The Crystal Structure of Domain 1 of Receptor Protein-tyrosine Phosphatase μ*

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Receptor-like protein-tyrosine phosphatases (RPTPs) play important roles in regulating intracellular processes. We have been investigating the regulation and function of RPTPμ, a receptor-like PTP related to the Ig superfamily of cell adhesion molecules. Recently, the crystal structure of a dimer of the membrane proximal domain of RPTPα (RPTP1) was described (Bilwes, A. M., den Hertog, J., Hunter, T., and Noel, J. P. (1996) Nature 382, 555–559). Within this crystal structure, the catalytic site of each subunit of the dimer is sterically blocked by the insertion of the N-terminal helix-turn-helix segment of the dyad-related monomer. It was proposed that dimerization would lead to inhibition of catalytic activity and may provide a paradigm for the regulation of the RPTP family. We have determined the crystal structure, to 2.3 Å resolution, of RPTPμ D1, which shares 46% sequence identity with that of RPTPα D1. Although the tertiary structures of RPTPα D1 and RPTPμ D1 are very similar, with a root mean square deviation between equivalent Ca atoms of 1.1 Å, the quaternary structures of these two proteins are different. Neither the catalytic site nor the N-terminal helix-turn-helix segment of RPTPμ D1 participates in protein-protein interactions. The catalytic site of RPTPμ D1 is unhindered and adopts an open conformation similar to that of the cytosolic PTP, PTP1B (Barford, D., Flint, A. J., and Tonks, N. K. (1994) Science 263, 1397–1404). We propose that dimerization-induced modulation of RPTP activity may not be a general feature of this family of enzymes.

Numerous cellular events are regulated by reversible phosphorylation of tyrosine residues, including growth, differentiation, the cell cycle, cell-cell adhesion, and cell-matrix contacts (1). Phosphotyrosine levels are controlled by the coordinated and competing actions of protein-tyrosine kinases and protein-tyrosine phosphatases (PTPs). The PTPs comprise a diverse family of transmembrane receptor-like and nontransmembrane, cytosolic enzymes (1). Receptor-like PTPs are likely to play crucial roles in transducing transmembrane signals and evidence is accumulating to link these enzymes to the control of phenomena mediated by cell adhesion (1). By interacting with ligands through their extracellular domains, the intracellular catalytic activity of RPTPs may be regulated, hence controlling the levels of cellular phosphotyrosine. The receptor-like PTPs RPTPμ, RPTPκ, and RPTPλ, which have extracellular segments sharing structural similarities with Ig superfamly cell adhesion molecules, have been shown to mediate cell aggregation via homophilic interactions (2–4). For RPTPμ, the homophilic binding site(s) reside on the immunoglobulin domain, whereas the intracellular segment consists of a juxtamembrane, cadherin-like domain, and two PTP domains (5). A role for RPTPμ in regulating cell junctions and cytoskeletal organization is suggested by the finding that the RPTPμ is associated with cadherin-catenin complexes at adherens junctions (6). Similar results have been demonstrated for RPTPκ (7) and RPTPλ (4).

The crystal structure of PTP1B in a complex with a phosphotyrosyl peptide substrate (8), together with the results of a number of kinetic studies (9), have revealed important insights into the catalytic mechanism of members of the PTP family. The signature motif, (I/V)HCXAGXXR/S/TG, which defines this family of enzymes, contains the catalytically essential Cys and Arg residues. It forms a rigid cradle structure that coordinates the phosphate moiety of the substrate and positions the cysteine residue to act as a nucleophile. This motif lies at the base of a cleft on the surface of the enzyme, which is defined at one end by the invariant residue Tyr and at the other by Phe182, which is in a loop containing the invariant general acid Asp181 within the highly conserved motif WPD. It produces a profound conformational change in the enzyme such that on engagement of Tyr(P) the WPD loop flips down onto the substrate and the covalent intermediate by a water molecule, possibly activated by Asp181. This creates a predominantly hydrophobic recognition pocket that surrounds and interacts with the phenyl ring of the Tyr(P) side chain and allows Asp181 to act as a catalytic acid. Catalysis proceeds via nucleophilic attack by the invariant Cys (Cys215) on the substrate phosphorous atom and protonation of the tyrosyl-leaving group of the substrate by Asp181 resulting in formation of a cysteinyl-phosphate covalent intermediate. The reaction is then completed by hydrolysis of the covalent intermediate by a water molecule, possibly activated by Asp181 (10).

Recently, a model for the regulation of RPTP activity was proposed by Bilwes et al. (11) on the basis of their crystal structure of domain 1 of RPTPα. In contrast to the cytosolic

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1 The abbreviations used are: PTP, protein-tyrosine phosphatases; RPTP, receptor protein-tyrosine phosphatase; D1, domain 1 (membrane proximal domain) of RPTPs; DTT, dithiothreitol; NCS, noncrystallographic symmetry.
PTPs, PTP1B and Versinia PTP, which are monomeric (12, 13), the crystal structure of domain 1 (D1) of RPTPα revealed a homodimeric organization that would result in an inhibited PTP (8, 11). The dimer results from a structural segment at the N terminus of the RPTPα D1 catalytic domain, consisting of a turn connecting helices α1' and α2' that wedges into the dyad-related catalytic site. The insertion of residues of the helix-turn-helix segment of one subunit into the catalytic site of the dyad-related subunit would be predicted to inhibit catalytic activity. This occurs first, as a result of sterically blocking substrate access to the catalytic site, and second, by constraining the WD loop in an open conformation that is unable to adopt the closed conformation necessary for catalysis (8). Distinct features of tertiary structure of RPTPα D1 that are absent from PTP1B are thought to facilitate this dimeric interaction. These include first, a two-residue insertion in the loop connecting α1' and α2', that inserts into the dyad-related catalytic site, and second, a β-strand (βx), immediately N-terminal to α1' that forms a distinctive two-strand β-sheet with βy. The β-sheet between βx and βy, together with conserved residues within α1' and α2', contribute to defining the conformation of the helix-turn-helix segment of RPTPα D1 (11).

Interestingly, a sequence analysis of the PTP family suggested, on the basis of residue conservations, that the tertiary structural features of RPTPα D1 would be shared with D1s of other PTPs but not cytosolic PTPs, such as PTP1B, or D2 of RPTPs (11). The crystal structure of RPTPα D1 lead Bilves et al. (11) to propose a dimerization model for the regulation of RPTPα and, as a result of the sequence similarity with other RPTP D1s, it was suggested that this model could provide a paradigm for the regulation of RPTPs generally (11). The model proposes that ligand binding to the extracellular segment would modulate dimerization and hence the catalytic activity of the PTP domain.

We have been pursuing an investigation of the function of the Ig superfamily cell adhesion molecule related-phosphatase RPTPμ. At present little is known about the regulation of RPTPμ activity. Although it associates through its intracellular segment with the cadherin-catentin complex (6), the effects on PTP activity remain unclear. We have initiated a structural analysis of RPTPμ to provide further insights into the mechanisms for control of its enzymatic activity. In this regard, the possibility that the structure of RPTPα is indicative of a general mechanism for ligand-induced inhibition of RPTP activity is exciting. Here, we describe the crystal structure of domain 1 of human RPTPμ and show that although it is present in the crystal as a dimer, unlike RPTPα D1, the active site of RPTPμ is in an open, uninhibited conformation. Therefore our data indicate that regulation of PTP activity by dimerization is not a general feature of these enzymes.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification—Residues 874–1168 of human RPTPμ D1 were cloned into a modified version of the pET28a vector (Novagen Inc.) that incorporates an N-terminal hexa-His tag immediately preceding Gly874. The protein was overexpressed in Escherichia coli (strain B834) grown at 18 °C for 16 h. Protein purification was achieved with the following steps. Bacterial cells were lysed in 25 mM Tris-HCl (pH 8.0), 500 mM NaCl, 20 mM imidazole, 2 mM benzamidine, 0.01 mM phenylmethylsulfonyl fluoride, 1 mM µg/ml leupeptin, 1 µg/ml aprotinin, 1 µg/ml pepstatin, 1 µg/ml DNase, 5 µg/ml lysozyme and the cleared lysate loaded onto a nickel-nitriotriacetic acid-agarose column (Novagen Inc.) equilibrated in 50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 2 mM benzamidine, and 20 mM imidazole. RPTPμ D1 was eluted using 50 mM imidazole. Peak fractions were dialyzed against a buffer of 25 mM Tris-HCl (pH 8.0), 50 mM NaCl, 2 mM DTT, 2 mM EDTA, 2 mM benzamidine. Precipitated impurities were removed by centrifugation and

the filtered solution loaded onto a Mono Q column (Pharmacia Biotech Inc.) equilibrated in dialysis buffer. RPTPμ D1 was collected in the flow-through, brought to 1.0 M ammonium sulfate, and applied to a phenyl TSK column (ToyoSIL Corp.) equilibrated in 1.0 M ammonium sulfate, 25 mM Tris-HCl (pH 8.0), 50 mM NaCl, 2 mM EDTA, 2 mM DTT, 2 mM benzamidine. The enzyme was eluted by applying a linear gradient of 300 ml to a buffer of 25 mM Tris-HCl (pH 8.0), 50 mM NaCl, 2 mM EDTA, 2 mM DTT, 2 mM benzamidine. Peak fractions were loaded onto a Superdex 75 gel filtration column (Pharmacia Biotech Inc.). The protein, at greater that 99% purity, was concentrated to 7.5 mg/ml and crystallized at 4 °C in conditions of 750 mM sodium citrate (pH 5.5), 2 mM DTT, 0.5 mM EDTA.

Size Exclusion Chromatography—100 µl of RPTPμ D1 at 7.5 mg/ml was loaded onto a Superdex 75 prep gel filtration column (Pharmacia Biotech Inc.) equilibrated with 50 mM Tris-HCl (pH 7.5), 200 mM NaCl and run at a flow rate of 1 ml/min.

Structure Determination—X-ray crystallographic data were collected to 2.3-Å resolution and processed using DENZO and SCALEPACK (14). The structure was determined by molecular replacement using the CCP4 integrated version of AmoRe (15, 16) using a polyanaline model of RPTPα (11), including side chains of residues identical with those of RPTPα, as a search model. Crystallographic refinement was performed using X-PLOR (17), applying strict noncrystallographic symmetry (NCS) restraints. Calculated phases using the model were improved by several cycles of combined solvent flattening (solvent content 0.50) and NCS averaging using the PHASES package (18). Iterative rounds of simulated annealing refinement, combined with NCS averaging and solvent flattening, were applied to improve the quality of the maps and the protein model. During the final stages of refinement, the NCS restraints were removed. Analysis of the coordinates and electron density maps was performed with O (19). The final model consists of residues 879–1156 of RPTPμ D1 that are visible within the electron density and 340 water molecules (Table I). The structure of RPTPα D1 consists of residues 877–1156 (RPTPα numbering (20)).

RESULTS AND DISCUSSION

In the crystal structure of RPTPμ D1, the subunits associate to form a dimer with 2-fold symmetry (Fig. 1). It is most likely that the dimerization of RPTPμ D1 is a consequence of crystallization, since size exclusion chromatography studies revealed that the protein was monomeric with an apparent molecular mass of ~28 kDa in concentrated solutions of ~7.5 mg/ml (Fig. 2). This is in contrast to RPTPα D1, which forms monomers, dimers, and higher oligomers in solution at protein concentrations of 0.1–5.0 mg/ml (11). The oligomeric state of RPTPμ D1 is entirely different from that of RPTPα D1, since the dimer interface of RPTPμ D1 does not include the N-terminal helix-turn-helix segment or the catalytic site (Fig. 1). Crystal packing analysis also shows no obstruction of the catalytic site of RPTPμ D1. The interactions between the two

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2 D. Barford and N. Hanlon, unpublished data.
dimeric subunits of RPTP\(\alpha\) are hydrophobic in nature and mediated by residues Thr\(^{1025}\) and Ile\(^{1027}\) from one subunit and residue Ile\(^{1050}\) and the aliphatic moiety of Glu\(^{1052}\) from the other. These residues are not conserved within other RPTPs (Fig. 3). Within RPTP\(\mu\) D1, the catalytic site is unhindered and adopts an open conformation similar to that of the apo form of PTP1B (12). Residues of RPTP\(\mu\), equivalent to those of the RPTP\(\alpha\) helix-turn-helix that inserts into the dyad related catalytic site, are exposed to solvent (Fig. 1).

In contrast to the differences in quaternary structure between RPTP\(\alpha\) and RPTP\(\mu\), the tertiary structures of domain 1 of these two RPTPs are very similar with a root mean square deviation between equivalent Ca atoms of 1.1 Å after superimposition (Fig. 4). The major differences in tertiary structure are present within more mobile surface loops. Of particular note, the tertiary arrangements of the N-terminal helix-turn-helix segment and N-terminal \(\beta\)-strand, \(\beta\)\(x\), and associated \(\beta\)-sheet with \(\beta\)\(y\), of RPTP\(\mu\) are essentially identical to that of the equivalent structural elements of RPTP\(\alpha\). These features distinguish RPTP D1s from the catalytic domains and N-terminal flanking sequences of cytosolic PTPs and are important in promoting the dimeric organization of RPTP\(\alpha\) D1. Interestingly, Asp\(^{227}\) and Asp\(^{228}\) of RPTP\(\alpha\) D1, which are present within the helix-turn-helix segment that inserts into the dyad-related catalytic site, superimpose less well with equivalent residues of RPTP\(\mu\) D1 (Ala\(^{895}\) and Glu\(^{896}\), respectively). The Ca atoms of these residues differ by 1.8 Å after global superimposition (Fig. 4), although this region of the polypeptide chain is well ordered.

Among RPTPs, conservation of amino acids within the N-terminal helix-turn-helix that contribute to the dimer interface of RPTP\(\alpha\) D1 is low (Fig. 3). Of the five residues implicated in this dimeric interface, only Glu\(^{234}\) is conserved between RPTP\(\mu\) D1 and RPTP\(\alpha\) D1. Residues of RPTP\(\mu\) that form interactions at their respective dimer interfaces are indicated with vertical arrows (top) and stars (bottom), respectively. The residues that form the dimer interface of RPTP\(\mu\) are poorly conserved throughout the family, whereas residues of the RPTP\(\alpha\) D1 interface are poorly conserved within the helix-turn-helix segment, but well conserved within the catalytic site. Invariant residues are in white type on a black background, and highly conserved residues are boxed.
D1 and RPTPα D1. However, catalytic site residues that form the dimer interface of RPTPα D1 are either invariant or highly conserved between different RPTPs (Fig. 3). Thus, it is unlikely that invariant catalytic site residues would have the potential to interact with all of the structurally diverse N-terminal helix-turn-helix segments within the RPTP family. Moreover, if this were possible, it would imply that RPTP heterodimers may form as the catalytic sites of any given RPTP would not distinguish between the helix-turn-helix segments of other RPTPs.

The structure of D1 of RPTPs determined by Bilwes et al. (11) indicated the existence of a dimer in which a helix-turn-helix segment on the N-terminal side of one catalytic domain occupied the catalytic site of the opposing PTP domain in the dimer. This observation led the authors to propose the exciting possibility that ligand-induced dimerization of RPTPs in general may lead to inhibition of activity. They used this structural model to interpret previous observations regarding the signaling properties of an EGF receptor-CD45 chimera (21). Ablation of expression of CD45, the prototypic RPTP, from T or B cells disrupts normal signaling responses to engagement of antigen receptor. However, in some T cell lines, signaling can be restored by expression of membrane-targeted constructs containing the catalytic domain of CD45. In the instance of the chimeric molecule in which the extracellular and transmembrane segments of CD45 were replaced with those of the EGF receptor, signaling was restored only in the absence of EGFR. It was proposed that in the presence of growth factor, dimerization of RPTPs may lead to inhibition of activity. They used this structural possibility that ligand-induced dimerization of RPTPs in general may lead to inhibition of activity. However, the fact we do not observe dimers of RPTP D1 in solution, unlike that for RPTPα D1, and that within the RPTP D1 crystal, which would mimic the restricted diffusion of RPTP in the plasma membrane, inhibition of catalytic sites is not observed, suggests that the N-terminal helix-turn-helix of RPTP D1 does not play a role in regulating the activity of this phosphatase.

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