The crystal structure of a human PP2A phosphatase activator reveals a novel fold and highly conserved cleft implicated in protein-protein interactions

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Running title: The novel fold of PTPA

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Protein phosphatase 2A (PP2A) is a heterotrimeric Ser/Thr phosphatase that is involved in regulating a plethora of signaling pathways in the cell, making its regulation a critical part of the cell’s well-being. For example, three of the non-catalytic PP2A subunits have been linked to carcinogenic events. Therefore, the molecular basis for the complicated protein-protein interaction pattern of PP2A and its regulators is of special interest. The PP2A phosphatase activator (PTPA) protein is highly conserved from humans to yeast. It is an activator of PP2A and has been shown to be essential for a fully functional PP2A but its mechanism of activation is still not well defined. We have solved the crystal structure of human PTPA to 1.6 Å. It reveals a two domain protein with a novel fold comprised of 13 α-helices. We have identified a highly conserved cleft as a potential region for interaction with peptide segments of other proteins. Binding studies with ATP and its analogs are not consistent with ATP being a cofactor/substrate for PTPA as had previously been proposed. The structure of PTPA can serve as a basis for structure-function studies directed at elucidating its mechanism as an activator of PP2A.

The heterotrimeric protein phosphatase 2A (PP2A) is a major serine/threonine phosphatase constituted of a highly conserved 36 kDa catalytic subunit (PP2Ac), a regulatory subunit (PP2Ab) and a 65 kDa structural subunit (PP2Aa) that serves as a scaffold bringing the other two subunits together. The core enzyme is a dimer of PP2Ac and PP2Aa that forms a complex with one of several PP2Ab subunits, each belonging to one of three distinct structural families. PP2A is involved in the regulation of numerous processes in the cell such as DNA replication, translation and transcription as well as RNA splicing, cell cycle progression and development. It is highly regulated through the holoenzyme composition, post-translational covalent modifications and through binding to several cellular and viral proteins (for reviews, see [1-7]). PP2A is believed to be involved in cancer development. The β form of the PP2Aa is mutated in 15% of colon and lung tumors [8] and specific PP2Abs have recently been suggested to play a part in cellular transformations [9, 10].

PTPA is a PP2A interacting protein once known as phosphotyrosyl phosphatase activator (PTPA) but now as protein phosphatase two A phosphatase activator. The reactivation of PP2Ac by PTPA in vitro was at first only identified as an increase in phosphotyrosyl phosphatase activity [11, 12] when in fact it appears to activate the phosphoserine/threonine specific activity of PP2Ac from a poorly active and substrate unspecific metal dependent state [13, 14]. PTPA is an essential protein as revealed by its high evolutionary conservation as well as the lethality of the deletion of the two PTPA homologs in yeast, Rrd1 and Rrd2, in certain nutritional backgrounds [15]. Rrd1/2 also interact with other less abundant type 2A phosphatases [16, 17]. Interestingly, the effect of deleting Rrd1/2 is more severe than deletion of the yeast PP2Ac, most likely due to the fact that the different type 2A phosphatases in yeast can
partly take over each others roles [18]. However, they all interact with the PTPA subunits [16], an interaction that appears to be critical for the 2A phosphatase activity in the cell.

The exact role of PTPA in cells remains unclear although several proposals have been put forward. It has, for example, been suggested to be involved in the introduction metals to the active site of PP2Ac, potentially serving as a metal chaperone [14]. PTPA was just recently suggested to have peptidyl prolyl \textit{cis/trans} isomerase (PPIase) activity which acts specifically on Pro190 in human PP2Ac [19]. This isomerase activity, seen \textit{in vitro}, could potentially control the folding of PP2Ac and thereby its activity. Rd1 and Rd2, have been suggested to act directly as regulatory subunits, as a part of a novel heterotrimeric complex with PP2Ac, providing a complex with altered substrate specificity [17]. These suggestions do not necessarily contradict each other, and the protein could, for example, be a third partner of a novel PP2A complex and at the same time catalyse the prolyl isomerization activity, bringing about a conformational change that makes metal binding more feasible. Nevertheless, the time is ripe for a conclusive assignment of PTPA’s physiological function and the structure of the protein presented in the present study will help in defining appropriate experimental strategies directed towards this goal as well as providing a structural framework for understanding the function of this important protein.

MATERIALS AND METHODS

\textbf{Materials} - Tripeptide (VPH) and pentapeptide (EVPHE) were synthesized by Elim Biopharmaceuticals, Inc, USA. Peptide LNEVPHEGPMCAL was synthesized by Bachem, Germany. All other chemicals were commercially available.

\textbf{Expression and purification} - Residues 22-323 of the 323 residue long hPTPA protein were expressed in BL21 (DE3) from the pET based vector pNIC-Bsa4 as a N-terminal 6xhistidine fusion. A Tev protease site was present between the His tag and the protein. The presence and integrity of the gene in the vector was confirmed by sequencing. The protein was purified using a 1 mL Hitrap chelating HP column and a superdex 75 gelfiltration column (Amersham, UK). The \textgreater 98% pure protein (as estimated from SDS page) was concentrated to 35.4 mg/mL in 20 mM HEPES, 300 mM NaCl, 10% glycerol, 0.5 mM TCEP and a mass of 37.30 kDa was confirmed by mass spectrometry (HPLC-ESI-MS). The same methods were used for purifying the selenomethionine labeled protein. The incorporation of 7 selenomethionines was confirmed by mass spectrometry.

\textbf{Crystallization and Data collection} - Hanging drop vapor diffusion was used to produce hPTPA crystals by making 2 \textmu L 1:1 protein:reservoir solution drops. Crystals of hPTPA were grown in 1.7-2.1 M (NH4)2SO4, 0.2 M NaCl and 0.1 M sodium cacodylate, pH 6.1-6.8 at 20\degree C for 1 week. The crystals grew in clusters and the size of a single crystal varied from 1.3 mm x 0.3 mm x 0.05 mm to 0.2 mm x 0.1 mm x 0.05 mm. Crystals of selenomethionine labeled protein were obtained through seeding with native crystals. The drops to be seeded were made by mixing 1 \textmu L 17.7 mg/mL protein and 1 \textmu L reservoir solution. Data was collected on native crystals that grew in 1.9 M (NH4)2SO4, 0.2 M NaCl and 0.1 M sodium cacodylate, pH 6.3 and selenomethionine labeled protein crystals that grew in 1.4 M (NH4)2SO4, 0.2 M NaCl and 0.1 M sodium cacodylate, pH 6.3. For data collection, the crystals were flash frozen in liquid nitrogen after adding a cryo solution of 20% glycerol, 2.1 M (NH4)2SO4, 0.3 M NaCl and 0.1 M sodium cacodylate (pH of drop) directly to the drop. Soaks of native crystals were performed by adding the cryo solution with ligand or peptide to the crystallization drop. Soaks were performed in 8 mM ATP and AMPPCP with 8mM MgCl2, 40 and 80 mM tripeptide (VPH) and 20mM pentapeptide (EVPHE). The concentration of the pentapeptide was relatively low due to the severe effect the peptide had on pH. Cocry stallizations were performed by adding the cryo solution with ligand or peptide to the crystallization drop. Soaks were performed in 8 mM ATP and AMPPCP with 8mM MgCl2, 40 and 80 mM tripeptide (VPH) and 20mM pentapeptide (EVPHE). The concentration of the pentapeptide was relatively low due to the severe effect the peptide had on pH. Cocry stallizations were performed by adding the cryo solution with ligand or peptide to the crystallization drop. Soaks were performed in 8 mM ATP and AMPPCP with 8mM MgCl2, 40 and 80 mM tripeptide (VPH) and 20mM pentapeptide (EVPHE). The concentration of the pentapeptide was relatively low due to the severe effect the peptide had on pH. Cocry stallizations were performed by adding the cryo solution with ligand or peptide to the crystallization drop. Soaks were performed in 8 mM ATP and AMPPCP with 8mM MgCl2, 40 and 80 mM tripeptide (VPH) and 20mM pentapeptide (EVPHE). The concentration of the pentapeptide was relatively low due to the severe effect the peptide had on pH. Cocry stallizations were performed by adding the cryo solution with ligand or peptide to the crystallization drop.
selenium sites in the asymmetric unit. RESOLVE [22] was used to carry out solvent flattening. ARP/wARP [23] was used to build the initial model and to place solvent molecules. Repeated rounds of manual model building using Coot [24] and refinement using Refmac [25] gave the final model consisting of residues 22-322 of hPTPA with 7 residues of the Tev-linker along with 4 sulfates and one glycerol molecule. The two first residues of the N-terminus were built in two conformations. The structure was refined to an R and R_{free} of 0.153 and 0.180, respectively and to a good stereo geometry. All residues of the model are within the most favored or additionally allowed regions of the Ramachandran plot. The coordinates and structure factors have been submitted to the PDB and have the accession code 2G62.

### RESULTS

A construct of residues 22-323 of human PTPA (hPTPA) was produced in *E. coli* as a N-terminal 6xHis fusion with a Tev cleavage site between the His-tag and the protein. It was crystallized in 1.9 M (NH₄)₂SO₄ at pH 6.3 and the structure was determined at 1.6 Å resolution and refined to good stereo geometry. The structure of hPTPA constitutes a novel fold as the DALI fold recognition server (http://www.ebi.ac.uk/dali/) [27] was not able to detect any structures with an overall fold similar to that of hPTPA.

The fold of the structure is shown in Fig. 1A. It is a two domain protein where the core of the larger domain is constituted by 5 long helices (helices 3-7 (see Fig. 1 C for helix numbering)) forming a helix bundle and the smaller domain is formed by 4 shorter helices (helices 9-11). The two domains are connected by helix 8. The C- and N-terminal helices (helices 1, 2 and 13) cover one phase of the protein. Helices 7 and 8 are the basis of a very conserved loop that is not visible in the current model. All three loops A, B and C shown in Fig. 2, connecting helix pairs 3/4 , 5/6 and 7/8, respectively are conserved and are all located on the same side of the protein as is the less conserved loop D connecting helix pair 11/12.

Two notable clefts are found in the structure. Cleft 1 is narrow and formed in between the two domains (Fig 1A), while the larger cleft 2 is in the large domain lined by the most C-terminal helix 13 (Fig. 2). Both these clefts constitute potential candidates for active sites/protein-protein interaction surfaces. Binding site analysis by the structure-function relationship server Profunc (http://www.ebi.ac.uk/thornton-srv/databases/profunc) [28] finds cleft 2 to be the most likely surface on the protein to participate in interaction with other molecules. Electron density for a glycerol molecule (used as a cryo protectant) and several water molecules were found in the central region of cleft 2. The conserved loops B, C and D all face cleft 2 and conserved loop A is situated above it (Fig. 2). That is, cleft 2 is highly conserved (Fig. 3).

The last 7 residues of the Tev-linker region can be seen in the present model. The backbone and side chains of the first and second visible residues of the N-terminus were built in two conformations. A peptide of the N-terminal Tev-linker from a neighboring molecule in the crystal lattice interacts with one end of cleft 2 where a phenylalanine enters into the conserved hydrophobic pocket formed by residues Ile278, Leu291, Met294, Val287 and Val281 (Fig. 3).

To explain the structural basis for the observed ATP/Mg²⁺ dependent PPIase activity of PTPA reported by Jordens et al. [19] we performed binding studies with potential ligands to PTPA in solution and in the crystal phase. Crystal soaks of native hPTPA crystals with 8 mM ATP and the ATP analog AMPPCP did not reveal any significant density implying poor binding in the crystal. As Pro190 of PP2Ac had been assigned as the scissile proline for the recently suggested PPIase activity of PTPA [19] peptides corresponding to this region were designed. Trimer and a pentamer peptides corresponding to Val189-His191 and Glu188-Glu192 of PP2Ac, respectively were soaked into the crystal. A high concentration of the trimeric peptide was used (40 mM ATP and the ATP analog AMPPCP did not reveal any significant density implying poor binding in the crystal. 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Leu198 of PP2Ac was also performed. None of the peptide treated crystals revealed a bound peptide. However, a new crystal form of PTPA was obtained in the cocrystallization attempts. This new crystal form belongs to space group P2₁ and has 2 molecules in the asymmetric unit while the native crystal form belongs to space group P2₁2₁2₁, with 1 molecule in the asymmetric unit.

Interestingly, the Tev-linker still interacts with a neighboring molecule in the P2₁ crystal packing obtained from cocrystallization experiments, but with an altered conformation. It is possible that the change of space group is induced by competition with the peptide binding in solution, but in the refined structure, of the cocrystallized protein, no obvious electron density for a bound peptide was seen.

To shed further light on the interactions of PTPA with its potential substrates a thermal stability shift assay was performed using the thermofluor methodology [26]. No detectable interaction was observed using ATP or its analogs AMPPCP, AMPNNP, AMPCPP or ATPγS (results not shown). Unfortunately, the binding studies using the peptides did not behave normally in the thermofluor experiment, probably due to interactions of the fluoro-probe with the peptide, excluding conclusive interpretation of the data.

**DISCUSSION**

hPTPA has a novel two domain fold constituted by 13 α-helices. A highly conserved cleft (cleft 2 in Fig. 2) is present in the protein where a peptide from the Tev-linker region of a neighboring molecule in the crystal binds. The high degree of conservation of this cleft along with the binding of the Tev-linker (Fig 3.) supports the notion that this cleft plays a functional role in interactions with other molecules.

The PTPA structure is not similar to the structure of any of the three types of PPIases known today: cyclophilins, FKBPs or parvulins (for a PPIase review see [29]), supporting the claim of Jordens et al. that PTPA is a new type of PPIase [19]. Unfortunately, this also means that it is more challenging to explain the PPIase activity of PTPA using the structural information. The definition of detailed structure-function relationships has also proven somewhat difficult for the established PPIases [29]. The NMR-data of Jordens et al. gives strong support for binding of a synthetic peptide corresponding to residues L186-L198 of PP2Ac to PTPA [19]. The authors do not show that Pro190, which they assign as the proline isomerized by PTPA, is crucial for this binding, or in fact that this residue is isomerized. We attempted to soak a trimer and a pentamer peptide corresponding to Val189-His191 and Glu188-Glu192 of PP2Ac, respectively, into the crystals of native hPTPA but were unable to detect any significant electron density indicative of binding. Cocryystallization with the peptide, corresponding to Leu186-Leu198 of PP2Ac used by Jordens et al. was also unsuccessful even though a new crystal form of the protein was obtained. The lack of binding could potentially be explained by poor accessibility in the crystal lattice or by direct competition with the Tev-linker peptide for the binding site in cleft 2. Alternatively, it might be that the kinetics of binding in the experiments of Jordan et al., only allows for transient interactions of reaction components in the PPlase reaction.

The activity of PTPA has been reported to be ATP/Mg²⁺ dependent in vitro [13, 19] but it is not clear what role ATP has for this activity. ATP and Mg²⁺ can on their own activate phosphoryrosyl phosphatase function of PP2A [14]. The recently observed PPlase activity was stimulated by ATP/Mg²⁺, indicating a direct interaction of ATP/Mg²⁺ with PTPA. However, ATP in excess of Mg²⁺ is inhibitory [19] raising the possibility that magnesium ions might play a dominant role. The lack of ATP binding in the crystal, as well as in the thermal stability shift assay is intriguing, indicating that ATP might not be a PTPA ligand. Furthermore, nucleotide binding proteins crystallized at high concentrations of ammonium sulfate usually have sulfate ions bound in the nucleotide binding pocket, where they mimic the physico chemical properties of the phosphate groups of nucleotides. hPTPA is crystallized in 1.9 M ammonium sulfate, but the crystal binding sites of the four SO₄²⁻ molecules that are present in the structure do not have the characteristics of a ATP binding site and the residues that coordinate them are generally not conserved. Together our data makes it unlikely that the involvement of ATP in PTPA activity observed in vitro is due to direct ATP binding to PTPA. Furthermore, Profunc, the protein function prediction server (http://www.ebi.ac.uk/thornton-
7 different splice variants of hPTPA have been identified but only 2 of those have been detected in tissue; PTPAα, the most abundant one presented here, and PTPAβ. PTPAβ has a 35 residue long insertion [30] between helices 2 and 3. This insertion appears feasible from a structural perspective and could possibly be involved in changing the protein-protein interaction pattern of PTPA.

The yeast PTPA homologs Rrd1 and Rrd2 interact with the conserved Tap42 protein (α4 in humans) and with the type 2A phosphatases [16, 17]. Tap42 also interacts with type 2A phosphatases and this complex is a crucial part in mediating the signal through the TOR pathway, a nutrient responsive signaling pathway [31-33]. Fellner et al. have shown that Rrd2 is necessary for the full and correct activity of PP2A in yeast and Tap42 seems necessary for correct function of Slt4 (a type 2A phosphatase called PP6 in humans) and Pph21/2 (yeast PP2Ac) [34]. Neither Zheng and Jiang nor Van Hoof et al. find it likely that Rrd1/2 carries out its function through a traditional PP2A dimer or a trimer [16, 17]. Recently, Zeng and Jiang suggested that Rrd1 or Rrd2 were a third partner in the Tap42-type 2A phosphatases complex. They propose that the heterotrimeric complex of Tap42-Rrd2-Pph21/2 administers the PP2A activity absent in Rrd1/2 depleted cells and therefore claim that it is a novel heterotrimeric PP2A complex with a distinct substrate specificity.

Therefore, PTPA is likely to form a number of biologically important protein-protein interactions and the structure of PTPA can now serve as a basis for work directed at defining these interactions. The most obvious candidate protein-protein interaction surface is the highly conserved cleft 2 (Fig. 2). It is possible that this cleft could constitute the binding site for PP2Ac and/or α4. The interaction formed by the Tev-peptide segment with PTPA might model a real peptide interaction in a complex containing PP2Ac and would then constitute the first experimentally derived model for an interaction in a PP2A complex. The structure does not provide any clues to how the protein could catalyze the proposed PPIase reaction, or alternatively, serve as a metal chaperone. However, several fully conserved residues are present in cleft 2 as well as in the disordered loop C located in cleft 2, which should be interesting candidates for site directed mutagenesis studies directed at defining, or alternatively, discarding these activities.

In summary, we have described the unique fold of hPTPA where conserved residues are clustered in cleft 2, that along with the peptide binding, supports the notion that this cleft constitutes a major surface for interaction with other protein components. The structural data together with binding studies indicate that ATP might not be a cofactor/substrate as previously proposed. Together, this data should provide an excellent starting point for more detailed studies of the structure-function relationship of PTPA.

REFERENCES


FOOTNOTES

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Figure legends

Table 1: Data collection statistics. Values in parenthesis correspond to highest resolution shell.

Figure 1. A. A cartoon representation of the structure of hPTPA colored from N (blue)- to C (red)-terminus. A narrow cleft (cleft 1) is present between the greenish-yellow (helix 7) and the orange (helix 11) helices. B. A topology diagram of the structure, where the helices represented by circles have the same coloring as in A. C. i) The secondary structure elements, ii) hydrophobicity (pink: hydrophobic, grey: intermediate cyan: hydrophilic), iii) accessibility (blue: accessible, cyan: intermediate, white: buried) and iv) intermolecular participation (in red if shortest distance between interacting residues is less than 3.2 Å) of the residues in hPTPA. Part C of this figure was made using the Espript utility (http://escript.ibcp.fr/ESPript/ESPript) [35].

Figure 2. A Cα trace rotated ca. 90º relative to the cartoon representation in Fig 1.A, colored from N (blue) to C (red) terminus. A major cleft is marked cleft 2. Loops A-C are labeled LA-LC. Note that part of loop C is missing in this model.

Figure 3. A stereo view of a space filling model of hPTPA. A peptide from the Tev-linker of a neighboring molecule (in grey) in the crystal lattice interacts with the base of cleft 2. The space filling model is oriented in approximately the same way as the Cα trace Fig 2, and the coloring of it is according to degree of conservation. Color coding: non conserved residues- blue<green<yellow<orange<red- completely conserved residues. The sequences used in the alignment were from the following organisms: Drosophila melanogaster, Saccharomyces pombe, Caenorhabditis elegans, Saccharomyces cerevisiae (Rrd2), Saccharomyces cerevisiae (Rrd1), Kluyveromyces lactis, Neurospora crassa, Arabidopsis thaliana, Leishmania major, Dictyostelium discoideum, Entamoeba histolytica, Plasmodium yoelii yoelii, Cryptosporidium hominis, Theileria parva, Mus musculus and Gallus gallus. The pdb file containing similarity scores of each residue was made using the Endscript utility (http://escript.ibcp.fr/ESPript/ENDscript/index.php) [35].
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Figure 1
The crystal structure of a human PP2A phosphatase activator reveals a novel fold and highly conserved cleft implicated in protein-protein interactions
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