line and sporadic activating mutations reported for MTC and MEN2B phenotypes lie within RET-KD (Fig. 4B); they are harder to rationalize than the Hirschsprung disease mutations because they have multiple effects on RET function. The best studied example is the germ line mutation M918T found in 95% of MEN2B patients. As with the equivalent mutation in the RON (76) and Met (77) receptors, M918T has an unexpectedly large effect on RET function. It both abolges the requirement for A-loop phosphorylation and tors, M918T has an unexpectedly large effect on RET function. It

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\text{flexibility, which may favor maintenance of an active RET-KD conformation.}
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CONCLUSION

Crystals of both phosphorylated and non-phosphorylated forms of RET-KD contain molecules with the structural characteristics of an active tyrosine kinase. Enzyme kinetic parameters show only very modest catalytic enhancement resulting from phosphorylation, and we have concluded that RET does not employ cis-inhibition to control inappropriate activity. In crystals, RET-KD molecules associate as dimers with mutually occluded binding sites. Several transforming mutations map close to the dimer interface, suggesting that they destabilize this arrangement of RET-KD. This dimer persists in both phosphorylated and non-phosphorylated RET-KD and in the longer RET-JM-KD, suggesting that it may be a preferred association that defines a trans-inhibited RET dimer. Structures of RET-KD with the inhibitors PP1 and ZD6474 demonstrate the mode of binding and the basis for inhibitor resistance in some RET-KD mutants.

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