Structural and Functional Basis of PP5 Ser/Thr Phosphatase Substrate Specificity

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The serine/threonine phosphatase PP5 regulates hormone and stress-induced cellular signaling by association with the molecular chaperone Hsp90. PP5-mediated dephosphorylation of the cochaperone Cdc37 is essential for activation of Hsp90-dependent kinases. However, the details of this mechanism remain unknown. We determined the crystal structure of a Cdc37 phospho-mimetic peptide bound to the catalytic domain of PP5. The structure reveals PP5 utilization of conserved elements of phosphoprotein phosphatase (PPP) structure to bind substrate and provides a template for many PPP-substrate interactions. Our data demonstrates that despite a highly conserved structure, elements of substrate specificity are determined within the phosphatase catalytic domain itself. Structure-based mutations *in vivo* reveal that PP5-mediated dephosphorylation is required for kinase and steroid hormone receptor release from the chaperone complex. Finally, our data shows that hyper- or hypoactivity of PP5 mutants increases Hsp90 binding to its inhibitor. This provides a mechanism to enhance the efficacy of Hsp90 inhibitors by regulation of PP5 activity in tumors.

Hsp90 | PP5 | Cdc37 | chaperone | phosphatase

Protein phosphatase 5 (PP5/Ppp5) has pleiotropic roles in cellular signaling including DNA damage repair, proliferation of breast cancer cells, circadian cycling, response to cytotoxic stresses, Rac-dependent potassium ion channel activity and activation of steroid hormone receptors (e.g. glucocorticoid receptor (GR) and estrogen receptor (ER)) (1, 2). It is a member of the phosphoprotein phosphatase (PPP) family of serine/threonine phosphatases, whose members share a highly conserved catalytic core and catalytic mechanism, dependent on two metal ions, commonly Mn^{2+}. Most PPP family members exhibit high, non-specific phosphatase activity. Specificity is provided by a large cohort of regulatory and other interacting proteins that function to inhibit basal activity and recruit substrates, thereby finely tuning the enzymes (3). This combinatorial approach enables a small number of catalytic subunits to have the breadth of specificity equivalent to that seen in kinases, which are greater in number by an order of magnitude. Structures of complexes between regulatory and catalytic domains have illuminated the importance of regulatory subunits in facilitating substrate recruitment (3). However, to date there is no structural information describing how a substrate binds at the active site of a PPP, therefore a central question remains how local interactions between the substrate and the catalytic domain contribute to the molecular basis of dephosphorylation.

PP5 is unique amongst the PPP family as it has a low basal activity due to an autoinhibitory N-terminal TPR domain (4). Its activity is promoted by a number of cellular factors, including fatty acids and the molecular chaperone Hsp90 (5), both of which release autoinhibition by interacting with the TPR domain (6, 7). Many established PP5 substrates are dependent on Hsp90 for their activation (known as Hsp90 clients). In addition to a requirement for Hsp90's chaperone activity, it is likely that these PP5 substrates require Hsp90 to act as a molecular bridge to bring the catalytic domain of PP5 in close proximity enabling dephosphorylation, as has been demonstrated for the Hsp90 cochaperone, Cdc37 (8). In such cases Hsp90 performs a role similar to that observed by the regulatory subunits of the PP1 and PP2A family (3).

The cochaperone Cdc37 regulates the activation of Hsp90 client-kinases by distinguishing between client and non-client kinases (9) and recruiting them to Hsp90 (10). Many of these kinases are oncogenes therefore the molecular details of their activation are of considerable interest in cancer therapy. Activation is dependent on a cycle of Cdc37-Ser13 phosphorylation by the constitutively active kinase CK2 (11, 12), and dephosphorylation by PP5 (8). The mechanisms by which Cdc37 phosphorylation and dephosphorylation, and any accompanying conformational changes, regulate kinase activation is not understood.

In order to understand the molecular determinants of the phospho-Ser13 Cdc37-PP5 interaction, we determined the 2.3 Å crystal structure of a Cdc37 phospho-mimetic peptide, bound to the catalytic domain of PP5. The structure reveals how PP5 utilizes conserved elements of PPP structure to bind substrate while *in vitro* and *in vivo* analysis indicate that, despite being highly

Significance

The activity of many proteins is dependent on molecular chaperones and their accessory proteins, cochaperones. The ability of a cohort of kinases, many of which are oncogenic, to transduce signals is promoted by the Hsp90 chaperone and Cdc37 cochaperone. This requires the removal of a phosphate from Cdc37 by the phosphatase PP5. We present the crystal structure of the PP5 phosphatase with Cdc37 trapped in the active site. This reveals how PP5 can associate with different substrates and previously unknown determinants of specificity. Our findings show kinase release from the chaperone complex is critically dependent on this dephosphorylation and that combined inhibition of both Hsp90 and PP5 could provide an effective therapeutic strategy for treating cancers addicted to these kinases.

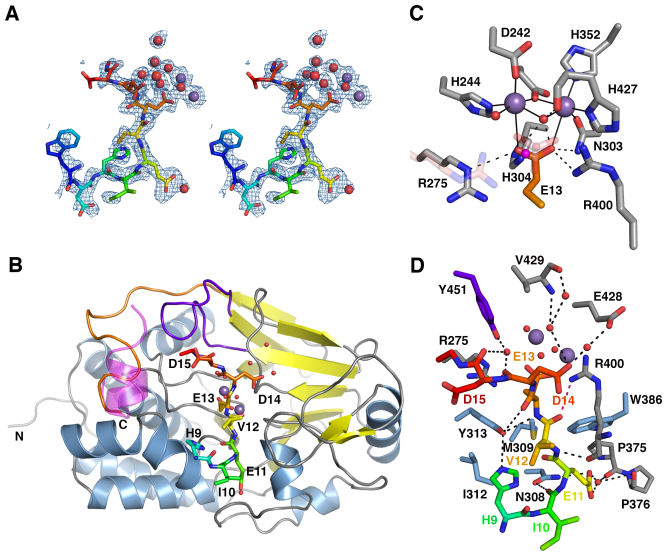

Fig. 1. Structure of Cdc37 phosphomimetic substrate bound in the PP5 catalytic cleft (A) Stereo image of the 2mFo-DFc electron density map contoured at 1σ for the Cdc37 phosphomimetic peptide. The peptide is coloured from blue (Trp7) to red (Asp15). Water molecules (red spheres) and Mn^{2+} ions (purple spheres) are shown. (B) The Cdc37-bound PP5 catalytic domain. Cdc37 is shown as sticks (coloured as (A)); PP5 is in cartoon representation. Cdc37-Trp7 and Asp8 do not make contact with the catalytic domain and are omitted for clarity. Sheets, helices and loops are coloured yellow, blue and grey respectively; the β12-13 loop is purple; the PP5 specific C-terminus is orange. The location of the αJ-helix from the *apo* PP5 catalytic domain structure (1S95) is shown in transparent magenta, generated by an rms fit of 1S95 and the chimera structure. The rest of 1S95 is omitted for clarity (see Fig. S1D for the complete fit).(C) Interactions between residues of the catalytic site of PP5 (grey) and the phosphomimetic Glu13 of Cdc37 (orange) including water molecules (red) and Mn^{2+} ions (purple). The new water molecule found in the phosphomimetic structure is coloured magenta. Differences in the PO_4^{3-} bound apo structure (PDB 1S95) are shown in transparent pink. (D) Hydrogen bonds (black dashed lines) between the Cdc37 peptide and PP5 (coloured as (B)). The catalytic residues interacting with E13 are omitted for clarity. Those involving the catalytic residues R275 and R400 are shown in pink.

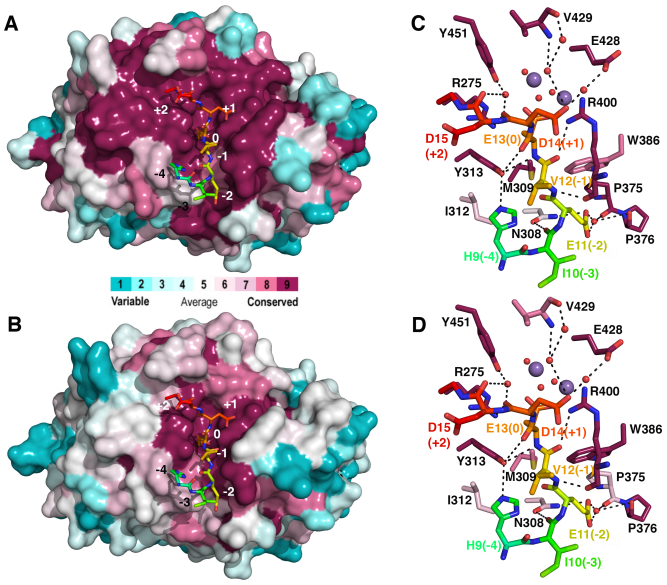

Fig. 2. Substrate main-chain conformation is likely to be conserved throughout PP5 and PPP family members (A, B) Surface representation of PP5 and (C, D) Cdc37 substrate-binding residues coloured according to sequence conservation using Consurf (see Methods). (A) and (C) show conservation between PP5 homologues; (B) and (D) show conservation amongst PPP family phosphatases. Cdc37 substrate is shown as sticks, coloured as Figure 1A and labeled from -4 through +2 to identify location relative to phosphomimetic S13E (position 0).

conserved, elements of substrate specificity are determined within the phosphatase catalytic domain itself.

Results

Crystal Structure of the PP5-Cdc37 Complex

Cocrystallisation of the PP5 catalytic domain with Cdc37 peptides in which the phospho-mimetic mutation Ser13→Glu was introduced to trap the substrate in the phosphatase active site, yielded crystals with either no, or very low, peptide occupancy. *In vivo* and *in vitro* PP5-mediated dephosphorylation of phospho-Cdc37-Ser13 requires Hsp90 to act as both a PP5-activator and a PP5-substrate recruiter. In order to obtain a construct suitable for crystallisation, we circumvented the need for Hsp90 by utilizing a chimera construct in which the TPR domain of PP5 was deleted and a peptide comprising residues 5-20 of Cdc37, including the Ser13→Glu mutation, was covalently linked to the catalytic domain of PP5 via a flexible linker of 9 residues (Fig. S1a). This construct yielded crystals that diffracted to 2.3 Å (Table S1).

In the resulting structure there is clear electron density for the Cdc37 peptide from Trp7 to Asp15 (Figure 1a), bound in an extended conformation across the dimetallic active site of PP5 (Figure 1b). The entire catalytic domain of PP5 is also clearly defined in the electron density with the exception of residues 491-499 (see below) and several side-chains on the surface of the protein remote from the catalytic centre. The overall orientation of the substrate peptide is identical to that of the microcystin (13), okadaic acid (14), nodularin and tautomycin (15) classes of PPP toxin inhibitors (Fig. S1b). Three putative substrate-binding clefts radiate from the catalytic centre of the PPP family member, PP1 (16). In PP5, two of these are occupied by Cdc37. Substrate residues N-terminal of S13E lie in the 'hydrophobic groove' while the C-terminal end of the substrate occupies the 'C-terminal groove' (Fig. S1c).

There are no significant global structural changes in the PP5 catalytic domain on binding the Cdc37 substrate peptide. The RMSD between the Cdc37-bound and PO_4^{3-}-bound (PDB 1S95) (17) structures is 0.391 Å (Fig. S1d). With the exception of the N- and C-termini of the domain and regions involved in crystal packing, the principal differences in main-chain and side-chain orientation lie around the substrate-binding cleft. This includes Asn308, Ile312, Pro376, Gly401 and Val402, all of which provide either van der Waals or hydrogen bond contacts with the peptide. The largest difference between the apo and holo crystal structures is the absence of density for the αJ-helix of the PP5-specific C-terminal subdomain. This may be a result of the design of the chimera as both the αJ-helix and the substrate are parallel to each other (Figure 1B). Nonetheless the location of this subdomain in the PO_4^{3-}-bound enzyme is not compatible with the direction of the substrate peptide C-terminal to Asp15. This suggests the αJ-helix plays a role in regulation of PP5 activity, not only through stabilizing the auto-inhibitory state of the TPR domain (6), but also by steric exclusion of the substrate.

The phospho-mimetic residue S13E is bound deep in the active site of the PP5 catalytic domain and the carboxylate group occupies the same location as the bound phosphate in the holo structure (Figure 1c). A new water molecule fills the cavity left by one of the missing oxygen atoms of the phosphate. The conformation of almost all residues involved in coordinating Cdc37-S13E, and the active site waters and Mn^{2+} ions, is unchanged compared to the phosphate-bound holo structure (17). The exception is Arg275 for which the guanadinium group has flipped. The conformation of the Cdc37 peptide is therefore that of a

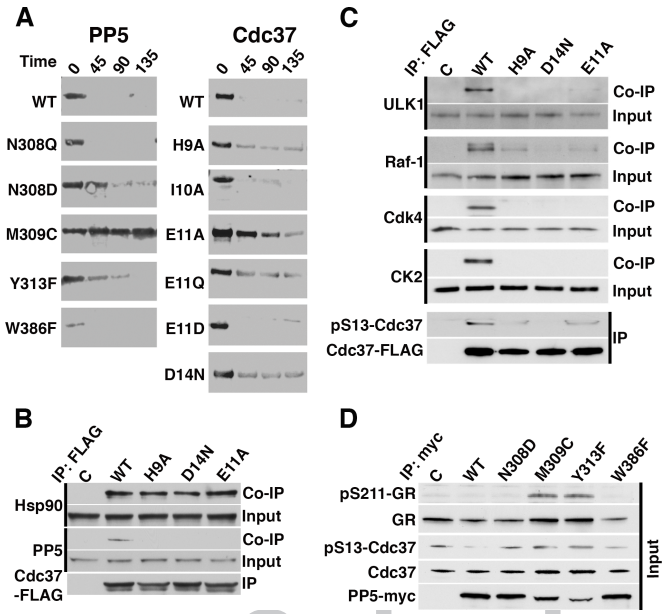

Fig. 3. Structure-based mutations reveal the functional importance of specific interactions between PP5 and substrate *in vitro* and *in vivo* (A) Dephosphorylation of phospho-Cdc37-Ser13 in the context of purified, full-length WT and indicated mutants of Cdc37 and PP5, in the presence of Hsp90. Activity was assessed using a phospho-specific antibody over time (minutes). (B) HEK293 cells were transiently transfected with empty plasmid (C), WT Cdc37-FLAG, or indicated mutants. Cdc37-FLAG proteins were immunoprecipitated (IP). Co-IP of Hsp90 and PP5 were examined by immunoblotting, (C) HEK293 cells were transiently transfected with empty plasmid (C), WT Cdc37-FLAG or indicated mutants. Cdc37-FLAG proteins were immunoprecipitated (IP) and the level of phospho-Ser13 examined by immunoblotting. Co-IP of CK2 and Hsp90 client kinases CDK4, Raf-1, and ULK1 were also examined by immunoblotting.(D) Total protein lysates were prepared from HEK293 cells transiently transfected with empty plasmid (C), WT PP5-c-myc, or indicated mutants. Cdc37, phospho-Ser13-Cdc37, GR and phospho GR-211 protein levels were examined by immunoblotting.

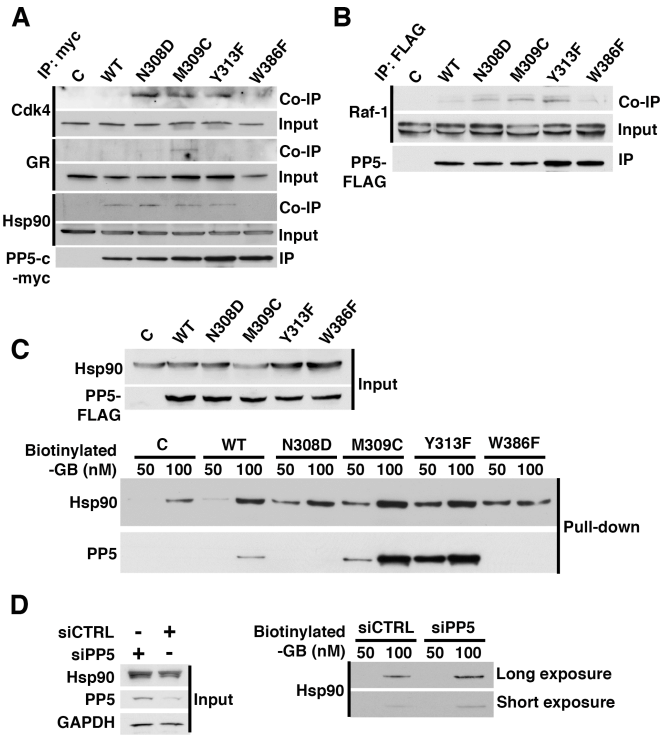

Fig. 4. Inactive mutants of PP5 cause client proteins to be stalled in the Hsp90 chaperone complex *in vivo* (A) Empty plasmid (C), WT PP5-c-myc, or indicated mutants were transiently transfected in HEK293 cells. PP5 proteins were immunoprecipitated (IP). Co-IP of Hsp90, CDK4, GR were examined by immunoblotting. (B) Immunoprecipitation (IP) of empty plasmid (C), PP5-FLAG WT, or mutants from transiently transfected HEK293 cells were immunoblotted for co-IP of Raf-1. (C) Empty plasmid (C), PP5-FLAG WT or mutants were transiently expressed in HEK293 cells. Lysates were incubated with 50 or 100 nM biotinylated-ganetespib (GB). Using streptavidin beads, Hsp90 and PP5 bound to the drug were isolated and immunoblotted. (D) HEK293 were transiently transfected with control and PP5 siRNA. Lysates were incubated with indicated amounts of biotinylated-ganetespib (GB). Using streptavidin beads, Hsp90 bound to the drug was isolated and immunoblotted.

trapped substrate, since all residues and active site waters are in orientations that are suitable for the in-line nucleophilic attack proposed for the catalytic mechanism (17).

The majority of H-bonds that determine the substrate conformation are from the substrate backbone amide groups, with all residues except His9 and Glu11 making contributions (Figure 1d). In the phosphatase domain, these interactions are contributed by PP5-Arg275, Asn308, Tyr313, Arg400 and Tyr451. Of these residues, Arg275 and Arg400 are also involved in the catalytic mechanism, and Tyr451 is part of the β12-13 loop, whose conformation is sensitive to the binding of some toxin inhibitors (16). The main-chain of the phosphomimetic Cdc37-S13E is coordinated via both its amino and carbonyl groups to the hydroxyl of PP5-Tyr313.

The peptide adopts an 'in-out-in-out' conformation, with the side-chains of His9, Glu11 and Glu14 orienting towards, and Ile10, Val12 and Asp15 orienting away from the surface of the PP5 catalytic domain. The side-chains of His9, Glu11 and Asp14 make direct or water-mediated H-bonds to PP5. Cdc37-His9 takes a highly unusual rotamer conformation to make a direct H-bond with the hydroxyl group of Tyr313. Cdc37-Glu11 is buried in a deep groove and makes water-mediated hydrogen bonds with the backbone carbonyl groups of PP5-Pro375 and Pro376. The carboxylate group of Asp14 makes water-mediated interactions with the main-chain of Val429 and the side-chain of Glu428 within the PP5 acidic groove. Significant van der Waals interactions are contributed from PP5-Trp386, Met309, Ile312 and Asn308.

Conservation Analysis of the PP5–Cdc37 Interaction

In addition to almost complete conservation of the catalytic residues within the PPP family of serine/threonine phosphatases (3), the substrate-binding cleft in PP5 is strongly conserved, both amongst PP5 homologues (Figure 2a) and between PPP family members (Figure 2b). A detailed analysis reveals that the interactions that dictate Cdc37 backbone conformation involve either PP5 main-chain groups, whose orientation is likely to be invariant in PP5 homologues, or side-chains with greater than 98% sequence identity (out of 69 homologues analysed) (Figure 2c, Table S2, Fig. S2a). In addition, since sequence and structural conservation of PPP catalytic domains is extremely high (Fig. S2b, S2c), interactions involving backbone groups are likely to be conserved not only amongst PP5 homologues, but also between PPP family members (Figure 2d). This striking conclusion also holds for the side-chains that interact with the substrate main-chain: these residues have greater than 79% sequence similarity amongst 87 PPP family members analysed (Figure 2d, Table S2, Fig. S2c). The only exception to this strong conservation is PP5-N308: while there is a predominant ability of the residue at this position to form hydrogen-bonds (94% of PP5 homologues, 99% of PPP family members) Asn308 is not highly conserved, either within the PPP superfamily or even the PP5 family (Fig. S2a, S2b). Therefore there is a greater likelihood of differences in the backbone conformation of the substrate in the -3 position. We conclude from this analysis that it is possible that many PPP substrates that occupy the hydrophobic and C-terminal grooves

will adopt the main-chain conformation seen for Cdc37, at least from the -2 to +2 position.

The Cdc37 side-chains Glu11 and Asp14 at the -2 and +1 positions respectively both contribute water-mediated hydrogen bonds with PP5, and sit in grooves that are conserved and spacious relative to the size of the side-chain itself. Analysis of the hydrophobic nature of these grooves (Fig. S2d) and crystal structures of the toxin inhibitors of the PPP family illustrate that both polar and non-polar groups can be found in each of these sites (13-15). Therefore it is likely that PP5, and indeed members of the PPP superfamily in general, can accommodate substrates with side-chains of different sizes and properties at the -2 and +1 positions, and that water molecules will play a role in moulding the substrate to the enzyme.

Mutational Analysis of PP5-Substrate Interactions *in Vitro* and *in Vivo*

In order to test our structure and conservation-based predictions of the relative importance of residues in both the enzyme and the substrate for determining specificity, we introduced a series of mutations in full-length PP5 and Cdc37 and assessed their activity in a dephosphorylation reaction in the presence or absence of Hsp90 (Figure 3a, Fig. S3a). PP5 residues previously identified as involved in coordination of phosphate or Mn^{2+} ions, or proposed to have a role in the mechanism of dephosphorylation, were not modified. To confirm that the chosen mutations did not disrupt the catalytic mechanism of PP5, all PP5 mutants were initially tested for their ability to dephosphorylate *p*NPP, a commonly used small molecule for assaying non-specific phosphatase activity (Fig. S3b). With the exception of W386F (hyperactive) and Y313F (approximately half as active as the wild-type (WT)), all other mutants displayed similar kinetics of dephosphorylation to the WT, indicating that the mutations did not cause significant structural disruption to the active site.

Mutations of PP5 residues that are involved in direct coordination (N308D, Y313F) or providing van der Waals interactions (M309C) to the Cdc37 substrate main-chain result in loss of activity, indicated by phospho-Cdc37-Ser13 detection using purified proteins *in vitro*, whereas the conservative mutation N308Q has no effect on Cdc37-Ser13 dephosphorylation (Figure 3a). For the substrate, all of the changes to residues that interact, either directly or through water-mediated interactions with PP5 (H9A, E11A/Q, and D14N) results in loss of dephosphorylation. By contrast mutation of the conserved residue Ile10 to alanine, whose side-chain is oriented away from the surface of PP5, has no effect on dephosphorylation. A careful balance of polar and charged groups is essential in directing the PP5-Cdc37 interaction since the mutations PP5-N308Q and Cdc37-E11D have minimal effect on dephosphorylation, whereas altering the electrostatic environment with the mutations PP5-N308D or Cdc37-E11Q, cause loss of activity.

To investigate the consequences of these mutations for the substrate - phosphatase interaction *in vivo*, FLAG-Cdc37 wild-type and mutants (H9A, E11A, D14N) (Figure 3b,3c) were transiently expressed and immunoprecipitated from HEK293 cells.

Cdc37 mutants that were not dephosphorylated *in vitro* (H9A, E11A, D14N), lose their interaction with PP5 *in vivo* while retaining wild type-like interaction with Hsp90 (Figure 3b). Reciprocal co-immunoprecipitation experiments show that this is a consequence of the direct interaction between PP5 and Cdc37, as the Cdc37 mutations do not alter the interaction between PP5 and Hsp90 (Fig. S3c).

However, loss of interaction with PP5 does not result in an increase of phospho-Cdc37-Ser13. Instead phosphorylation of Cdc37 is diminished (Figure 3c). A positive feedback loop between CK2 and Cdc37 regulates the activation of the former, and the phosphorylation of the latter (18, 19). His9, Glu11 and Asp14 form part of the highly conserved N-terminal region of Cdc37 (Fig. S3d) that is required for client-kinase binding to Cdc37 (9, 20-23). These mutations disrupt this feedback mechanism, diminishing or preventing phosphorylation of Cdc37 by CK2 because they prevent recruitment of CK2 to Cdc37 (Figure 3c), despite not overlapping with the CK2 consensus sequence (SXXE/D) (Fig. S3d) or residues implicated in direct association with the client kinase B-Raf (9). Consequently recruitment of ULK1, Raf-1 and Cdk4, known Hsp90 client-kinases, is also affected, as this in turn is dependent on Cdc37 phosphorylation (Figure 3c). These results emphasize the essential nature of the polypeptide sequence surrounding Cdc37-Ser13 in client-kinase activation, and expand the known residues in this N-terminal region that are essential for productive client-kinase chaperoning (9, 20).

Mutations in the catalytic cleft of myc-tagged PP5 (N308D, M309C, Y313F, W386F) strongly diminish dephosphorylation of phospho-Cdc37-Ser13 in cells, when compared to myc-tagged WT PP5 (Figure 3d), as seen *in vitro*, as well as impairing PP5's ability to dephosphorylate phospho-GR-Ser211, another known target of PP5 discussed in detail later (2). This data is in agreement with our previous work in which overexpression of PP5 in HCT116 colon cancer cells enhanced Cdc37 dephosphorylation (8).

Implications for Hsp90-Dependent Activation of Client Proteins

PP5-mediated dephosphorylation of Cdc37 is an essential step in the activation of Hsp90-dependent kinases. Dysregulation of this step, by overexpression of PP5 with concomitant reduction in phospho-Cdc37-Ser13, results in reduced Raf-1 activity in HCT116 cells and in yeast both the overexpression and deletion of the orthologue, Ppt1, causes reduced v-Src activity (8). In order to understand how Cdc37 dephosphorylation affects the activation mechanism of Hsp90-dependent kinases we investigated the impact of PP5 mutants on chaperoning of the kinase clients Cdk4 and Raf-1. The PP5 mutants were immunoprecipitated and their interaction with Cdk4 and Hsp90 was assessed by immunoblotting. Cdk4 did not co-immunoprecipitate with WT PP5, presumably due to its dynamic interaction within the Hsp90 chaperone cycle. However, the N308D, M309C and Y313F PP5 mutations, that prevent the dephosphorylation of Cdc37 *in vitro* and *in vivo*, trap the kinase in the Hsp90-PP5 complex (Figure 4a). We also observed similar results with PP5 mutants' interaction with Raf-1 (Figure 4b). Co-immunoprecipitation of Hsp90 with PP5 mutants at steady state shows that they bind to Hsp90 with similar affinity, with the exception of the W386F mutant, which is hyperactive, resulting in a transient interaction (Figure 4a). These results provide mechanistic details that elaborate our previous work (8) demonstrating that kinase release from the chaperone complex is critically dependent on Cdc37 dephosphorylation by PP5.

Hyperactivity or hypoactivity of PP5 mutants enhances Hsp90 binding to drug

It is well established that increased binding of Hsp90 to its inhibitors is associated with sensitivity of cells to Hsp90 drugs (24, 25). We have previously shown that overexpression of the yeast PP5 ortholog (Ppt1) sensitizes cells to the Hsp90 inhibitor geldanamycin (8). We reasoned that PP5 mutants might influence Hsp90 inhibitor association therefore, we expressed FLAG-PP5 WT and mutants in HEK293 cells followed by biotinylated-ganetespib (biotinylated-GB) pulldown experiments. Our data showed that the hypoactive (N308D, M309C and Y313F) and hyperactive (W386F) PP5 mutants significantly enhanced Hsp90 binding to biotinylated-GB (Figure 4c). Although PP5 mutants bound with similar affinity as the WT PP5 to Hsp90 at steady state (Figure 4a), these mutants differentially impacted Hsp90 binding to its inhibitor ganetespib. Additionally, once Hsp90 was bound to biotinylated-GB, it formed different complexes with the PP5

mutants (Figure 4c). We obtained further evidence to support our hypothesis by down regulating PP5 using siRNA (Figure 4d, Fig. S4). These data suggest that the activity of PP5 is a determining factor for Hsp90 affinity to its inhibitor.

Implications for PP5 Specificity

Having established that particular residues in the catalytic cleft are necessary for Cdc37 recognition, the question arises whether these residues are able to confer specificity of phosphatase activity, i.e. does their mutation have differential effects on different substrates? PP5 also dephosphorylates GR, another Hsp90 client, at Ser203, Ser211 and Ser226 and subsequently controls its activity and nucleo-cytoplasmic translocation (2). To examine specificity we therefore investigated the consequences of mutations in the PP5 catalytic cleft on its ability to dephosphorylate GR-Ser211. Mutations of PP5-M309C and Y313F prevent dephosphorylation of GR-Ser211, as observed with Cdc37 (Figure 3d). In addition, an equivalent trapping of GR on Hsp90-containing PP5 complexes is observed for the M309C mutation (Figure 4a). The Y313F mutation does not result in a trapped GR-complex, but as this mutant has an activity profile that is distinct from the other mutants (Fig. S3b) this may be a result of unpredicted structural changes in the vicinity of the substrate-binding site. However, unlike the case for Cdc37, the PP5-N308D mutant had no effect on dephosphorylation of GR-Ser211. In the PP5-Cdc37 structure the side-chain of Asn308 provides interactions with the backbone carbonyl of Cdc37-Ile10 and the carboxylate of Glu11, as well as van der Waals contacts to Glu11. The equivalent position for GR phospho-Ser211 substrate is Asn209 (sequence TNEpSPWR), therefore the PP5-N308D mutant may provide similar electrostatic interactions to WT PP5. In agreement with our structural analysis, the contrasting effect of this mutation on different substrates confirms that the binding groove on PP5 for the substrate -2 position provides a degree of specificity while providing an environment that allows a variety of side-chains to bind (Figure 1d, Fig. S2d).

Discussion

The structure presented here defines the interaction of the Cdc37 substrate with the PPP Ser/Thr phosphatase PP5. The conformation of the substrate is primarily dictated by interactions between its backbone and residues of PP5 that line the active site. Interactions between the side-chains of the Cdc37 substrate and PP5 are largely water-mediated and lie within spacious pockets that form part of the Y-shaped channel that surrounds the catalytic site. This channel is highly conserved in sequence and structure throughout the PPP superfamily and, together, the use of the main-chain of the substrate and the versatility provided by water-mediated interactions to accommodate a range of side-chains reveal how different members of this superfamily can accommodate a multitude of unrelated substrates with highly divergent sequences. In addition these two factors suggest that the substrate conformation observed here may provide a template for many substrates that occupy the hydrophobic and C-terminal grooves of the PPP superfamily.

Our structure illustrates the fundamental difference in the mechanism of substrate binding at the catalytic sites of kinases and phosphatases and shows how this is achieved on an atomic level. For kinases the sequence of the substrate is critical in ensuring specificity through highly conserved interactions dictated by the substrate side-chains (26, 27), while Ser/Thr phosphoprotein phosphatases allow sequence plasticity by providing an environment in which a variety of side-chains can be accommodated within a conserved substrate backbone conformation.

While this sequence plasticity is a key feature of PPP substrate recognition our biochemical and cell biology data indicate that, contrary to the current widespread assumption in the field, the catalytic subunit can confer some degree of local specificity. Nonetheless regulatory subunits are still fundamental in controlling the primary specificity of a Ser/Thr PPP through their role in recruiting substrates and increasing their local concentration; thus phosphatases other than PP5 cannot dephosphorylate Cdc37 because they are unable to form the complete tripartite interaction required for efficient dephosphorylation (8).

Our results delineate mechanistic details of one step in the Hsp90-dependent kinase activation cycle for Cdk4 and Raf-1. While the requirement for PP5-mediated dephosphorylation of Cdc37 was previously known to be essential for activation of several Hsp90 client kinases (8), its role at a molecular level was not understood. The results presented here identify this dephosphorylation step as a requirement for release of these clients from the Hsp90 chaperone. Previous results suggest that association of PP5 with Hsp90 chaperone complexes is accompanied by structural changes since, within a purified Hsp90-Cdc37-Cdk4 complex, the phospho-Ser13 Cdc37 substrate is inaccessible to dephosphorylation by the non-specific lambda phosphatase but readily accessible to dephosphorylation by PP5 (8). This is further supported by recent results indicating that residues in the vicinity of the Ser13 are responsible for binding client kinases (9, 22, 23) where it is likely to be buried. A conformational change would therefore be required to allow PP5 access to its substrate. It may be these conformational changes, rather than the absence of the phosphorylation itself, that are the trigger for client release, as eventual dephosphorylation by lambda phosphatase, which occurs after prolonged incubation with the complex, does not cause release of the Cdk4 client from the Hsp90-Cdc37 complex (8).

While phosphorylation of Cdc37 Ser13 is essential for client kinase activation *in vivo* (11, 12) and *in vitro* (28) its mechanistic role remains enigmatic. In the cell the interaction of client kinase with Cdc37 and its subsequent recruitment to Hsp90 is likely to always occur with the phospho-Ser13 Cdc37, as CK2 is constitutively active. Phospho-Ser13 Cdc37 has a more stable and compact conformation than wild-type (29), in which the phospho-Ser13 is accessible to dephosphorylation by both calf intestine alkaline phosphatase (29) and lambda phosphatase (8). However, at least for the kinase B-Raf, this conformation is not a requirement for either client versus non-client recognition (9) or subsequent association with Hsp90 as a stable complex between Hsp90, Cdc37 B-Raf can be formed from the individual proteins *in vitro* in the absence of phosphorylation (22, 23) although phospho-Ser13 enhances its stability (22). This is consistent with recent data indicating that the primary interaction of Cdc37 with B-Raf is via a distinct C-terminal domain of Cdc37 and is remote from this site of phosphorylation (9, 23). Nonetheless our results augment the set of known, non-overlapping residues, residing in a relatively short stretch of the Cdc37 polypeptide that encompasses Ser13, responsible for interactions that regulate Cdc37 chaperone function, namely CK2 phosphorylation (11, 12), client-kinase recognition *in vitro* (9), client-kinase complex formation *in vivo* (20), and PP5 dephosphorylation.

PP5-mediated dephosphorylation of Cdc37 is one of a number of modifications that control progression of the chaperone cycle, either by affecting cochaperone or client association. Phosphorylation of Y197 on Hsp90 dissociates Cdc37 from Hsp90 immune complexes, while phosphorylation of Y313 stimulates association with Aha1 (21), the cochaperone required for the enhancement of Hsp90's ATPase activity and completion of one round of the chaperone cycle (30). Indeed these two cochaperones are not found in the same immune complexes (21), highlighting the degree of control imposed by these post-translational modifications (PTMs).

We have identified the trapping of GR, a Cdc37-independent Hsp90 client, on the Hsp90 chaperone when PP5 activity is abrogated, showing that PP5 activity exerts influence on the chaperone

cycle regulation beyond kinases alone. Serine/threonine phosphorylation of Hsp90 itself is, in general, found to result in reduced affinity with clients (31-33). Client kinase release is also triggered by tyrosine phosphorylation of Cdc37 on Y298 by the Src family kinase, YES, and tyrosine phosphorylation of Hsp90 on Y627 dissociates Cdk4 (21). The latter modification also dissociates Aha1 and PP5 from Hsp90 (21), and as such may be a mechanism for terminating one cycle of chaperone activity. Together these events emphasize the intricacy of the client activation process and the many layers of control provided by serine/threonine and tyrosine phosphorylation and its reversal.

Finally, this study demonstrates that both hyper- and hypoactivity of PP5 mutants enhances Hsp90 binding to its inhibitor ganetespib. Previous works have shown that PTM of co-chaperones influence their activity. It remains to be seen whether PP5 activity is also regulated by PTMs. Targeting the potential enzymes that catalyze those PTMs may influence cell sensitivity to Hsp90 inhibitors.

Methods

See SI Materials and Methods for details. Briefly, the chimera protein, comprising the catalytic domain of PP5 (residues 175-499) and a peptide comprising residues 5-20 of Cdc37, with the mutation S13E, joined by a 9-residue flexible linker was generated by PCR. The protein was purified to homogeneity using standard chromatographic techniques, and crystallised using hanging drop vapour diffusion. Data were processed using XDS (34) & Scala (35). The structure was solved by molecular replacement of PDB 1S95 using Phaser (36), and refined using Phenix Refine (37) with manual rebuilding in Coot (38). Data collection and refinement statistics are summarized in Table S1. *In vitro* dephosphorylation was assessed using anti-phosphoSerine 13 antibody (Sigma). Immunoprecipitation assays in transiently transfected HEK293 cells were carried out using anti-FLAG (Sigma) or anti-myc (ThermoScientific) antibody conjugated beads. Biotinylated ganetespib (Synta Pharmaceuticals) and streptavidin agarose (ThermoScientific) were used for drug binding assays. siRNA knock-down of PP5 (Origene) was carried out using standard methods.

Author Contributions

JO cloned constructs, purified and crystallised the protein, solved and refined the structure, and carried out *in vitro* biochemical assays; LM cloned the chimera construct; DD, JS, MW and DB carried out *in vivo* cell biology assays; all authors contributed to study design, data analysis and interpretation; JO, DD, MM and CKV wrote the manuscript.

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