PROTEIN STRUCTURE REPORT

The crystal structure of human receptor protein tyrosine phosphatase κ phosphatase domain 1

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Abstract

The receptor-type protein tyrosine phosphatases (RPTPs) are integral membrane proteins composed of extracellular adhesion molecule-like domains, a single transmembrane domain, and a cytoplasmic domain. The cytoplasmic domain consists of tandem PTP domains, of which the D1 domain is enzymatically active. RPTP κ is a member of the R2A/IIB subfamily of RPTPs along with RPTP μ , RPTP ρ , and RPTP λ . Here, we have determined the crystal structure of catalytically active, monomeric D1 domain of RPTP κ at 1.9 Å. Structural comparison with other PTP family members indicates an overall classical PTP architecture of twisted mixed β -sheets flanked by α -helices, in which the catalytically important WPD loop is in an unhindered open conformation. Though the residues forming the dimeric interface in the RPTP μ structure are all conserved, they are not involved in the protein–protein interaction in RPTP κ . The N-terminal β -strand, formed by β _x association with β _y, is conserved only in RPTPs but not in cytosolic PTPs, and this feature is conserved in the RPTP κ structure forming a β -strand. Analytical ultracentrifugation studies show that the presence of reducing agents and higher ionic strength are necessary to maintain RPTP κ as a monomer. In this family the crystal structure of catalytically active RPTP μ D1 was solved as a dimer, but the dimerization was proposed to be a consequence of crystallization since the protein was monomeric in solution. In agreement, we show that RPTP κ is monomeric in solution and crystal structure.

Keywords: crystal structure; protein tyrosine phosphatase κ ; RPTP κ ; catalytic phosphatase D1 domain

Reversible phosphorylation of tyrosine residues is a key regulatory mechanism in numerous eukaryotic cellular events such as proliferation, differentiation, gene expression, and migration (Neel and Tonks 1997). The 107 human PTPs (protein tyrosine phosphatases) identified are classified into the phospho-tyrosine-specific, “classical” PTPs, and the dual-specificity PTPs that dephosphorylate phospho-tyrosine, -threonine, and -serine residues. The 38 classical PTP family

members include receptor-like transmembrane forms and nontransmembrane cytosolic forms (Andersen et al. 2001; Alonso et al. 2004). These PTPs are divided into two major subfamilies: 17 nonreceptor (or cytoplasmic) PTPs and 21 receptor-like (transmembrane) PTPs. Transmembrane receptor-like PTPs contain a variable sized extracellular region, a single transmembrane segment, and an intracellular region containing either one membrane proximal PTP domain (D1), or a D1 domain and a second membrane distal PTP domain (D2). The first, membrane proximal, phosphatase domain, D1, harbors most, or in some cases, all of the catalytic activity, whereas D2 is highly conserved with little or no activity (Neel and Tonks 1997).

Receptor-like PTPs play an essential role in transducing transmembrane signals, and some of these are suggested to

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be involved to the control of phenomena mediated by cell adhesion (Neel and Tonks 1997; Schnekenburger et al. 2005). RPTP κ belongs together with RPTP μ , RPTP ρ , and RPTP λ to the R2A/IIb subfamily of receptor protein tyrosine phosphatases (McAndrew et al. 1998; Andersen et al. 2001) (Fig. 1A). RPTP κ possesses an extracellular region, a single transmembrane region, and two intracellular tandem catalytic domains (Jiang et al. 1993). The extracellular domain of the RPTP κ precursor protein contains an immunoglobulin-like domain and four fibronectin type III-like repeats, preceded by a signal peptide and a region of ~ 150 amino acids with similarity to the *Xenopus* A5 antigen, a putative neuronal recognition molecule (McAndrew et al. 1998). The purified extracellular domain of RPTP κ functions as a substrate

for adhesion by cells expressing RPTP κ and induces aggregation of coated synthetic beads (Drosopoulos et al. 1999).

RPTP κ is widely expressed in the spleen, prostate and ovary, brain, lung, skeletal muscle, heart, placenta, liver, kidney, and intestine (Yang et al. 1997; Shen et al. 1999), and the expression is induced by TGF- β and by high cell density (Yang et al. 1996). RPTP κ has been shown to stimulate cell motility and neurite outgrowth, and is required for both the anti-proliferative and the pro-migratory effects of TGF- β , suggesting a role in regulation, maintenance, and restitution of cell adhesions (Wang et al. 2005). These functions might be regulated through dephosphorylation of β - and γ -catenin at adherens junctions (Fuchs et al. 1996).

Figure 1. (A) Structure-based sequence alignment of RPTP κ , RPTP λ , RPTP ρ , and RPTP μ (PDB codes: RPTP μ , 1RPM; RPTP κ , 2C7S). Secondary structure elements were determined using the program ICM (Molsoft) using the nomenclature in (Barford et al. 1994). (Red) α -Helices; (green) β -strands; (magenta) 3_{10} -helices. The asterisks indicate residues involved in dimerization in the R2A/IIb subfamily of receptor protein tyrosine phosphatases. (B) Sedimentation coefficient distribution for 1.2 mg/mL RPTP κ in 10 mM HEPES, 25 mM NaCl (pH 7.5) (red), and 50 mM HEPES, 150 mM NaCl, 5 mM DTT (blue). (C) Molecular weight distribution for 1.2 mg/mL RPTP κ in 10 mM HEPES, 25 mM NaCl (pH 7.5) (red), and 50 mM HEPES, 150 mM NaCl, 5 mM DTT (blue).

Expression of RPTP κ is absent or down-regulated in >20% of melanoma cell lines and in some unmanipulated melanoma biopsies (Novellino et al. 2003). Furthermore, the human RPTP κ gene lies in a region frequently deleted in hematological neoplasms, melanomas, ovary carcinomas, and many other solid tumors (Nakamura et al. 2003). RPTP κ plays a dual role as tumor suppressor and tumor promoter in mammary epithelial cells (Wang et al. 2005). RNA interference (RNAi) experiments showed that down-regulation of RPTP κ results in acceleration of cell cycle progression, enhancement of the cellular response to epidermal growth factor (EGF), and abrogates TGF- β -mediated anti-mitogenesis, whereas overexpression of RPTP κ results in inhibition of basal and EGF-induced proliferation and ERbB receptor signaling in cancer cells. Therefore, RPTP κ is a key regulator of EGFR tyrosine phosphorylation and function in human keratinocytes (Yang et al. 1996; Xu et al. 2005). Here, we report the crystal structure of the catalytic D1 domain of human RPTP κ at high resolution along with the self-association analysis using analytical ultracentrifugation (AUC).

Results and Discussion

It was suggested that RPTP activity was regulated by dimerization, and the first evidence for dimerization as a regulatory mechanism of RPTP activity came from studies with a chimeric protein consisting of the extracellular domain of the epidermal growth factor receptor (EGFR, a prototypical RPTK) and the intracellular domain of the RPTP CD45 (Desai et al. 1993). The crystal structure of the N-terminal membrane-proximal PTP domain of RPTP α (RPTP α -D1) provided structural support for dimerization-induced inhibition of RPTP activity (Bilwes et al. 1996). Nonetheless, CD45 structure shows that the cytoplasmic region of CD45 does not dimerize, and the dimeric interaction as observed for RPTP α D1 would be impossible given the D1–D2 intramolecular domain orientation (Nam et al. 2005).

Among the R2A/IIb subfamily of receptor protein tyrosine phosphatases the crystal structure of RPTP μ has been solved (Hoffmann et al. 1997). In the crystal structure of RPTP μ D1, the subunits associate to form a dimer with twofold symmetry. The dimerization was proposed to be a consequence of crystallization since the protein was monomeric in solution. The dimer also showed no obstruction to the catalytic site. However the crystal structure of RPTP α was shown to be a crystallographic dimer in which the active site of one domain is occluded by a helix-turn-helix wedge motif of its dyad-related partner (Bilwes et al. 1996). This occurs first, as a result of sterically blocking substrate access to the catalytic site, and second, by constraining the WPD loops in an open conformation that is unable to adopt the closed conformation necessary for catalysis. However, biochemical evidences suggest that the transmembrane region is

essential for dimerization in vivo (Jiang et al. 2000); therefore, it has been proposed that the D1 domain wedge motif may only serve to stabilize the RPTP α dimeric state and occlude the related D1's active site, whereas the transmembrane region most likely provides the energetic driving force for dimerization.

RPTP κ D1 domain is a monomer

The receptor PTP κ D1 protein was purified and found to be active (data not shown). The homogeneity of the purified sample was analyzed using analytical ultracentrifugation using various buffers (Fig. 1B,C). When the buffer 10 mM HEPES (pH 7.5), 25 mM NaCl without DTT was used, two main species were observed in almost equal amounts, giving $s_{20,w}$ values of 3.09S and 4.81S (Fig. 1B). Upon conversion to a molecular weight distribution in SEDIT, the two peaks gave molecular weights of 33 kDa and 65 kDa (Fig. 1C). Therefore the RPTP κ protein is present as a monomer and dimer and smaller amounts of higher species. In contrast, in buffer 50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM DTT, RPTP κ protein is 97% monomer, giving an $s_{20,w}$ value of 3.14S. Therefore it is clear that the presence of reducing agents and higher ionic strength prevent the self-association behavior of RPTP κ .

Quality of the model

We solved the monomeric structure of the receptor RPTP κ D1 domain, and it has been refined to 1.9 Å, low *R*-factor values, and satisfactory geometry (Table 1; Fig. 2). The structure of RPTP κ determined here contains residues Met865 to Phe1156 of the catalytic D1 domain. The entire structure was very well defined in the electron density. The

Table 1. Crystallographic data and refinement statistics

	RPTP κ
Data collection	
Space group	P4 ₃ 2 ₁ 2
Cell dimensions (Å)	<i>a,b</i> = 91.373, <i>c</i> = 108.415
Resolution (Å)	1.9
Total observations (unique, red.)	208,173 (32,866, 6.1)
Completeness (outer shell)	96.3 (87.7)
<i>R</i> _{merge} (outer shell)	0.079 (0.2871)
<i>I</i> / σ (outer shell)	11.14 (4.19)
Refinement	
<i>R</i> _{work} (<i>R</i> _{free}) (%)	19.97 (21.86)
Protein atoms (water)	2307 (137)
Hetero groups:	acetate
RMSD bond length (Å)	0.011
RMSD bond angle (°)	1.202
Ramachandran	
Allowed, disallowed (%)	100, 0

Figure 2. Three-dimensional structure of RPTP κ . (Red) α -Helices; (green) β -strands; (magenta) 3_{10} -helices. The helix-turn-helix segment ($\alpha 1'$, $\alpha 2'$) and the β -strand formed by association of βx and βy are labeled. The corresponding conserved residues involved in dimerization of RPTP μ are shown in ball-and-stick representation.

asymmetric unit contains one protein molecule as well as an acetate moiety bound to the active-site cysteine mimicking binding of a phosphate moiety. The crystal packing showed no obstruction to the catalytically important WPD loop, which is distinct to the RPTP α .

Overall structure and active-site properties

The overall structure of RPTP κ resembles closely the structure of RPTP μ , which has 79.9% sequence identity and 1.05 Å RMSD. The main secondary structure elements were found to be conserved in both structures, containing overall classical PTP architecture of twisted mixed β -sheets flanked by α -helices. While the catalytically important WPD (Trp¹⁰⁴⁹–Ala¹⁰⁵⁸) loop conformation is similar to RPTP μ in an unhindered open conformation, it is more open than the cytosolic PTP1B (PDB). As in all tyrosine phosphatases the active-site cysteine (Cys1083) is located in the well-conserved phosphate binding loop, and an acetate molecule bound to it might mimic binding of a phosphate moiety. In PTP1B residues Met258 and Gly259 form an open cleft, allowing direct access of substrates to the active site, whereas in RPTP α , bulkier residues are found in this position causing steric hindrance, and they are key determinants of substrate selectivity (Peters et al. 2000; Iversen et al. 2001). In RPTP κ , Ile1123 and Asn1124 are present in the corresponding positions, and Asn1124 seems to be likely to accommodate the phosphopeptides with a small amino acid in the pY+1 position.

Secondary structure comparisons with other PTPs

In RPTP μ , though the crystal packing shows no obstruction of the catalytic sites, the interaction between the two dimeric subunits are hydrophobic and mediated by Thr1025 and Ile1027 from one monomer and Ile1050 and Glu 1052 from another (Hoffmann et al. 1997). It is interesting to note that the corresponding conserved residues Thr1025, Ile 1027, Ile 1050, and Glu 1052 in RPTP κ are not involved in the protein–protein interaction, but they are solvent-exposed in the structure. Similar to other PTPs, RPTP κ also lacks the second tyrosine pocket seen in PTP1B formed by Arg24, Arg254, and Glu262. The helix-turn-helix wedge, which is involved in dimerization in RPTP α , is not well conserved among the RPTPs but the $\alpha 1$ and $\alpha 2$ helices are conserved between the RPTP μ and RPTP κ except the turn (Fig. 1), which forms a small distortion in the helix $\alpha 2'$. The N-terminal β -strand, formed by βx association with βy , is conserved between RPTP μ , RPTP κ , and RPTP α , and most of the RPTPs. This feature was suggested to distinguish RPTPD1s from the cytosolic PTPs, and this is conserved in the RPTP κ structure forming a β -strand at the N-terminal (Fig. 1).

Conclusion

The structure of RPTP α showed the dimeric form of RPTP, which led investigators to propose an interesting suggestion that the regulation of the RPTP could be occurring through ligand-induced dimerization (Jiang et al. 1999). However, the crystal structure of RPTP μ dimeric conformation showed that neither the catalytic site nor the N-terminal helix-turn-helix is involved in protein–protein interactions similar to RPTP α . Combining the RPTP μ structural data along with the biochemical evidence, Hoffmann et al. (1997) suggested that the regulation through dimerization could be specific to the RPTP α subfamily and their close homologs but not a general mechanism for all RPTPs. Our results clearly show that the difference in buffer condition can induce multimeric forms of phosphatase D1 domain in solution. The RPTP κ monomer D1 domain is the first monomeric crystal structure in this family that is similar to RPTP μ , and the regulation through dimerization proposed for RPTP α is not observed in RPTP κ . However crystal structures represent only a particular conformation of a molecule, and therefore the regulatory mechanism of RPTP κ in cells remains to be studied.

Materials and methods

Cloning

A sequence encompassing the catalytic domain of RPTP κ (residues Met865–Phe1156 of GenBank entry gi|18860902)

was amplified by PCR and subcloned into a pET-21a-derived vector. The vector includes a TEV-cleavable (*) N-terminal 6xHis tag (MHHHHHSSGVDLGATENLYFQ*SM).

Expression and purification

Escherichia coli BL21(DE3) cells transformed with the expression constructs were grown at 37°C in Luria-Bertani medium containing 100 µg/mL kanamycin until the OD₆₀₀ reached 0.3 and then transferred to 18°C. Protein expression was induced at an OD₆₀₀ of 0.8 using 1 mM isopropyl-thio-β-D-galactopyranoside. Cells were harvested after 3 h by centrifugation at 4000g for 10 min and then lysed in 50 mM HEPES (pH 7.5), 500 mM NaCl, 1 mM PMSF, and 0.5 mM TCEP using an EmulsiFlex high-pressure homogenizer, and the cell extract was centrifuged at 60,000g for 30 min at 4°C. The supernatant was loaded onto 5 mL of the Nickel-sepharose affinity resin and washed with 10 volumes of loading buffer (50 mM HEPES at pH 7.5, 500 mM NaCl, 5 mM imidazole, 0.5 mM TCEP, 5% glycerol) and 10 volumes of wash buffer (50 mM HEPES at pH 7.5, 500 mM NaCl, 20 mM imidazole, 0.5 mM TCEP, 5% glycerol), then eluted with elution buffer (50 mM HEPES at pH 7.5, 500 mM NaCl, 250 mM imidazole, 0.5 mM TCEP, 5% glycerol) at 0.8 mL/min. The eluted protein was further purified by gel filtration S75 column equilibrated in 10 mM HEPES (pH 7.5), 25mM NaCl, 5 mM DTT. The purified proteins were homogeneous, as assessed by SDS-PAGE and electrospray mass spectrometry, which also confirmed the predicted mass of the proteins. Protein was concentrated to 7–10 mg/mL using a 10-kDa cutoff concentrator (Vivascience).

Analytical ultracentrifugation (AUC)

Sedimentation velocity was carried out on RPTP κ using a Beckman Coulter Optima XLI analytical ultracentrifuge. Two different concentrations of RPTP κ were run, 1.2 mg/mL and 2.8 mg/mL, with the 1.2 mg/mL sample being run in two different buffers: (1) 10 mM HEPES (pH 7.5), 25 mM NaCl, and (2) 50 mM HEPES (pH 7.5), and 150 mM NaCl, 5 mM DTT. Samples were run at 50,000 rpm at a temperature of 10°C, and scans were taken using absorbance optics at 2-min intervals, with a detection wavelength of 297 nm. Data were analyzed using the c(s) model of SEDFIT (Schuck 2000). Subsequent sedimentation coefficient values obtained from these peaks were converted to $s_{20,w}$ values (corrected to conditions of standard temperature and buffer) in SEDNTERP.

Crystallization, data collection, structure solution, and refinement

Crystals were obtained using the vapor diffusion method and a protein concentration of 10 mg/mL by mixing 100 nL of the concentrated protein with 100 nL of a well solution containing 0.20 M NaNO₃, 20.0% PEG 3350, 10.0% ethylene glycol. The crystal belongs to the space group P4₃2₁2 with unit cell dimensions $a, b = 91.37$ Å and $c = 108.45$ containing one molecule in the asymmetric unit. The RPTP κ data set was collected at the beamline X10 at SLS to a resolution of 1.9 Å. Data collection statistics and cell parameters are listed in Table 1. The structures were solved with molecular replacement using Phaser with the human protein phosphates RPTP μ (PDB ID 1RPM) as a search model. Iterative rounds of restrained refinement with TLS against maximum likelihood targets using

Refmac5 were interspersed by manual rebuilding of the model using Coot. The structure was deposited in the Protein Data Bank (PDB) with accession number 2C7S.

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