

Molecular basis for TPR domain-mediated regulation of protein phosphatase 5

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Protein phosphatase 5 (Ppp5) is a serine/threonine protein phosphatase comprising a regulatory tetratricopeptide repeat (TPR) domain N-terminal to its phosphatase domain. Ppp5 functions in signalling pathways that control cellular responses to stress, glucocorticoids and DNA damage. Its phosphatase activity is suppressed by an autoinhibited conformation maintained by the TPR domain and a C-terminal subdomain. By interacting with the TPR domain, heat shock protein 90 (Hsp90) and fatty acids including arachidonic acid stimulate phosphatase activity. Here, we describe the structure of the autoinhibited state of Ppp5, revealing mechanisms of TPR-mediated phosphatase inhibition and Hsp90- and arachidonic acid-induced stimulation of phosphatase activity. The TPR domain engages with the catalytic channel of the phosphatase domain, restricting access to the catalytic site. This autoinhibited conformation of Ppp5 is stabilised by the C-terminal α J helix that contacts a region of the Hsp90-binding groove on the TPR domain. Hsp90 activates Ppp5 by disrupting TPR–phosphatase domain interactions, permitting substrate access to the constitutively active phosphatase domain, whereas arachidonic acid prompts an alternate conformation of the TPR domain, destabilising the TPR–phosphatase domain interface.

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Introduction

Reversible phosphorylation of proteins on serine and threonine residues contributes to the regulation of virtually all functions of the eukaryotic cell. Protein phosphatase 5 (Ppp5), a ubiquitous member of the PPP family of serine/threonine protein phosphatases, participates in several stress-activated cellular signalling pathways that regulate growth arrest, apoptosis and response to ionising radiation-induced

DNA damage (Zuo *et al*, 1998; Morita *et al*, 2001; Ali *et al*, 2004; Wechsler *et al*, 2004; Zeke *et al*, 2004). Similar to other PPP family members (PP1, PP2A, Ppp4), Ppp5 is potently inhibited by the tumour promoters okadaic acid, microcystin, cantharadin, calyculin A and tautomycin (Chen *et al*, 1994; Borthwick *et al*, 2001). However, whereas PP1, PP2A and Ppp4 exist as dimers or trimers with a catalytic subunit bound to regulatory subunit(s), Ppp5 uniquely comprises a regulatory N-terminal tetratricopeptide repeat (TPR) domain fused to a C-terminal phosphatase catalytic domain (reviewed in Cohen, 1997; Andreeva and Kutuzov, 1999; Chinkers, 2001).

The discoveries that Ppp5 forms complexes with glucocorticoid receptors and the Ask1 protein kinase implicated the phosphatase as a regulator of signalling networks initiated by glucocorticoids (Chen *et al*, 1996; Zuo *et al*, 1999) and oxidative stress (Morita *et al*, 2001). Ppp5 interacts with the TPR acceptor site of the heat shock protein Hsp90 and competes with TPR containing immunophilins in binding to some glucocorticoid receptor complexes (Chen *et al*, 1996; Silverstein *et al*, 1997). Suppression of Ppp5 expression with antisense oligonucleotides enhances glucocorticoid-mediated phosphorylation of p53 on Ser15 with consequent expression of p21^{WAF1/Cip1} and concomitant G₁ growth arrest in a lung carcinoma cell line (Zuo *et al*, 1998, 1999; Urban *et al*, 2003). Oxidative stress-mediated activation of apoptosis signal-regulating kinase 1 (Ask1), which promotes apoptosis via the JNK and p38 MAP kinase cascades, was inhibited by over-expression of Ppp5 (Morita *et al*, 2001). This negative regulation of Ask1 by Ppp5 is likely to involve dephosphorylation of a phospho-threonine residue within the activation loop of Ask1. Ppp5 also suppresses the transient increase in Ask1 activity produced by hypoxia (Zhou *et al*, 2004). In a further link between Ppp5 and Ask1, rapamycin, an inhibitor of the mammalian target of rapamycin (mTOR), induces sustained activation of Ask1, associated with inhibition of Ppp5 activity (Huang *et al*, 2004).

Recently, two important studies have demonstrated roles for Ppp5 in the DNA damage response. Ppp5 associates with both ataxia telangiectasia mutated (ATM) and DNA-PK (Ali *et al*, 2004; Wechsler *et al*, 2004), two PI3K-related kinases whose activation in response to genotoxic stress initiates DNA damage-induced checkpoints and DNA repair, respectively. Ppp5 regulates the phosphorylation states of these kinases and their downstream signalling events. Significantly, a fragment of DNA-PK containing an autophosphorylation site (Thr2609) interacts with the TPR domain of Ppp5, suggesting that DNA-PK–Ppp5 interactions may be dependent on DNA-PK phosphorylation states (Wechsler *et al*, 2004). A variety of other proteins have also been reported to associate with Ppp5. These include the atrial natriuretic peptide receptor (Chinkers, 1994), the TPR containing Cdc16 and Cdc27 subunits of the anaphase promoting complex (Ollendorf and Donoghue, 1997), the human homologue of *Arabidopsis thaliana* blue-light photoreceptor, cryptochrome 2 (Zhao and Sancar, 1997), the A subunit of PP2A (Lubert *et al*, 2001) and the Hsp90-dependent haem-regulated

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eIF2 α kinase (Shao *et al*, 2002). The G α_{12} /G α_{13} subunits of heterotrimeric G proteins also interact with Ppp5, suggesting that Ppp5 is a downstream effector of G α_{12} /G α_{13} signalling to ion channels (Yamaguchi *et al*, 2002).

Unlike PP1 and PP2A, the *in vitro* basal activity of Ppp5 is extremely low. Numerous studies have demonstrated that the TPR domain and C-terminal 13 residues cooperate to maintain the phosphatase in an autoinhibited state, with suppressed phosphatase activity. Removal of either the TPR domain or C-terminal 13 residues by limited proteolysis or deletion mutagenesis potently activates the enzyme some 10- to 50-fold (Chen and Cohen, 1997; Sinclair *et al*, 1999; Kang *et al*, 2001). These findings indicate that the isolated phosphatase domain is constitutively active, and that the TPR domain and C-terminal segment coordinately suppress catalytic activity. How Ppp5 is stimulated *in vivo* remains unclear, although a number of Ppp5 activators have been identified from *in vitro* studies. For example, polyunsaturated fatty acids, such as arachidonic acid, and saturated and unsaturated fatty acyl CoA esters stimulate the phosphatase activity of Ppp5 through interaction with its TPR domain (Chen and Cohen, 1997; Skinner *et al*, 1997; Ramsey and Chinkers, 2002). Furthermore, proteins that interact with the TPR domain also stimulate phosphatase activity, notably the C-terminal domain of Hsp90 (Ramsey and Chinkers, 2002) and G α_{12} /G α_{13} subunits (Yamaguchi *et al*, 2002).

The crystal structure of the TPR domain of Ppp5 revealed how the three TPR motifs, each comprising a pair of antiparallel α -helices, are organised to form a superhelical structure presenting an amphipathic groove that provides a binding site for interacting proteins (Das *et al*, 1998). Basic residues within this groove interact with acidic residues in the TPR acceptor site at the C-terminus of Hsp90 (Russell *et al*, 1999; Ramsey *et al*, 2000). More recently, the crystal structure of the catalytic domain of Ppp5 (residues 169–499) was reported (Swingle *et al*, 2004). The tertiary structure of the catalytic core and proposed catalytic mechanism are very similar to those of PP1 (Egloff *et al*, 1995; Goldberg *et al*, 1995) and calcineurin/PP2B (Griffith *et al*, 1995; Kissinger *et al*, 1995) with which Ppp5 shares approximately 40% identity (Barton *et al*, 1994).

An understanding of how the TPR domain and C-terminal segment of Ppp5 cooperate to suppress catalytic activity requires structural information of the full-length protein. Here we describe the crystal structure of the autoinhibited conformation of human Ppp5. The structure reveals an extensive interface between the TPR and phosphatase domains, augmented by the C-terminus of the protein, that blocks access to the catalytic site, thereby inhibiting activity. The TPR–phosphatase domain interface includes a region of the Hsp90-binding groove of the TPR domain, suggesting that TPR–Hsp90 interactions would dissociate the TPR domain from the phosphatase catalytic site, activating the phosphatase. In contrast, long-chain fatty acids activate Ppp5 by stabilising an alternate conformation of the TPR domain that disrupts contacts with the phosphatase domain.

Results and discussion

Structure determination

The structure of Ppp5 was solved by means of molecular replacement with the initial phasing obtained by determining

the position of the catalytic domain of the molecule using the structure of the catalytic subunit of PP1 as a search object. Details of the structure determination procedure are described in Materials and methods. Four Ppp5 molecules exist within the asymmetric unit and three are well defined, except for the N-terminal six residues and the TPR–phosphatase domain linker residues (152–157) that are disordered. The fourth Ppp5 molecule has a much less ordered structure, the electron density for its TPR domain being particularly weak, with breaks in density corresponding to both side-chain and main-chain features. In the crystal, Ppp5 molecules are assembled into dimers, with the dimer interface formed by the antiparallel association of the edge β -strand (β 14) of the catalytic domain β -sandwich, creating a continuous β -sheet shared between two molecules. The TPR domain does not participate in the dimer interactions. The physiological relevance of this dimer is unknown. In solution, the protein behaves as a monomer, eluting at the expected position for a monomeric 58 kDa protein on an S200 size exclusion column. Moreover, the crystal structure of the isolated catalytic domain of Ppp5 is a monomer (Swingle *et al*, 2004).

Description of overall structure

Ppp5 is organised into two distinct domains: the N-terminal TPR domain is connected via a partially disordered linker region to the phosphatase domain (Figure 1). Immediately C-terminal to the phosphatase domain, the polypeptide forms a subdomain of 20 residues terminating in a short two-turn α -helix (α J) that is unique to Ppp5. The TPR domain is composed of three tandem TPR motifs arranged in parallel (Das *et al*, 1998). As each TPR motif of 34 residues is generated from a pair of antiparallel α -helices, the TPR domain is composed of a series of antiparallel α -helices (termed α A and α B, connected by short turns) rotated relative to one another by a constant 24°. This topology creates a right-handed superhelical structure generating a pronounced concave surface on one side with a contrasting convex surface on the other. The architecture of the catalytic phosphatase domain of Ppp5 is essentially identical to that of PP1 and

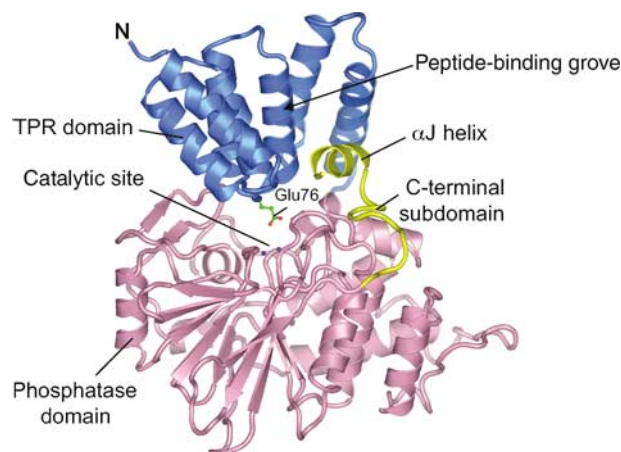


Figure 1 Structure of human Ppp5. Ribbon representation of Ppp5 with the TPR and phosphatase domains coloured blue and pink, respectively. The C-terminal subdomain including the α J helix is in yellow. Metal ions of the binuclear centre are shown as blue spheres. The figures were produced using PYMOL (<http://www.pymol.org>).

PP2B, other members of the PPP Ser/Thr-specific protein phosphatase family (Egloff *et al*, 1995; Goldberg *et al*, 1995; Griffith *et al*, 1995; Kissinger *et al*, 1995). The architecture of these proteins is characterised by a central β -sandwich surrounded on one side by seven α -helices and on the other by a subdomain consisting of three α -helices and a three-stranded mixed β -sheet. Loops connecting β -stands from the β -sandwich with the flanking α -helices contribute conserved catalytic residues that function to coordinate two metal ions at the centre of the catalytic site. This binuclear metal centre is responsible for mediating the dephosphorylation reaction by coordinating the scissile phosphate group of the phosphorylated substrate adjacent to a metal-activated nucleophilic water molecule. The catalytic centre lies within a pronounced surface channel created by the association of the two β -sheets of the β -sandwich, which presumably allows engagement with the phosphoprotein substrate (Figure 1). Such a feature is common to the catalytic domains of PP1 and PP2B. Inhibitory toxins of PP1, and the intrasteric inhibitory domain of PP2B, associate with the equivalent channel in these phosphatases. In Ppp5, the C-terminal αJ helix packs onto one side of the β -sandwich and functions to deepen the catalytic site channel. Pairwise comparison of the four independent copies of Ppp5 within the asymmetric unit indicates that the catalytic domains of the four molecules have essentially identical conformations (mean pairwise r.m.s.d.

between equivalent C α atoms is 0.15 Å). However, there are small differences in the relative orientations of the TPR domains relative to the phosphatase domains, suggesting flexibility at the domain interface.

Previously determined structures of the isolated TPR (Das *et al*, 1998) and catalytic domains of Ppp5 (Swingle *et al*, 2004) indicated that these domains form autonomously folded structures. Within the context of the autoinhibited state of Ppp5, there are small changes in conformation of these individual domains. In the catalytic domain, these differences are confined to a small readjustment of the C-terminal αJ helix. Otherwise, the structures are identical and equivalent C α atoms superimpose with an r.m.s.d. of 0.4 Å (Figure 2A). Similarly, the conformations of the three core TPR motifs of the TPR domain are the same between the isolated domain and the full-length protein (equivalent C α atoms superimpose with an r.m.s.d. of 0.6 Å). However, the seventh helix ($\alpha 7$) of the isolated TPR domain, which forms an extended helix that packs antiparallel to the third TPR domain (Das *et al*, 1998), is rotated by $\sim 5^\circ$ in Ppp5, and the C-terminal five turns of $\alpha 7$ unwind to form the linker connecting the TPR and phosphatase domains, together with the N-terminus of the phosphatase domain (Figure 2B).

TPR domain inhibits Ppp5 by blocking the catalytic channel

We crystallised Ppp5 in the absence of activating ligands where Ppp5 is between 5 and 15% of the activity of either the fully activated protein or the constitutively active isolated catalytic phosphatase domain, implying that our structure represents the autoinhibited conformation of Ppp5. In this state, the TPR and phosphatase domains engage to form an extensive interface, which blocks access to the phosphatase catalytic site, thereby explaining the ability of the TPR domain to inhibit Ppp5 phosphatase activity (Figures 1 and 3A). Comparison with the structure of the isolated catalytic domain shows that there are no changes in conformation of the phosphatase catalytic site on association with the TPR domain, indicating that TPR-induced inhibition of catalytic activity is not due to distortion of the phosphatase catalytic domain. Specifically, the interactions between the TPR and phosphatase domains involve contacts between the turns connecting the αA and αB helices of TPR motifs 1–3 (intra-repeat turns), which form a contiguous ridge that inserts into the catalytic channel of the phosphatase domain, reminiscent of how toxins inhibit PP1 (Goldberg *et al*, 1995; Maynes *et al*, 2001; Kita *et al*, 2002) (Figures 1 and 3A). Specific contacts between the TPR and phosphatase domains are relatively few. Of particular importance, however, are hydrogen bonds formed between the carboxylate side chain of Glu76, which projects from the intrarepeat turn of TPR-2 (second TPR motif of the TPR domain), and two catalytic site residues, Arg275 and Tyr451 (Figure 3B). Both Arg275 and Tyr451 are conserved in all members of the PPP Ser/Thr phosphatase family (Barton *et al*, 1994). Arg275 coordinates the phosphate group of the phosphoprotein substrate, whereas Tyr451 may also be involved in substrate recognition and/or metal ion coordination (Egloff *et al*, 1995). Consistent with our structural data that Glu76 participates in stabilising the autoinhibited state of Ppp5, a previous study found that substituting Ala for Glu76 activates Ppp5 some 10-fold (Kang *et al*, 2001). Apart from a hydrogen bond between the phenolic hydroxyl group

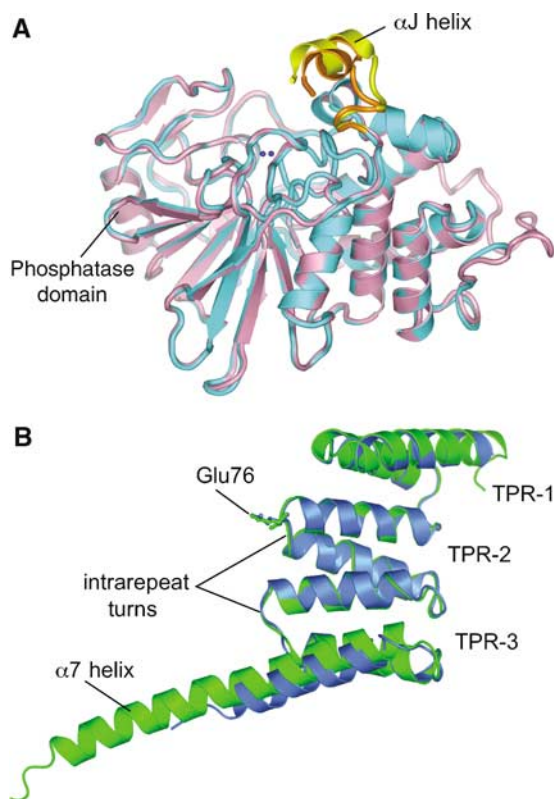


Figure 2 The TPR domain does not alter the conformation of the phosphatase domain. (A) Superimposition of the isolated phosphatase domain of Ppp5 (cyan) onto the Ppp5 phosphatase domain (salmon) within full-length Ppp5 shows a small shift of the C-terminal subdomain αJ helix (yellow: full-length Ppp5; orange: isolated Ppp5 domain). (B) Superimposition of the TPR domain of full-length (blue) and isolated protein (green).

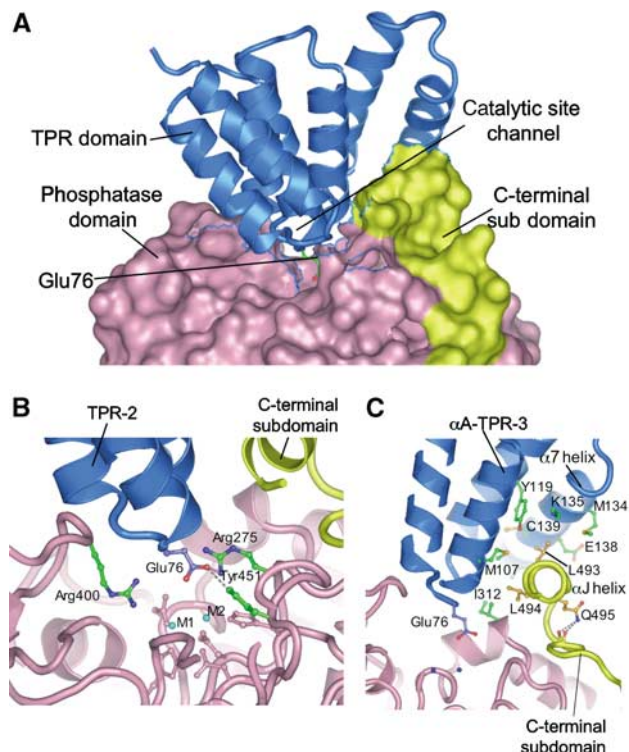


Figure 3 Phosphatase-TPR domain interactions. (A) The phosphatase domain and C-terminal subdomain are represented with a molecular surface, and the TPR domain as ribbon. Intra-TPR turns form a ridge that inserts into the phosphatase domain catalytic channel, with Glu76 of TPR-2 projecting towards the binuclear metal centre. (B) Glu76 of the TPR-2 interacts with Arg275 and Tyr451 at the catalytic site. Metal ions are indicated as M1 and M2 and side chains of metal ion-binding residues are coloured pink. (C) The α J helix forms hydrophobic contacts with the TPR domain. Detailed interactions involving Leu493 and Leu494 of the α J helix with TPR-3 and α 7 of the TPR domain are shown. The amide side chain of Gln495 donates hydrogen bonds to the main-chain carbonyls of 489 and 490, stabilising the position of the α J helix.

of Tyr313 of the phosphatase domain, and a main-chain carbonyl oxygen atom of Leu108 of α A of TPR-3, other TPR-phosphatase domain interactions are predominantly nonpolar in character. The most extensive set of nonpolar interactions are formed between the aliphatic side chains of two Leu residues, Leu493 and Leu494, lying on one face of the C-terminal α J helix, which pack into a pocket on the TPR domain generated by the α A and α B helices of TPR-3 and TPR domain α 7 helix. These interactions are likely to be responsible for the small shift in conformation of the α J helix in the autoinhibited state compared with the constitutively active isolated phosphatase domain (Figures 2A and 3C).

Previous biochemical data have revealed that both the TPR domain and the extreme C-terminal 10 residues function in a coordinated manner to maintain the autoinhibited state of Ppp5 (Sinclair *et al*, 1999; Kang *et al*, 2001). Removal of either the TPR domain or the C-terminal 13 residues leads to a constitutively active phosphatase that is essentially insensitive to fatty acid stimulation. Our structure shows that the C-terminal residues do not restrict access to the catalytic site themselves, but function to promote the autoinhibited conformation of Ppp5 by stabilising the interactions between the TPR and phosphatase domains. Consistent with this notion,

removal of the C-terminal 13 residues renders Ppp5 more susceptible to trypsin cleavage within the linker region connecting the TPR and phosphatase domains, suggesting that the α J helix functions to maintain Ppp5 in a constrained conformational state (Sinclair *et al*, 1999). In a detailed analysis of the contribution of C-terminal residues to the repressed state, Kang *et al* (2001) found that deletion of five or more residues is required to destabilise the autoinhibited state, leading to a 10-fold activation of the enzyme. Examination of the Ppp5 structure indicates that the amide side chain of Gln495 (the fifth residue from the C-terminus) stabilises the tight turn immediately preceding the α J helix by donating hydrogen bonds to the carbonyl oxygen atoms of Tyr489 and Ala490 (Figure 3C).

TPR-mediated inhibition resembles inhibition of PP1 by toxins and PP2B by its autoinhibitory domain

The mechanism of TPR-mediated inhibition of the Ppp5 phosphatase domain bears striking resemblance to mechanisms of inhibition of PP1 by natural product toxins and the autoinhibition of PP2B by its C-terminal regulatory segment. Superimposition of the phosphatase domains of the PP1-toxin complexes and PP2B onto the Ppp5 phosphatase domain exemplifies the nature of these similarities (Figure 4). Inhibition of phosphatase activity is achieved by blocking access to the catalytic binuclear metal centre. In Ppp5, the carboxylate group of Glu76 of the TPR domain contacts the side chain of one of the catalytic Arg residues (Arg275 in Ppp5) and Tyr451, being within 4.2 Å of the binuclear metal centre, although direct contacts between the negatively charged group and the two metals are not made. A similar structural feature is observed in the PP1-toxin complexes and in the autoinhibited state of PP2B. In the PP1-microcystin complex, a Glu residue of microcystin contacts Arg96 of the PP1 catalytic site (equivalent to Arg275 of Ppp5) (Goldberg

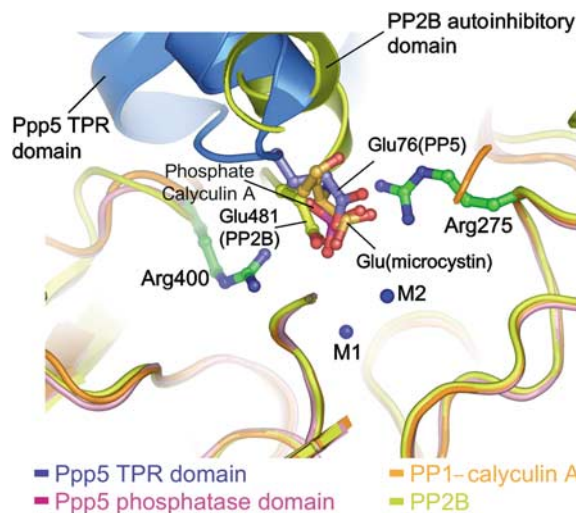


Figure 4 Inhibition of Ppp5 by the TPR domain Glu76 residue resembles inhibition of PP1 by toxins and autoinhibition of PP2B. Superimposition of the phosphatase domains of Ppp5, PP1 in complex with toxins microcystin and calyculin A and the autoinhibited state of PP2B shows a conserved anionic group that contacts the invariant catalytic site Arg-phosphate binding and Tyr residues of these phosphatases. For clarity, only the glutamate residue of microcystin and the phosphate group of calyculin A are shown.

et al, 1995), whereas in PP2B, Glu481 of the C-terminal autoinhibitory segment is shifted slightly relative to the position of Glu76 of Ppp5, and contacts the neighbouring catalytic Arg site residue, Arg254 (equivalent to Arg400 of Ppp5) (Kissinger *et al*, 1995). The carboxylic acid in the C1 position of okadaic acid contacts Arg96 of the PP1 catalytic site (Holmes *et al*, 2002). Finally, the anionic group of the calyculin A toxin is a phosphate moiety, and in the PP1-calyculin A structure this group contacts both catalytic site Arg residues of PP1 (Kita *et al*, 2002). Anionic groups on inhibitory toxins of PP1 and autoinhibitory segments of Ppp5 and PP2B interact with their respective phosphatase catalytic sites in a manner that partially mimics the mode of interaction between the phosphate group of a phosphorylated substrate and the catalytic site.

Bacterially expressed and native mammalian Ppp5 are potently inhibited by both okadaic acid and microcystin (IC₅₀ values of 1–7 and 1–15 nM, respectively) (Chen *et al*, 1994; Borthwick *et al*, 2001; Dean *et al*, 2001; Honkanen and Golden, 2002), and microcystin coupled to Sepharose was found to deplete 80% of Ppp5 from cauliflower extracts (Meek *et al*, 1999). These findings indicate that both toxins interact with the catalytic site of Ppp5 and, by analogy to PP1-toxin structures, are likely to engage the catalytic channel, which interacts with the TPR domain in the autoinhibited Ppp5 structure. Inhibition of Ppp5 by toxins will therefore be mutually exclusive with the suppression of phosphatase activity mediated by the TPR domain, and inhibition of Ppp5 by toxins can only be measured once the TPR domain has been displaced. Indeed Borthwick *et al* (2001) and Dean *et al* (2001) assayed the okadaic acid-mediated inhibition of Ppp5 by stimulating the enzyme with 100–200 μM polyunsaturated fatty acid, whereas Chen *et al* (1994) determined the toxin sensitivity of basal Ppp5 activity that results from limited proteolysis of Ppp5 causing removal of the inhibitory TPR domain and/or the C-terminal region. Plant Ppp5 is stimulated by arachidonic acid (Meek *et al*, 1999), suggesting that it has an autoinhibited structure similar to that of mammalian Ppp5. The efficient capture of plant Ppp5 on microcystin-Sepharose indicates that microcystin can displace the TPR domain from the phosphatase domain catalytic channel.

Activation of Ppp5 by TPR-mediated Hsp90 interactions

Ppp5 has been isolated from cells as a complex including both Hsp90 and Hsp70 (Chen *et al*, 1996; Silverstein *et al*, 1997; Zeke *et al*, 2004), and interactions between Ppp5 and Hsp90 are mediated by the Ppp5 TPR domain and C-terminus of Hsp90 (Russell *et al*, 1999; Ramsey *et al*, 2000). Interaction of Hsp70 with Ppp5 is also via the TPR domain of Ppp5 (Zeke *et al*, 2004) and therefore likely to involve the four C-terminal residues (EEVD) of Hsp70, which are identical to those in Hsp90. Biochemical and mutagenesis studies of Ppp5-TPR domain interactions with Hsp90 (Russell *et al*, 1999; Ramsey *et al*, 2000), together with the crystal structures of the TPR domains of Hop (Hsp70 and Hsp90 organising protein) with Hsp70 and Hsp90-based peptides (Scheufler *et al*, 2000) have provided insights into how the Ppp5 TPR domain interacts with Hsp70/90. In the structure of the TPR1 domain (which comprises three TPR motifs) of Hop in complex with a peptide based on the C-terminal seven residues of Hsp70 (Hsp70 peptide), the Hsp70 peptide interacts with the Hop

TPR domain in an extended conformation (Scheufler *et al*, 2000). Peptide-protein interactions are dominated by hydrogen bonds involving the carboxylate groups of acidic residues and the C-terminus of the Hsp70/90 EEVD motif interacting with conserved Arg and Lys residues lining the basic peptide-binding channel of Hop.

Arg, Lys and Asn residues responsible for Hop-Hsp70/90 interactions are conserved in other Hsp70/90-binding TPR domains, including Ppp5 (Scheufler *et al*, 2000), and studies from Chinkers and co-workers (Russell *et al*, 1999) demonstrated that Lys32, Lys97 and Arg101 are required for Hsp90-Ppp5 interactions. Lys97 and Arg101 are situated on adjacent helical turns of the αA helix of TPR-3, in close proximity to Lys32 on αA of TPR-1 (Figure 5). These residues together with Arg74 of αA of TPR-2 create a basic region at one side of the TPR peptide-binding channel for engagement of the Hsp70/90 acidic C-terminal region, and it is reasonable to assume that the Ppp5 TPR domain engages Hsp70/90 in a manner similar to that observed in the Hop-peptide complexes. The Hsp70 peptide binds to the Hop TPR domain such that the sequence of Hsp70 runs antiparallel with the αA helices of the TPR motifs. This places the C-terminus of the Hsp70 peptide close to the turns connecting adjacent TPR motifs (inter-repeat turns), whereas amino acids 6–7 residues N-terminal to the C-terminal Asp of Hsp70 (termed P-6 and P-7) are positioned adjacent to the intrarepeat turns (Scheufler *et al*, 2000) (Figure 5). The Ppp5 TPR domain engages the catalytic channel of the phosphatase domain mainly via these turns, whereas the basic region within the TPR peptide-

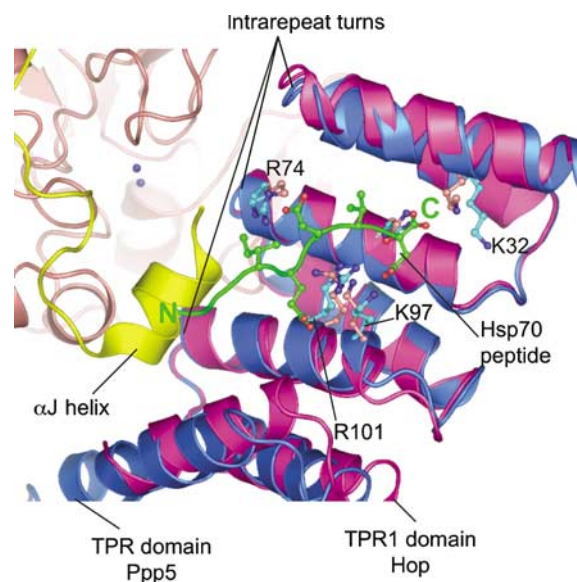


Figure 5 Model of Hsp90 activation of Ppp5 based on the structure of the Hop-Hsp70 peptide. The TPR domain (purple) of the Hop-Hsp70 peptide complex is superimposed onto the TPR domain of Ppp5 (blue). Peptide-binding basic residues are conserved in the TPR domains of both proteins, suggesting a related mechanism of peptide binding. The region of the Hsp70 peptide N-terminal to the IEEVD motif overlaps the position of the αJ helix, suggesting that optimal peptide binding to the Ppp5 TPR domain is mutually exclusive with TPR-phosphatase domain interactions. The N- and C-termini of the Hsp70 peptide are indicated by N and C, respectively. The C-terminus of Hsp90 (MEEVD) is predicted to bind to the Ppp5 TPR domain with a similar conformation as seen in the Hop-Hsp70 complex (see text for details).

binding channel formed from Lys32, Lys97 and Arg101 does not participate in the TPR–catalytic domain interface, but remains solvent exposed. Interestingly, in a site-directed mutagenesis study of Ppp5, Chinkers and co-workers (Kang *et al*, 2001) found that mutation of basic residues within the TPR peptide-binding groove had no effect on the basal activity of Ppp5, indicating that residues required (although not necessarily sufficient) to mediate TPR–Hsp90 interactions are distinct from those mediating autoinhibitory interactions with the phosphatase domain. The α J helix, C-terminal to the phosphatase domain, however, interacts with the concave channel of the TPR domain, close to the intrarepeat turns. This association would partially block one side of the peptide-binding channel, preventing optimal peptide–TPR domain interactions. Superimposing the Hop–Hsp70 peptide complex onto the TPR domain of Ppp5 reveals that the C-terminal region of the peptide-binding groove of the Ppp5 TPR domain is accessible to the peptide, but that the α J helix occludes the binding site for peptide residues P-5 to P-7, with these residues sterically overlapping the phosphatase α J helix (Figure 5). Acidic residues of the EEVD motif are required for Ppp5–Hsp90 interactions, supporting the notion that the C-termini of Hsp90 and Hsp70 interact with the TPR domains of Ppp5 and Hop by similar mechanisms (Ramsey *et al*, 2000). In addition to these residues, an acidic region (residues –9 to –13 relative to the Hsp90 C-terminus) is also required for Hsp90–Ppp5 association (Ramsey *et al*, 2000), demonstrating that optimal interactions between Hsp90 and Ppp5 overlap the α J helix–TPR contacts.

Hsp90 activates Ppp5 by disrupting phosphatase–TPR domain interactions

Our structure of Ppp5 suggests that high-affinity TPR–Hsp90 interactions are mutually exclusive with TPR-mediated autoinhibition of the phosphatase domain. Although a region of the Hsp70/90 peptide-binding site is accessible in the autoinhibited structure, high-affinity binding requires access to sites that are buried at the TPR–phosphatase domain interface. For Hsp70 or Hsp90 to engage Ppp5 with high affinity, the TPR domain would require to dissociate from the phosphatase domain, exposing the constitutively active catalytic site, stimulating phosphatase activity. Conversely, the phosphatase domain, by blocking a region of the Hsp90 peptide-binding site within the TPR domain, is predicted to antagonise Hsp90 binding, lowering the affinity of Hsp90 for full-length Ppp5 relative to the isolated TPR domain. To test these proposals, we performed a variety of kinetic and isothermal titration calorimetry (ITC) experiments. First we tested whether full-length Hsp90 and a peptide corresponding to the C-terminus of Hsp90 are capable of stimulating Ppp5 activity. Figure 6A and B shows the dose-dependent activation of Ppp5 by both Hsp90 protein and peptide. Full-length Hsp90 is a moderate activator of Ppp5, inducing \sim 7-fold stimulation of the enzyme with a half-maximal activation (AC_{50}) of \sim 2 μ M. The human Hsp90 α peptide (TSRMEEVD) induces a similar fold activation but with a greatly reduced AC_{50} constant of 57 μ M, indicating a significantly weaker association between Ppp5 and peptide. These results are in agreement with previous findings that a 12 kDa C-terminal domain of Hsp90 promoted a 10-fold stimulation of Ppp5 with an AC_{50} constant of 6 μ M (Ramsey and Chinkers, 2002). Using ITC, we measured the affinity of Hsp90 peptide for both

the isolated TPR domain and for Ppp5 (Figure 6C and D). Significantly, Hsp90 peptide binds the isolated TPR domain tightly, with a K_D of 40 nM, some 500-fold higher than its affinity for the full-length protein (K_D of 20 μ M).

These experiments suggest that the phosphatase domain partially blocks the Hsp90 peptide-binding site on the TPR domain in the context of the full-length protein, preventing optimal Hsp90–TPR domain interactions. Moreover, the relatively close correlation between the AC_{50} constant for the activation of Ppp5 by the Hsp90 peptide (57 μ M) and the dissociation constant describing Hsp90–peptide–Ppp5 interactions (20 μ M) suggests that binding of Hsp90 to Ppp5 is accompanied by activation of the phosphatase. Our findings that full-length Hsp90 is a more potent activator of Ppp5 than the C-terminal Hsp90 peptide indicate that residues N-terminal to the MEEVD motif, not present on our peptide, contribute to high-affinity Ppp5–Hsp90 interactions which could involve regions outside of the TPR domain. Consistent with the relative efficacies of Hsp90 and a short peptide to activate Ppp5, an acidic region (residues –9 to –13 relative to the Hsp90 C-terminus) is required for high-affinity Ppp5–Hsp90 interactions (Ramsey *et al*, 2000).

Arachidonoyl-CoA stimulates Ppp5 by inducing a structural change in the TPR domain

Soon after Ppp5's discovery, it was found to be stimulated by polyunsaturated fatty acids, with half-maximal activation at concentrations between 50 and 125 μ M (Chen and Cohen, 1997; Skinner *et al*, 1997). That this activation involved the TPR domain was determined from the findings that only full-length Ppp5, and not the constitutively active catalytic domain, is activated by arachidonic acid (Chen and Cohen, 1997; Sinclair *et al*, 1999). Additionally, it has been shown that the TPR domain interacts with arachidonic acid, which protects against proteolytic cleavage of the TPR domain by subtilisin and trypsin (Sinclair *et al*, 1999). Moreover, Ppp5 and the isolated TPR domain, but not the isolated catalytic domain, interact directly with phosphatidylinositol lipids (Chen and Cohen, 1997). These original findings required concentrations of activator much greater than their physiological levels; however, recent data have extended these results revealing that long-chain fatty acyl-CoA esters activate Ppp5 at physiological concentrations of \sim 1 μ M (Ramsey and Chinkers, 2002). The CoA moiety functions to enhance the overall solubility of such long acyl chains in an aqueous environment. The most hydrophobic compounds, with chain lengths of 16 carbons or more, were found to be the most effective activators (Ramsey and Chinkers, 2002).

Some TPR domains have been found to be structurally unstable (Taylor *et al*, 2001; Jinek *et al*, 2004), and recent NMR data suggest that the isolated Ppp5 TPR domain, which is substantially unfolded at physiological relevant temperatures, becomes ordered in the presence of the Hsp90 MEEVD peptide (MJ Cliff *et al*, unpublished). Significantly, in the crystal structure of the isolated TPR domain, the molecule was stabilised by association of the C-terminus of a symmetry-related molecule into the peptide-binding groove (Das *et al*, 1998). Because of their extended conformation, TPR proteins differ from conventional globular proteins in having a high surface area to volume ratio and a relatively small hydrophobic core. These observations prompted us to test whether a possible mechanism of fatty acid-mediated activa-

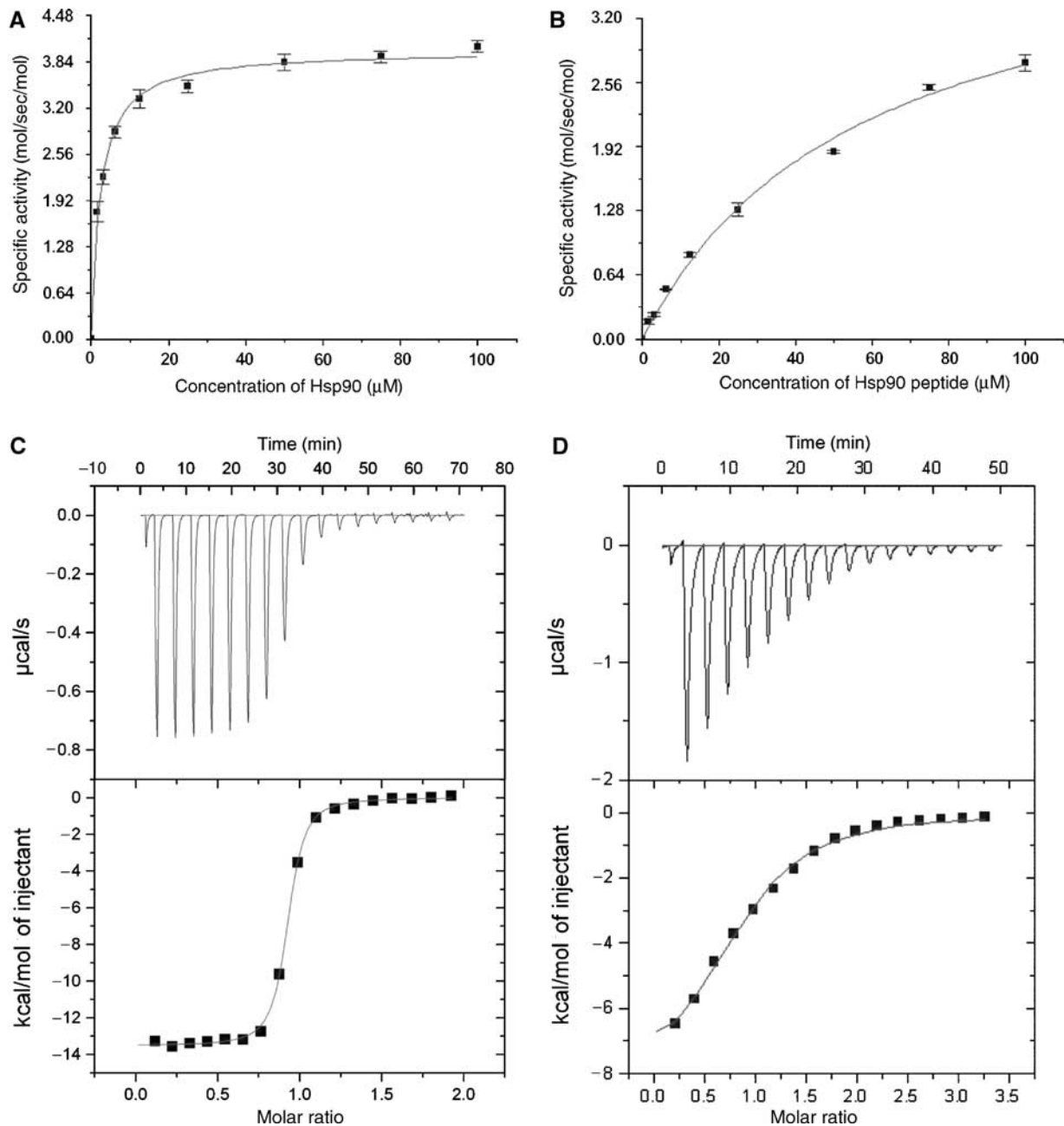


Figure 6 Hsp90 and Hsp90 peptides activate Ppp5 by binding to its TPR domain. (A) Dose-dependent activation of Ppp5 by full-length yeast Hsp90 and (B) Hsp90 C-terminal peptide. The data plotted are corrected for Ppp5 activity in the absence of activator. ITC plots for Hsp90 peptide interactions with (C) the isolated TPR domain (residues 19–146) and (D) full-length Ppp5.

tion of Ppp5 is due to fatty acid-induced disruption of the TPR structure, possibly by competing for the relatively weak hydrophobic interactions holding the fold together. Figure 7 shows the thermal-induced unfolding of the TPR domain as assayed by the α -helical content of the domain's circular dichroism signal. The addition of arachidonoyl-CoA promotes a significant reduction in α -helical content of the TPR domain below 300 K, and increases its melting temperature by $\sim 5^\circ\text{C}$. These results are consistent with the notion that arachidonoyl-CoA binds and stabilises an alternate conformation of the TPR domain with reduced helical content. Thus, one possible explanation for the ability of long-chain fatty acids to activate Ppp5 is that this induced conformational change results in the TPR domain being unable to form

the specific contacts with the catalytic site channel of the Ppp5 phosphatase domain and αJ helix, required for auto-inhibition.

Concluding remarks

The structure of the autoinhibited state of Ppp5 reveals how the N-terminal TPR domain docks onto the phosphatase domain catalytic site thereby hindering access to substrate. Contacts between the phosphatase domain and TPR domain involve the intrarepeat turns of the TPR protein, a mode of TPR–protein interaction not observed previously. Interactions between the C-terminal αJ helix and the groove of the TPR domain stabilise the autoinhibited state, explaining biochemical data that the TPR domain and C-terminal 13 residues

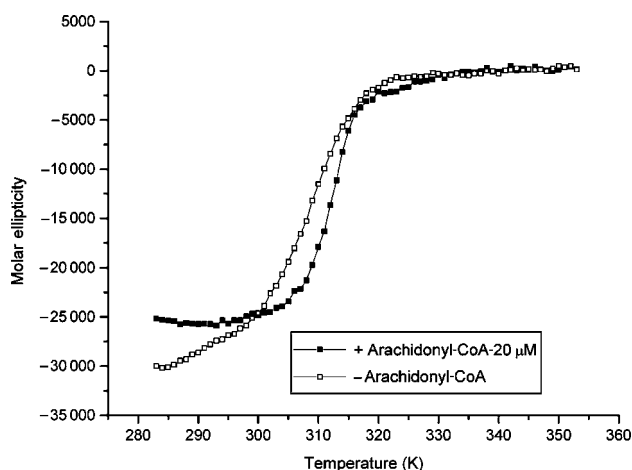


Figure 7 Arachidonoyl-CoA mediates TPR domain unfolding. Arachidonoyl-CoA affects the folding of the isolated TPR domain. The temperature dependence of the CD signal at 222 nm of amino acids 19–147 Ppp5 was recorded in the presence (closed symbols) and absence (open symbols) of 20 μ M arachidonoyl-CoA. The melting temperature is increased from 34.6 ± 0.3 to $39.9 \pm 0.2^\circ\text{C}$ by the addition of arachidonoyl-CoA, although the apo-state loses helical content ($\sim 15\%$ of CD signal at 222 nm).

Table I Data collection and processing statistics

Native Ppp5 data	
Data collection	ID14-EH1, ESRF
Wavelength (\AA)	0.979
Space group	$P2_1$
Unit cell (\AA)	$a = 51.74$, $b = 117.54$, $c = 200.41$, $\beta = 93.79^\circ$
Resolution (\AA)	30–2.9 (3.11–2.9)
Observations (N)	369 163 (53 949)
Unique reflections (N)	53 119 (10 083)
Redundancy	6.9 (5.4)
Completeness (%)	99.9 (99.9)
R_{merge}	0.118 (0.423)
$I/\sigma I$	3.4 (1.4)
Z	4
Refinement statistics	
Protein atoms (N)	15 082
R_{cryst}	0.247
R_{free}	0.291
R.m.s.d. bond lengths (\AA)	0.0086
R.m.s.d. bond angles (deg)	1.37

Highest shell values are in parentheses.

cooperate to suppress phosphatase activity. These interactions partially overlap the region of the TPR domain groove that is predicted to bind the C-terminus of Hsp90, indicating that sites within the TPR domain required for high-affinity Ppp5–Hsp90 interactions are blocked in the autoinhibited conformation. Hsp90 causes substantial activation of Ppp5 by competing for TPR–phosphatase domain contacts and allowing access to the catalytic site. The TPR domain of Ppp5 also interacts with other proteins, such as Ask1 (Morita *et al*, 2001), $G\alpha_{12}/G\alpha_{13}$ subunits (Yamaguchi *et al*, 2002) and DNA-PK (Wechsler *et al*, 2004), and it will be important to establish if these, and potentially other, TPR–protein interactions contribute to Ppp5 stimulation. The mechanism of Ppp5 autoinhibition by its N-terminal TPR domain, and activation by Hsp90, shares analogies to how SH2 domains regulate the activities of Src family kinases

(Sicheri *et al*, 1997; Williams *et al*, 1997; Xu *et al*, 1997), and the protein tyrosine phosphatases SHP1/2 (Hof *et al*, 1998). In these proteins, the unliganded SH2 domains maintain their respective proteins in an autoinhibited state, by inducing an inactive catalytic domain, as in Src, or by blocking access to the catalytic site (SHP1/2). Engagement of phosphotyrosine proteins to their SH2 domains disrupts SH2–catalytic domain interactions, with concomitant stimulation of activity. Understanding how a TPR domain confers ligand-dependent regulation of enzyme activity provides new insights into the roles and mechanisms of TPR motifs, which occur in > 150 human genes.

Materials and methods

Expression and purification of Ppp5, TPR domain and Hsp90

Human Ppp5 (residues 16–499) was amplified by PCR and cloned into the *Bam*HI and *Xho*I sites of the pGEX 6P-2 vector with a His₆ tag incorporated into the C-terminus of the protein. Expression of this protein was performed at 20°C overnight in *Escherichia coli* strain B834. Ppp5 was purified using a combination of glutathione–Sepharose affinity, S-Sepharose cation exchange (Amersham) and phenyl TSK hydrophobic interaction chromatography, prior to a desalting column, Ni-NTA agarose (Qiagen) and S200 gel filtration column (Amersham). The N-terminal His-tagged TPR domain (residues 19–146) was purified using Ni-NTA chromatography. His-tagged budding yeast Hsp90 was transformed into *E. coli* and purified ($> 90\%$) as described (Panaretou *et al*, 1998).

Crystallisation of Ppp5

Ppp5 was concentrated to 21 mg/ml. Crystals were grown using the under-oil batch method. Protein (1 μ l) was mixed with an equal volume of crystallisation buffer (10% (w/v) polyethylene glycol 6000, 4% 2-methyl-2,4-pentanediol (MPD), 0.1 M NaHepes (pH 8.5) and 3 mM DTT) in individual wells of a 72-well polystyrene tray, immersed under silicon oil and incubated at 14°C.

Data collection and structure determination

Crystals were incubated in a cryoprotection buffer (13% (w/v) polyethylene glycol 6000, 18% MPD, 0.075 M NaHepes (pH 8.5)) for 20 s, prior to freezing in a nitrogen gas stream at 100 K. Data (low- and high-resolution passes) from a single crystal were collected at ID14-EH1, ESRF, Grenoble. Data were processed, scaled and merged using MOSFLM, TRUNCATE and SCALA (CCP4, 1991) (Table I).

Ppp5 structure was solved by molecular replacement methods with the initial phasing obtained by determining the position of the catalytic domain of the molecule using the structure of the catalytic subunit of PP1 as a search object with CNS (Brünger *et al*, 1998). Assuming a solvent content close to 50% predicts four molecules of Ppp5 per asymmetric unit; however, only two distinct orientations of PP1 could be obtained in cross-rotation searches. A strong self-vector peak at $U = 0.444$, $V = 0.5$, $W = 0.3644$ on a native Patterson map indicated that two or more molecules per asymmetric unit shared identical orientations. We applied the translation vector to each of the two positioned PP1 catalytic subunits to generate the position of two other molecules. Attempts to position the TPR domain by molecular replacement methods failed; however, low-resolution refinement (8 \AA) and atomic positional refinement at 3 \AA , applying NCS restraints, and incorporation of the Ppp5 sequence improved phases sufficiently so that $2F_o - F_c$ maps revealed positions of three α -helices of the TPR domain. Iterative cycles of fitting TPR helices to electron density maps and refinement allowed the entire TPR domain to be located. The structure was refined using CNS (Brünger *et al*, 1998) and modelling building was performed using O (Jones *et al*, 1991).

Isothermal titration calorimetry

ITC was performed using a VP-ITC MicroCalorimeter (MicroCal). All protein and peptide samples were dialysed into a buffer comprising 25 mM Tris.HCl (pH 8.0), 50 mM NaCl, 1 mM MgCl_2 and 3 mM DTT. Titrations were performed by injecting 19 consecutive aliquots (15 μ l) of human Hsp90 α peptide (sequence

TSRMEEVD) into the ITC cell containing Ppp5 or the MEEVD peptide with the TPR domain (residues 19–146) at 25°C. ITC data were corrected for heats of dilution of the protein solution. Binding stoichiometry, enthalpy, entropy and binding constants were determined by fitting the corrected data to a bimolecular interaction model.

Activation of Ppp5 by HSP90 and peptide of HSP90

Cuvettes containing 50 mM pNPP, 0.5 mM MnCl₂ and 50 mM NaCl in 100 mM Tris, pH 8.0, and activator (either full-length yeast Hsp90 or TSRMEEVD human Hsp90 α peptide) were mixed at 22°C. The reaction was started by adding Ppp5 to a final amount of 0.5 μ g. Production of pNP was followed by monitoring the increase in the absorbance at 410 nm. Standard error values were obtained from assays performed in triplicate. Activity was expressed as moles of pNP released per mole of Ppp5 per second, using a molar extinction coefficient of 15.1 $\times 10^3$ mol pNP/L/cm (Ramsey and Chinkers, 2002).

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