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The phosphatase Ppt1 is a dedicated regulator of the molecular chaperone Hsp90

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Ppt1 is the yeast member of a novel family of protein phosphatases, which is characterized by the presence of a tetratricopeptide repeat (TPR) domain. Ppt1 is known to bind to Hsp90, a molecular chaperone that performs essential functions in the folding and activation of a large number of client proteins. The function of Ppt1 in the Hsp90 chaperone cycle remained unknown. Here, we analyzed the function of Ppt1 *in vivo* and *in vitro*. We show that purified Ppt1 specifically dephosphorylates Hsp90. This activity requires Hsp90 to be directly attached to Ppt1 via its TPR domain. Deletion of the *ppt1* gene leads to hyperphosphorylation of Hsp90 *in vivo* and an apparent decrease in the efficiency of the Hsp90 chaperone system. Interestingly, several Hsp90 client proteins were affected in a distinct manner. Our findings indicate that the Hsp90 multichaperone cycle is more complex than was previously thought. Besides its regulation via the Hsp90 ATPase activity and the sequential binding and release of cochaperones, with Ppt1, a specific phosphatase exists, which positively modulates the maturation of Hsp90 client proteins.

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Introduction

Ppt1 is a protein phosphatase from *Saccharomyces cerevisiae* that belongs to the PPP family of serine/threonine phosphatases (Becker *et al*, 1994; Chen *et al*, 1994; Chinkers, 1994). Although Ppt1 was discovered a decade ago, its function in yeast is still unclear. It does not appear to be essential for viability, as a *ppt1*Δ yeast strain shows no significant phenotype, even under a variety of stress conditions (Chen *et al*, 1994). The human homolog of Ppt1 is the phosphatase PP5, exhibiting a sequence identity of 63% (Chen *et al*, 1994). For both phosphatases, complex formation with the molecular chaperone Hsp90 was demonstrated *in vitro* and *in vivo* (Chen *et al*, 1996; Silverstein *et al*, 1997; Russell *et al*, 1999; Gavin *et al*, 2002; Ramsey and Chinkers, 2002; Yang *et al*, 2005). Interaction of PP5 and Hsp90 is mediated by the tetratricopeptide repeat (TPR) domain of PP5 (Chen *et al*, 1996; Silverstein *et al*, 1997; Yang *et al*, 2005). The presence of a TPR domain in PP5 and Ppt1 is unique among phosphatases (Cohen, 1997; Andreeva and Kutuzov, 1999; Chinkers, 2001). It consists of three motifs of degenerated 34-amino-acid repeats, each made up of two antiparallel α-helices connected by a short loop (Das *et al*, 1998). Consecutive motifs combine to a superhelical groove, which can bind TPR acceptor modules. The TPR domain interacts with a common TPR acceptor site, the C-terminal EEVD residues of Hsp90 (Silverstein *et al*, 1997; Scheufler *et al*, 2000; Ramsey *et al*, 2000; Yang *et al*, 2005). This is also the case in other Hsp90 partner proteins containing TPR domains like the large peptidyl prolyl *cis/trans* isomerase (PPIases) (Radanyi *et al*, 1994; Owens-Grillo *et al*, 1996) and the Hsp90/Hsp70 organizing protein Hop (Honore *et al*, 1992; Smith *et al*, 1993; Scheufler *et al*, 2000). In fact, PP5 and the large PPIases FKBP52 and Cyp40 compete for binding to mammalian heat-shock protein 90 (hHsp90) (Silverstein *et al*, 1997). The TPR domain resides in the N-terminal part of Ppt1/PP5, in front of the C-terminal catalytic domain (Chen *et al*, 1994; Yang *et al*, 2005).

The low basal phosphatase activity of PP5/Ppt1 is due to autoinhibition by the TPR domain and the C-terminal αJ subdomain (Chen and Cohen, 1997; Sinclair *et al*, 1999; Kang *et al*, 2001; Swingle *et al*, 2004). The recent determination of the three-dimensional structure of PP5 showed that the TPR domain of PP5 blocks the catalytic cavity (Yang *et al*, 2005). This autoinhibited conformation is stabilized by the C-terminal subdomain. Binding of a ligand to the TPR domain is thought to destabilize the TPR–phosphatase domain interaction and to ultimately lead to a complete activation of the phosphatase. For PP5, activation can be achieved by Hsp90, a C-terminal 12 kDa fragment of Hsp90 or an 8-amino-acid C-terminal peptide (Ramsey and Chinkers, 2002; Yang *et al*, 2005).

Hsp90 is a ubiquitous and abundant molecular chaperone (Young *et al*, 2001; Picard, 2002; Prodromou and Pearl, 2003; Pratt and Toft, 2003; Wegele *et al*, 2004; Whitesell and Lindquist, 2005). Under physiological conditions, a broad set of proteins depends on Hsp90 for reaching their native conformations. Many of these Hsp90 substrates are involved in signaling pathways, such as steroid hormone receptors and kinases (reviewed in Pratt and Toft (2003) and Wegele *et al* (2004)). Substrate activation by the Hsp90 multichaperone machinery is accompanied by the sequential binding and release of Hsp90 partner proteins or cochaperones, respectively (Smith, 1993).

The finding that a phosphatase is present in complexes of Hsp90 with its client proteins (Chen *et al*, 1996; Silverstein *et al*, 1997) raised the question of what the function of Ppt1

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is. Competition between PP5 and the PPIases FKBP52 or Cyp40 for binding to Hsp90 suggests a role of PP5/Ppt1 in the late steps of the Hsp90 multichaperone cycle (Silverstein *et al*, 1997). Three different functions seemed plausible: (i) *the dephosphorylation of components of the Hsp90 machinery*: Many components of the Hsp90 multichaperone cycle are phosphoproteins: p23/Sba1 (Johnson *et al*, 1994), Cdc37 (Shao *et al*, 2003), Hop/Sti1 (Lassle *et al*, 1997; Longshaw *et al*, 2000) and FKBP52 (Miyata *et al*, 1997); (ii) *the dephosphorylation of Hsp90*: Hsp90 itself is phosphorylated at several serin/threonine residues (Garnier *et al*, 2001); and (iii) *the unspecific dephosphorylation of proteins*.

Here, we analyzed the function of Ppt1 in the yeast heat-shock protein 90 (yHsp90) chaperone system, both *in vitro* and *in vivo*. Our results show that Ppt1 specifically dephosphorylates yHsp90. Furthermore, *ppt1*Δ yeast cells exhibit an insufficient maturation of the several client proteins of Hsp90 investigated here. This is the first evidence that a phosphatase specifically modulates the chaperoning properties of Hsp90 by modifying its phosphorylation state.

Results

The TPR domain is required for complex formation between Ppt1 and yHsp90

In the first set of experiments, we determined whether Ppt1 interacts with Hsp90 in a similar way as mammalian PP5/Hsp90 (Chen *et al*, 1996; Russell *et al*, 1999; Ramsey *et al*, 2000; Gavin *et al*, 2002; Yang *et al*, 2005). Employing isothermal titration calorimetry (ITC) (Figure 1A), we obtained a dissociation constant K_D for the complex of ~670 nM with a binding stoichiometry of 0.82 molecules Ppt1 per yHsp90 monomer. With yHsp90 being a dimer and Ppt1, like PP5 (Das *et al*, 1998; Yang *et al*, 2005), being a monomeric enzyme (data not shown), the ITC data show that two Ppt1 molecules bind to a yHsp90 dimer.

PP5 was found to bind the common TPR acceptor site of Hsp90, the C-terminal amino acids of Hsp90, and competes with other TPR-containing Hsp90 partner proteins (Chen *et al*, 1996; Silverstein *et al*, 1997; Ramsey *et al*, 2000; Yang *et al*, 2005). For a further analysis of the interaction of Ppt1 and yHsp90, fragments of Ppt1 were generated (Figure 1B) and used in a modified ELISA to determine the association of Ppt1 and Ppt1 fragments with yHsp90 (Figure 1C). Both Ppt1 and the TPR fragment retained FITC-labeled yHsp90. No interaction was observed for the phosphatase fragment, suggesting that the first 170 amino acids of Ppt1 are sufficient for the interaction of Ppt1 and yHsp90. For a truncation fragment of Hsp90, which contains only the C-terminal domain including the TPR acceptor site, the same result was observed as for the full-length yHsp90. This demonstrates that the C-terminal domain of yHsp90 is sufficient for the interaction with Ppt1. A longer fragment of Ppt1 (amino acids 118–513), exclusively lacking the TPR domain, showed no interaction with Hsp90 in a co-immunoprecipitation experiment (data not shown).

Therefore, the binding of Ppt1 and Hsp90 is, like that of PP5, based on the TPR domain binding to the C-terminal end of Hsp90. In a yeast two-hybrid experiment, the interaction of Hsp90 with the TPR fragment of Ppt1 was also confirmed *in vivo* (data not shown).

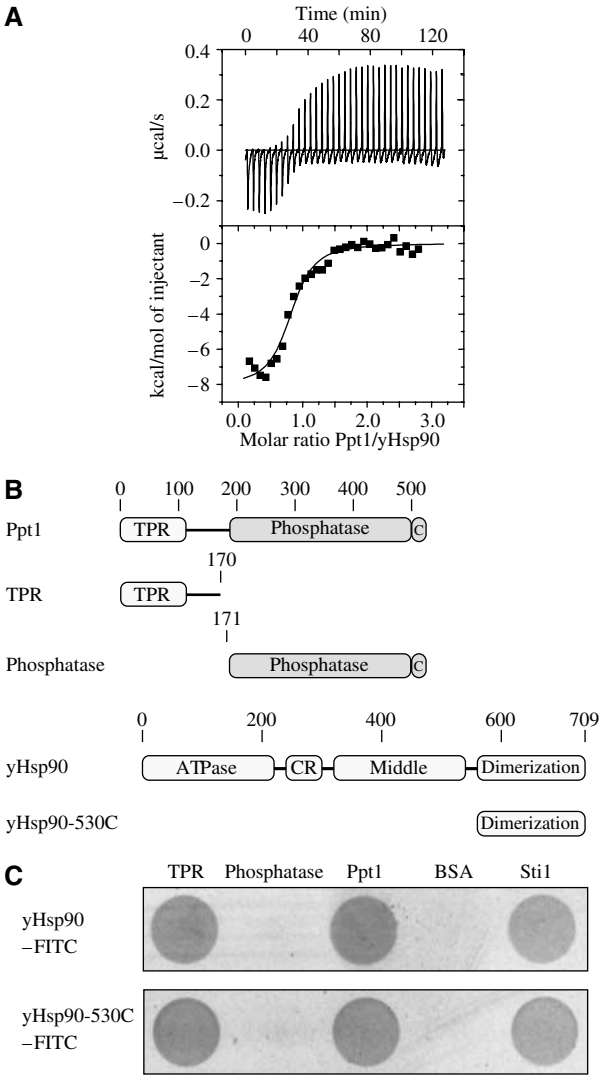

Figure 1 Interaction of Ppt1 and yHsp90. (**A**) Calorimetric analysis of the Ppt1–yHsp90 interaction. ITC was performed at 25°C. Data were analyzed using the Origin software package provided by the manufacturer. The upper panel shows the change in heat upon injection of Ppt1 (273 µM in the syringe) to yHsp90 (16 µM) in the cell. The lower panel shows the derived binding curve. The filled squares correspond to the integration of the peaks of the upper panel. (**B**) Schematic representation of the Ppt1 and yHsp90 fragments used. Ppt1 consists of two domains, the TPR (amino acids 12–113), and the catalytic domain (amino acids 188–513). The C-terminal subdomain is denoted as C. The TPR fragment ranges from amino acids 1 to 170, the phosphatase fragment from amino acids 171–513. (**C**) ELISA-based analysis of the interaction of the full-length Ppt1 and its fragments with the full-length yHsp90 (top panel) and the C-terminal yHsp90 fragment 530C (bottom panel). Proteins denoted in the top line were coated on the ELISA plate as described in Materials and methods. FITC-labeled yHsp90/530C was employed to detect interactions by recording the FITC fluorescence. BSA and Sti1 served as negative and positive control, respectively.

Enzymatic properties of autoinhibited Ppt1 and its catalytic domain

The enzymatic properties of Ppt1 were analyzed using the artificial phosphatase substrate *p*-nitrophenylphosphate (*p*NPP) (Ramsey and Chinkers, 2002). The pH optimum of Ppt1 was at pH 7.8 (data not shown), which is close to the published pH optimum of PP5 (Ramsey and Chinkers, 2002). The K_M value of Ppt1 for *p*NPP was determined by recording

the kinetics at increasing substrate concentrations. A representative example is shown in Figure 2A. The K_M value derived from the substrate titration curve was calculated to be ~27 mM and the turnover number k_{cat} was 7 min^{-1} (Figure 2B). These values are similar to data obtained for PP5 (Skinner *et al*, 1997; Kang *et al*, 2001). The high K_M value mirrors the problem that *p*NPP is an artificial substrate, which does not mimic all aspects of phosphorylated Ser/Thr residues in the context of a protein. Specific protein substrates of Ppt1 are likely to be dephosphorylated with higher affinity.

To test whether Ppt1 is regulated by autoinhibition similar to PP5 (Chen and Cohen, 1997; Sinclair *et al*, 1999; Kang *et al*, 2001; Swingle *et al*, 2004), a fragment of Ppt1 lacking the TPR domain (Figure 1B) was analyzed. The k_{cat} of this phosphatase fragment was ~47 min^{-1} (Figure 2C), and thus, ~7-fold higher than that of the full-length Ppt1. This corresponds to the stimulation observed for PP5. Binding of the full-length yHsp90 also resulted in a stimulation (data not shown) similar to that observed for PP5 (Yang *et al*, 2005).

Ppt1 specifically dephosphorylates Hsp90

The authentic *in vivo* substrate proteins of Ppt1 were unknown so far. The observed interaction of Ppt1 with yHsp90, however, suggested that components of the Hsp90 chaperone system could be potential Ppt1 substrates, especially as several are phosphoproteins. To assay whether Ppt1 was able to dephosphorylate these protein substrates, Hsp90 and its partner proteins were phosphorylated by casein kinase II (CKII) in the presence of [γ-^{32}P]ATP. The phosphorylated proteins were then used as substrates for Ppt1. Remarkably, for Hsp90, we observed a specific removal of radioactive phosphate in the presence of Ppt1 (Figure 3A). In sharp contrast, addition of Ppt1 to all other radiolabeled proteins of the yHsp90 chaperone cycle did not result in a significant decrease of phosphorylation (Figure 3B).

As Hsp90 is known to stimulate the phosphatase activity of PP5/Ppt1 (Ramsey and Chinkers, 2002; Yang *et al*, 2005), dephosphorylation of the Hsp90 cochaperones was further analyzed in the simultaneous presence of both Ppt1 and yHsp90. However, also under these conditions, Ppt1 was not able to dephosphorylate Hsp90 partner proteins (Figure 3D).

In addition, we tested the effect of Ppt1 on the phosphorylation state of Hsp104 and Ssa1, two chaperone proteins with C-terminal ends homologous to that of Hsp90. For Hsp104, we employed a mutant ($Hsp104_{TRAP}$) lacking ATPase activity (Bosl *et al*, 2005). The Hsp70 protein Ssa1 could not be phosphorylated *in vitro*. Therefore, we used Ssa1 isolated from *S. cerevisiae* and phosphorylation specific stains. For both proteins, the addition of Ppt1 did not result in changes of the phosphorylation signal (Figure 3C; data not shown).

Finally, two standard substrate proteins for phosphatases, casein and myelin basic protein (MBP), that had previously been demonstrated to be dephosphorylated by Ppt1 were analyzed as targets for Ppt1 (Figure 3E). Consistent with published data (Jeong *et al*, 2003), we see a slight influence of Ppt1 on the phosphorylation state of casein and MBP. In contrast, dephosphorylation of Hsp90 performed in parallel was complete within 90 min. Again, dephosphorylation was

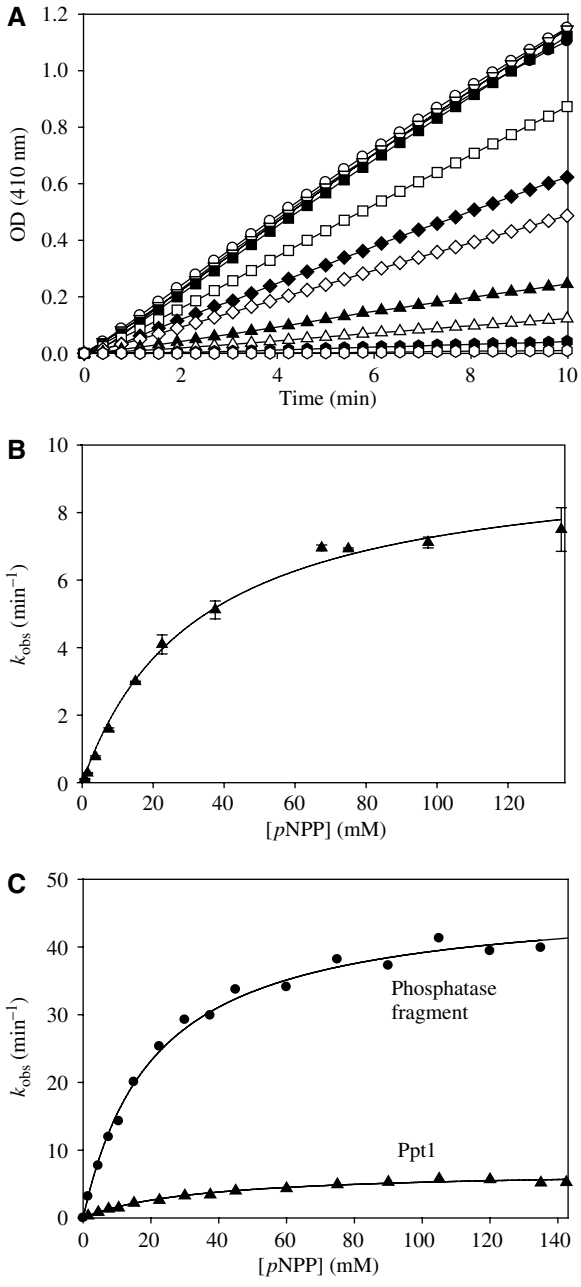

Figure 2 Analysis of the enzymatic properties of Ppt1 for the substrate *p*NPP. (**A**) Titration of Ppt1 with increasing concentrations of *p*NPP. The substrate titration was performed at the optimal pH value of 7.8 as described in Materials and methods. *p*NPP was added up to 150 mM. The symbols in (A) correspond to increasing concentrations of *p*NPP: open hexagon: 0.75 mM *p*NPP; filled hexagon: 1.5 mM *p*NPP; open triangle up: 3.75 mM *p*NPP; filled triangle up: 7.5 mM *p*NPP; open diamond: 15 mM *p*NPP; filled diamond: 22.5 mM *p*NPP; open square: 37.5 mM *p*NPP; filled square 67.5 mM *p*NPP; open triangle down: 75 mM *p*NPP; filled triangle down: 97.5 mM *p*NPP; open circle: 135 mM *p*NPP; and filled circle: 148 mM *p*NPP. This kind of substrate titration was used to obtain the reaction kinetics of Ppt1 as shown in (B). (**B**) Reaction kinetics of Ppt1 (1 μM) with *p*NPP. Shown is the average of a triple experiment (▲) together with the standard error (bars). The black curve corresponds to the regression of the Michaelis–Menten equation. The formation of *p*NP was recorded photometrically at 410 nm to obtain the reaction velocity as described in Materials and methods. (**C**) Comparison of the reaction kinetics of Ppt1 (▲) and the phosphatase fragment (●). Both titrations were performed as in (A). The k_{cat} of the phosphatase fragment (~47 min^{-1}) is approximately seven-fold higher than that of the full-length Ppt1 (~7 min^{-1}).

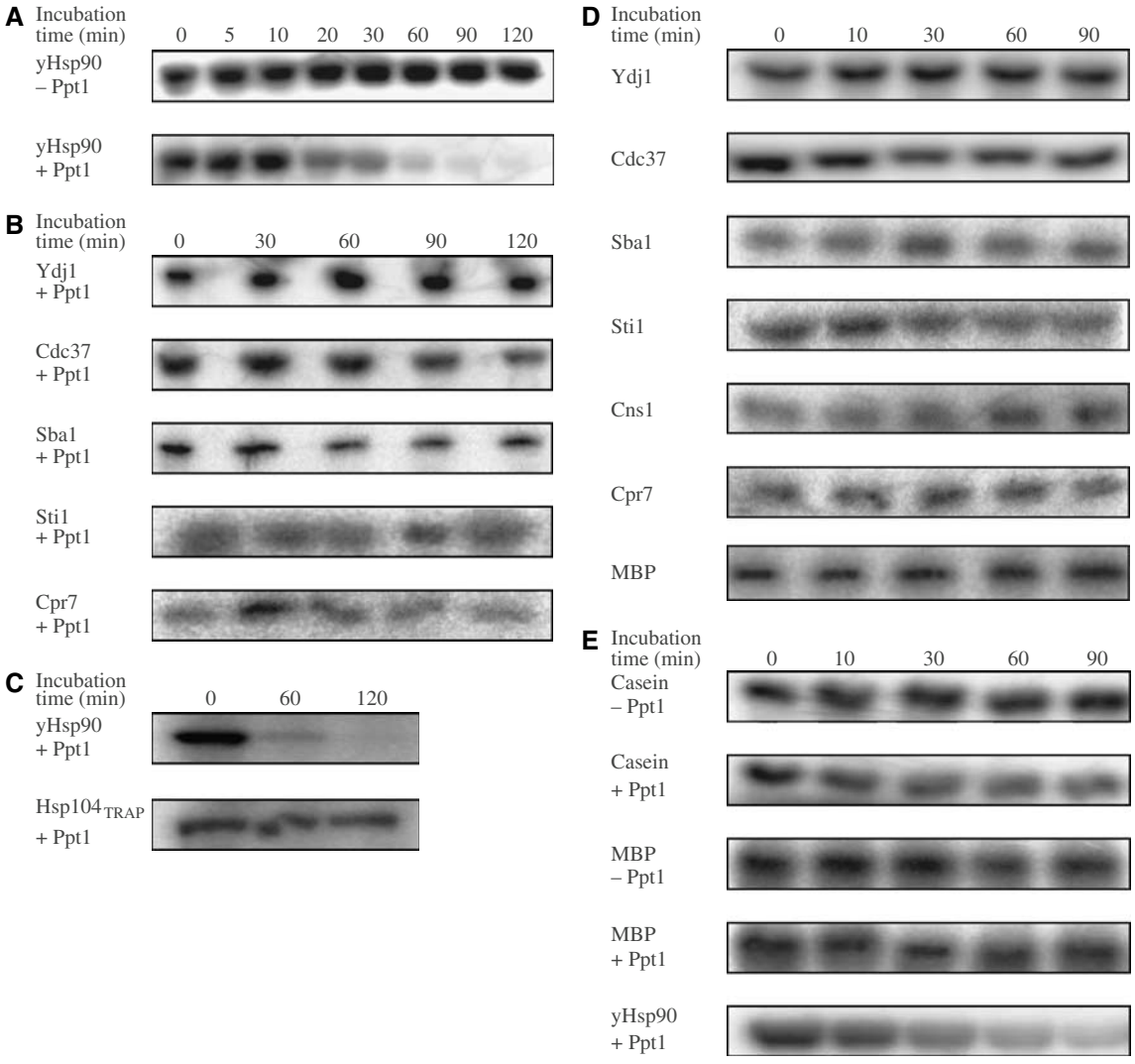

Figure 3 Analysis of the activity of Ppt1 towards phosphorylated proteins of the yHsp90 system. (**A**) Activity of Ppt1 (1 μM) towards yHsp90 (1 μM). Hsp90 was prepared using CKII and [γ-^{32}P]ATP as described in Materials and methods. After labeling, apyrase was added to hydrolyze the remaining ATP before Ppt1 was added. Ppt1 addition was omitted in the experiment shown in the top panel of (A) to demonstrate the stability of the phosphorylation. (**B**) Activity of Ppt1 towards partner proteins of yHsp90. Hsp90 partner proteins were prepared as in (A). (**C**) Activity of Ppt1 towards $Hsp104_{TRAP}$. The assay was performed as in (A). (**D**) Activity of Ppt1 towards partner proteins of yHsp90 in the presence of yHsp90. The assay was performed as in (B), except that prior to addition of Ppt1, yHsp90 was added. (**E**) Activity of Ppt1 towards casein and MBP. Proteins were labeled as described in (A). Ppt1 addition was omitted in the experiments shown in the top panels (denoted as −Ppt1). As a comparison, the yHsp90 dephosphorylation is shown in the lowest panel.

also not observed in the presence of both Ppt1 and Hsp90 (Figure 3D and data not shown).

Taken together, these experiments demonstrate that, surprisingly, the phosphatase activity of Ppt1 is specific for Hsp90. The presence of Hsp90 does not render Ppt1 capable of dephosphorylating other proteins.

The TPR interaction enables Ppt1 to dephosphorylate Hsp90

In a next set of experiments, we wished to analyze whether the TPR interaction between Ppt1 and Hsp90 is required for the observed dephosphorylation of the chaperone. Therefore, we investigated a deletion mutant of yHsp90 lacking the C-terminal TPR-binding motif (ΔMEEVD-Hsp90), which, as in the mammalian system (Ramsey *et al*, 2000), is incapable of binding Ppt1 or its TPR fragment (data not shown). Upon addition of Ppt1, no change in phosphorylation could be observed for ΔMEEVD-Hsp90 (Figure 4A). Under the same conditions, wild-type (wt) yHsp90 was almost completely dephosphorylated. Furthermore, Ppt1 was found to compete for binding with other TPR proteins for the MEEVD site of yHsp90. The presence of excess Sti1, a yHsp90 partner protein known to bind to the MEEVD residues of yHsp90 (Carrello *et al*, 1999), prevented dephosphorylation by Ppt1 (Figure 4B). An equimolar mixture of Sti1 and Ppt1 led to a reduction of the dephosphorylation by Ppt1 (data not shown). Similar results were obtained when an isolated TPR fragment of Ppt1 was added to the dephosphorylation reaction (Figure 4C). With increasing concentrations of the isolated TPR domain, the dephosphorylation of yHsp90 decreases.

Assays performed in the presence of a TPR-binding peptide showed that the peptide did not stimulate the dephosphorylation of yHsp90, but rather inhibited it (Figure 4D). This is

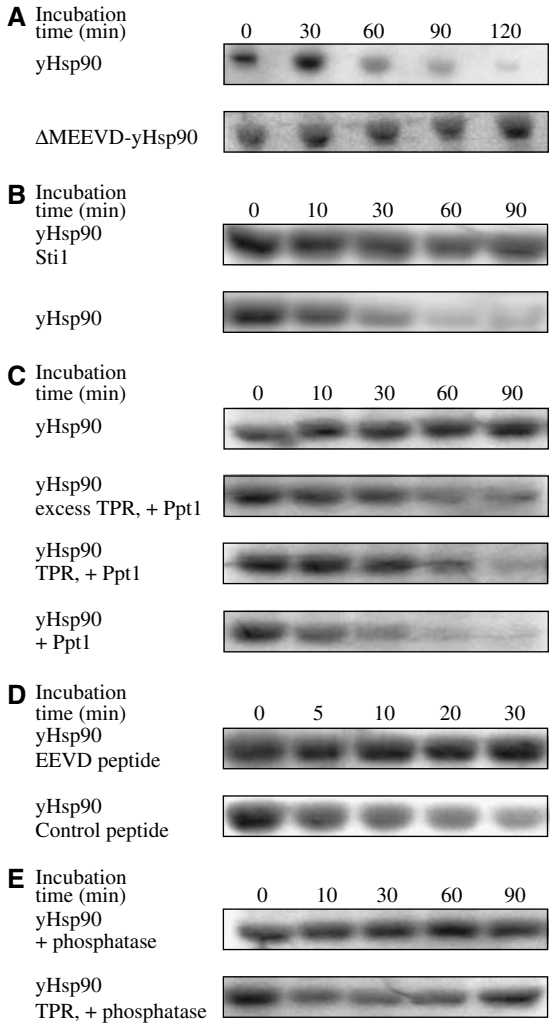

Figure 4 Investigation of the dependence of the TPR interaction in the yHsp90 dephosphorylation. (**A**) Activity of Ppt1 (1 μM) towards phosphorylated ΔMEEVD-yHsp90 (1 μM), a deletion mutant of the chaperone, lacking the C-terminal MEEVD residues necessary for binding of TPR domains. Phosphorylated proteins are always listed at the first position in the legend. (**B**) Competition of Ppt1 and Sti1 in binding to the common TPR acceptor site of yHsp90. The assay was performed as in (A). Prior to the addition of Ppt1 to phosphorylated yHsp90, Sti1 (10-fold excess) was added as indicated. (**C**) Inhibition of the phosphatase activity of Ppt1 in the presence of the TPR fragment of Ppt1 in different stoichiometries. In the experiment of the second panel, the TPR fragment was used in five-fold excess over Ppt1, while it was employed equimolar in the third panel. (**D**) Inhibition of the yHsp90 dephosphorylation by Ppt1 in the presence of an EEVD-containing peptide. Labeling of yHsp90 was performed as described in (A). Prior to addition of Ppt1, peptides were added in excess over yHsp90. Peptide sequences were PPAPEAEGPTVEEVD (EEVD peptide) and EVGLKRVVTKAMSSR (control peptide). (**E**) Analysis of the yHsp90 dephosphorylation by the phosphatase fragment of Ppt1 in the absence and presence of the TPR fragment.

best explained by saturation of the TPR domain of Ppt1 with the peptide and blocking of its interaction with yHsp90.

Finally, the activity of the isolated phosphatase domain towards yHsp90 was analyzed (Figure 4E). Although the phosphatase domain itself exhibits a ~7-fold higher activity in the *p*NPP assay, it did not dephosphorylate yHsp90. Furthermore, the activity of the phosphatase domain was

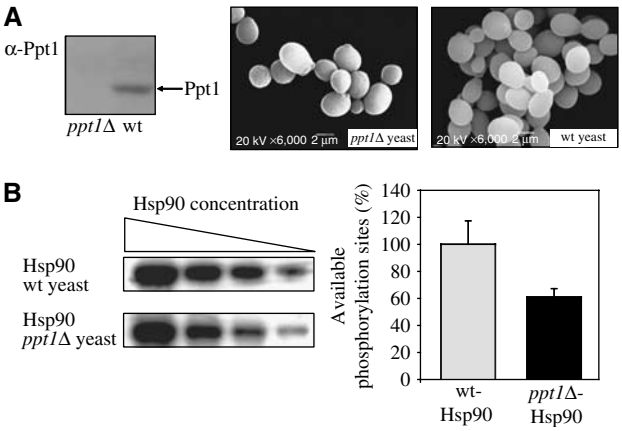

Figure 5 Analysis of the *in vivo* effect of *ppt1*Δ. (**A**) Immunoblot analysis for Ppt1 expression. Lysate of the *ppt1*Δ yeast strain (left) and the wt yeast (right) was investigated using a polyclonal rabbit serum directed against purified Ppt1 (α-Ppt1). The arrow indicates the position of Ppt1. The scanning electron microscopic images compare *ppt1*Δ yeast cells and wt yeast cells. (**B**) Radioactive phosphorylation of identical amounts of yHsp90 derived from a *ppt1*Δ yeast strain and the corresponding wt strain. Identical amounts of both proteins were treated with CKII and [γ-^{32}P]ATP to reveal the availability of phosphorylation sites. After the phosphorylation reaction was allowed to take place for 2 h, yHsp90 was separated from free ATP by SDS–PAGE. Aliquots of different protein quantities were analyzed and the relative amount of radioactivity was calculated. The graph shows the mean value corrected for the respective protein concentration. Three independent experiments gave similar results.

not restored when the isolated TPR fragment was added to the dephosphorylation reaction. These results are consistent with a model in which Ppt1 is only activated when bound to its substrate protein, Hsp90.

Hsp90 from ppt1Δ yeast cells shows difference in phosphorylation

In the next set of experiments, we investigated the relevance of Ppt1 for the Hsp90 system *in vivo*. A yeast strain with a genomic deletion of the *ppt1* gene was used and compared to the otherwise genetically identical wt strain. The absence of Ppt1 was verified by immunoblotting (Figure 5A). Deletion of the *ppt1* gene exhibits no obvious phenotype (Chen *et al*, 1994). In agreement with this observation, the *ppt1*Δ yeast cells show no morphological differences when compared to wt cells by scanning electron microscopy (Figure 5A). For investigation of the overall phosphorylation state of Hsp90, the chaperone was purified from both yeast strains, and subsequently phosphorylated with radioactive phosphate to uncover free phosphorylation sites. If Ppt1 is capable of dephosphorylating Hsp90 *in vivo*, Ppt1-sensitive phosphorylation sites of the chaperone should be occupied in yHsp90 derived from the *ppt1*Δ strain. Indeed, Hsp90 from the *ppt1*Δ strain contained only 60% of the radioactive phosphate detected for the protein from the wt strain (Figure 5B), strongly suggesting that Ppt1 dephosphorylates Hsp90 *in vivo*.

Ppt1 positively modulates the Hsp90 chaperone machinery in vivo

Hsp90 hyperphosphorylation in a *ppt1* knockout strain raised the question whether the phosphorylation state will affect the

activity of yHsp90 *in vivo*. To investigate this, we employed several well-established *in vivo* assays using substrates of the Hsp90 system, for example, the glucocorticoid hormone receptor (GR) (Smith, 1993) and viral Src kinase (v-Src kinase) (Brugge *et al*, 1981). In yeast, as in mammals, GR and v-Src depend on the Hsp90 chaperone system to reach their active state (Xu and Lindquist, 1993; Nathan and Lindquist, 1995; Nathan *et al*, 1997). These proteins can therefore be used as reporters for the activity of the yeast Hsp90 system. To assay this quantitatively, we transformed *ppt1*Δ and wt yeast with a plasmid harboring a constitutively expressed GR gene and β-galactosidase under the control of a GR-regulated promotor (Nathan and Lindquist, 1995). Thus, the amount of β-galactosidase activity obtained after induction with the hormone derivative deoxycorticosterone (DOC) corresponds quantitatively to the functionality of the yHsp90 system. In the *ppt1*Δ yeast, GR activity was reduced to ~55% of the wt activity, implying that the yHsp90 chaperone cycle requires Ppt1 for efficient maturation of GR in yeast (Figure 6A). To directly investigate whether the decrease of GR activity in *ppt1*Δ yeast is caused by the absence of the phosphatase activity of Ppt1, we reconstituted the expression of Ppt1 with an active site point mutant of Ppt1 (H311A). The active site of members of the PPP family of phosphatases is highly conserved. Among the residues involved in catalysis, histidine 311 is thought to function as a general acid (Swingle *et al*, 2004). Mutation of the corresponding histidine in various Ser/Thr phosphatases resulted in a complete loss of phosphatase activity (Zhuo *et al*, 1994; Shibasaki and McKeon, 1995; Zhang *et al*, 1996; Mondragon *et al*, 1997; Swingle *et al*, 2004). The levels of Ppt1 (H311A) expressed in *ppt1*Δ yeast cells were similar to Ppt1 in wt yeast (see also Figure 6C). However, the H311A mutant did not restore the GR activity to the level observed in wt yeast (Figure 6A).

To test whether the effect of Ppt1 is specific for GR maturation, firefly luciferase was employed as a further reporter of the yHsp90 system. Luciferase maturation *in vivo* depends on Hsp90 (Schneider *et al*, 1996; Nathan *et al*, 1997), and is easily detectable by the luciferase-catalyzed chemoluminescence of luciferin. Again, deletion of Ppt1 results in a marked decrease of luciferase activity to ~43% of the activity in wt yeast cells (Figure 6B). As a third reporter, we utilized v-Src kinase. When the yHsp90 system is functional, v-Src passes through the chaperone cycle and reaches its native conformation, which can be monitored by the detection of the autophosphorylation of v-Src with an antibody specific for phosphotyrosine residues in an immunoblot experiment (Nathan and Lindquist, 1995). Maturation of this kinase depends highly on Hsp90 (Brugge *et al*, 1981; Xu and Lindquist, 1993; Nathan *et al*, 1997). Most interestingly, in this study, autophosphorylation of v-Src was only observed in wt yeast and not in the *ppt1*Δ yeast strain (Figure 6C). Again, expression of the active site mutant of Ppt1 did not restore the maturation of v-Src to levels found in wt yeast. Thus, in the absence of Ppt1, when Hsp90 is hyperphosphorylated, the yHsp90 system is not functional for this substrate and the protein does not reach its native conformation.

Finally, we analyzed an endogenous yeast-specific Hsp90 client protein involved in pheromone signaling. Pheromone signaling occurs in yeast as part of the mating behavior. Upon binding of the α-mating factor, a signal-transduction cascade

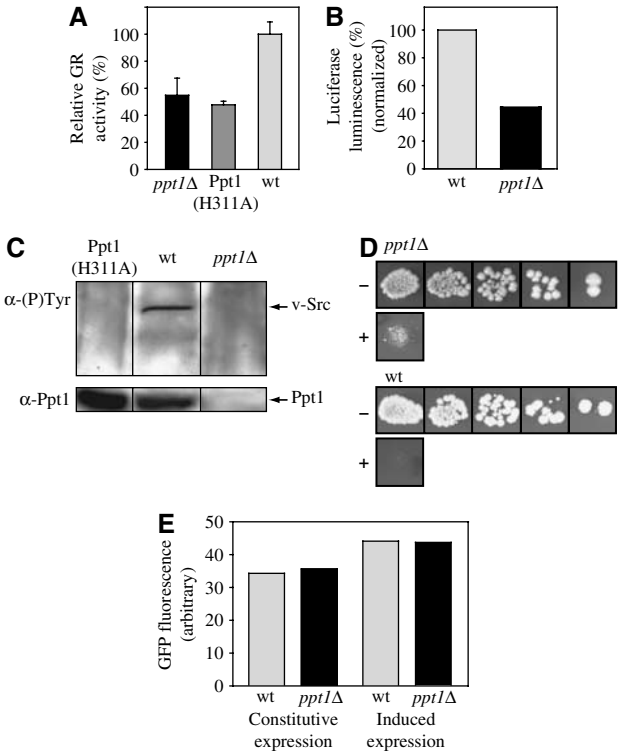

Figure 6 *In vivo* activation of yHsp90 client proteins. (**A**) *In vivo* activation of GR in *ppt1*Δ and wt yeast. The indicated yeast strains harboring a plasmid with the GR reporter β-galactosidase construct were grown in their logarithmic growth phase and identical cell numbers were induced with the GR ligand DOC (20 μM) for 90 min. Subsequently, cells were harvested and lysed. The β-galactosidase activity in the lysate, which corresponds to the GR activity, was determined with a chemoluminescence assay system as described in Materials and methods. The data shown represent the mean of five independent samples. The GR activity in the *ppt1*Δ yeast is reduced to ~55% of the wt level and to ~50% in *ppt1*Δ yeast cells expressing Ppt1 (H311A). (**B**) Analysis of luciferase activity in *ppt1*Δ and wt yeast cells. Identical numbers of yeast cells expressing firefly luciferase for 3 h were lysed and the lysate was supplemented with luciferin in a 96-well plate. Chemoluminescence was recorded using a Tecan Genios plate reader and normalized for the total protein concentration as determined by a Bradford assay (Bradford, 1976). Data shown are representative of multiple independent experiments. (**C**) Immunoblot analysis of v-Src activation in *ppt1*Δ, Ppt1 (H311A) and wt yeast. (Upper box) Immunoblot for autophosphorylated v-Src with a monoclonal antibody specific for phosphotyrosine residues (α-(P)-Tyr). The gel was loaded with identical quantities of cell lysate as indicated. The arrow points to the position of activated v-Src. (Lower box) Immunoblot of the lysates as shown above using a polyclonal serum directed against Ppt1. Data shown are representative of multiple independent experiments. (**D**) Growth behavior of *ppt1*Δ and wt yeast cells in response to the α-mating factor. Shown is a dilution series of identical numbers of yeast cells in the absence (−) and presence (+) of 5 μM α-factor in the media. (**E**) Analysis of eGFP production in *ppt1*Δ and wt yeast cells. Lysate of identical amounts of logarithmically grown yeast cells expressing eGFP constitutively or inducibly were prepared as described in Materials and methods. Induction of eGFP production was performed for 3 h. After eGFP oxidation, the fluorescence of the indicated cell lysates was recorded and normalized for the total protein concentration as determined by a Bradford assay (Bradford, 1976). Data shown are representative of multiple independent experiments.

is initiated by the kinase Ste11, leading to a cell cycle arrest (Rhodes *et al*, 1990). Yeast cells with mutations in Hsp90 fail to respond to α factor, which renders them capable of growth

in the presence of the pheromone because Ste11 is unable to reach its signal-transducing conformation (Louvion *et al*, 1998). Wt yeast cells respond to the addition of pheromone with a tight cell cycle arrest (Figure 6D). In contrast, in *ppt1*Δ yeast cells, this growth arrest was alleviated.

To test whether protein synthesis and folding was generally impaired in the *ppt1*Δ yeast cells, maturation of enhanced green fluorescent protein (eGFP), a small and rapidly folding protein that does not seem to depend on the Hsp90 system (Coxon and Bestor, 1995), was studied. In contrast to the proteins described above, GFP activity was identical in *ppt1*Δ yeast cells and in wt cells (Figure 6E).

Discussion

The physiological role of many Hsp90 cochaperones is not yet understood well. Some of the cofactors, like Sti1 (Prodromou *et al*, 1999; Richter *et al*, 2003) and Hch1/Aha1 (Panaretou *et al*, 2002), are regulators of the ATPase activity of Hsp90 (Obermann *et al*, 1998; Panaretou *et al*, 1998), others (PPIases, p23/Sba1, Cdc37) share the ability to interact with unfolded proteins (Bose *et al*, 1996; Freeman *et al*, 1996; Kimura *et al*, 1997). The identification of the phosphatase PP5 as a component of the Hsp90 chaperone system raised the question of a functional contribution of PP5 to the Hsp90 system. The TPR interaction of PP5 resembles that of some large PPIases, enzymes that catalyze isomerization of peptidyl prolyl *cis/trans* bonds (Fischer *et al*, 1998). However, Ppt1 is devoid of PPIase activity in standard assays, and it also does not exhibit chaperone activity in *in vitro* assays (data not shown), suggesting that the function of Ppt1 must be restricted to its phosphatase activity.

The unique feature of the PP5/Ppt1 family of phosphatases is the regulation of the phosphatase activity by the TPR domain (Sinclair *et al*, 1999; Ramsey and Chinkers, 2002; Yang *et al*, 2005). Compared to most other cofactors associating with Hsp90 via a TPR domain (Mayr *et al*, 2000), the affinity between Ppt1 and Hsp90 is at least 10-fold lower. This result can be explained by the autoinhibitory mechanism of this family of phosphatases, in which the TPR domain was found to be partially occupied by the C-terminal end of PP5 (Yang *et al*, 2005). In consequence, the isolated TPR domains of PP5 and Ppt1 bind to Hsp90 with higher affinity (Prodromou *et al*, 1999; Yang *et al*, 2005; and data not shown).

Here, we demonstrate for the first time that Ppt1 specifically dephosphorylates Hsp90, while it does not exhibit significant phosphatase activity towards Hsp90 partner proteins or any other protein tested. Further characterization of the Ppt1/Hsp90 interactions showed that disruption of the TPR interaction (between the Ppt1 TPR domain and the C-terminal residues of Hsp90) abolishes dephosphorylation of Hsp90. Although ligand binding to the TPR domain of PP5/Ppt1 stimulates its phosphatase activity (Skinner *et al*, 1997; Sinclair *et al*, 1999; Ramsey and Chinkers, 2002; Yang *et al*, 2005), this activation does not enable Ppt1 to dephosphorylate proteins *in trans*. A direct link of Ppt1 to its substrate protein, Hsp90, seems to be required.

In line with our *in vitro* results on the substrate specificity of Ppt1, we found that Ppt1 mediates dephosphorylation of Hsp90 *in vivo*. Hsp90 was observed to be hyperphosphorylated in *ppt1*Δ yeast cells. Previous *in vivo* studies using phosphatase inhibitors gave contradictory results concerning effects of phosphatases on Hsp90 client protein maturation. For the heme-regulated eIF2α kinase, inhibition of PP5 led to a more active substrate (Shao *et al*, 2002). In contrast, for v-Src kinase, phosphatase inhibition reduced the activity of this particular Hsp90 client (Mimnaugh *et al*, 1995). Our experiments using four different Hsp90 client proteins gave a consistent picture, demonstrating unequivocally that the maturation and activation of each client protein tested was handicapped in a Ppt1 deletion strain. Each of the three heterologous client proteins analyzed was affected in a distinct manner, whereas GFP used as an Hsp90-independent control protein was not impaired. While the amount of active GR and luciferase folding was reduced to about one-half compared to the Ppt1 wt strain, maturation of v-Src kinase was completely blocked in *ppt1*Δ yeast cells. We also investigated the effect of the Ppt1 deletion on an authentic yeast client protein of yHsp90, the kinase Ste11, which is involved in the α-mating factor pheromone signaling cascade. Deletion of Ppt1 prevented the Ste11-dependent complete cell cycle arrest observable in *ppt1* wt strains. It is also important to note that expression of an active site mutant of Ppt1 failed to restore client protein maturation, thus clearly demonstrating that the phosphatase activity of Ppt1 is specifically causative for the altered activation of Hsp90 client proteins.

Together, our results strongly imply that, *in vivo*, the efficacy of the Hsp90 chaperone machinery is significantly decreased in the absence of Ppt1 activity. Therefore, an additional layer of regulation has to be added to our current model of the Hsp90 chaperone system. It will be important to investigate the molecular details of the Hsp90 phosphoregulation in order to explain how Hsp90 phosphorylation determines the fate of different Hsp90 client proteins and how the phosphorylation state of Hsp90 is controlled. Since Ppt1 competes with other TPR domain proteins for binding to Hsp90, one could imagine that Hsp90 phosphorylation is governed by the ratio between TPR proteins, such as Sti1, and Ppt1 in the cells. Uncovering the molecular mechanisms underlying the Hsp90 phosphoregulation will be no easy task. The analysis is complicated by the sheer multitude of potential phosphorylation sites of Hsp90 (yHsp90 contains 45 serine and 40 threonine residues), and the possibility that several phosphorylation isoforms of Hsp90 might coexist in yeast cells.

Materials and methods

Materials

Kanamycin, *p*NPP, p-nitrophenolate (*p*NP), apyrase, casein and MBP were from Sigma (St Louis, MO), CKII from Roche Diagnostics (Mannheim, Germany), chloramphenicol from Roth (Karlsruhe, Germany), IPTG from ICN Biochemicals (USA), [γ-^{32}P]ATP from Hartmann Analytics (Darmstadt, Germany), α-mating factor from Bachem (Weil am Rhein, Germany) and all other chemicals from Merck (Darmstadt, Germany). Peptides were synthesized by Professor S Modrow (University of Regensburg, Germany). Rabbit α-Ppt1 antiserum was produced with purified protein (Pineda, Berlin, Germany) and the antibody 4G10 (phosphotyrosine specific) was from Upstate (Hamburg, Germany).

Yeast and bacterial strains, culture media

*ppt1*Δ− and wt yeast strains were from Euroscarf (Frankfurt, Germany; *ppt1*Δ: YGR123c::kanMX4: Accession number Y14753; wt: Accession number Y10000). Corresponding Mat-a strains were used for the α-mating factor assay. Yeast cells were transformed and

grown as described elsewhere (Ito *et al*, 1983; Nathan and Lindquist, 1995). *Escherichia coli* strain BL21 (DE3) Codon+ (Stratagene, La Jolla, CA) was used for recombinant protein expression as described elsewhere (Hainzl *et al*, 2004).

Proteins and constructs

Ppt1. The gene *ppt1* was introduced for expression in pET28b (Novagen, Madison, WI). Fragments of Ppt1: pET28b-Ppt1 was used as template to amplify the TPR fragment (bp 1–510) and the phosphatase fragment (bp 511–1542) by PCR. Point mutant Ppt1 (H311A): Ppt1 was subcloned in pRS316CUP. Mutagenesis of Ppt1 was performed using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Each coding region was sequenced to confirm the absence of mutations.

Purification of Ppt1. Ppt1 was purified by Ni-NTA, ResourceQ anion exchange and Superdex 75 pg size-exclusion chromatography (Amersham Biosciences, Freiburg, Germany). Ppt1 was dialyzed against 40 mM Tris–Cl, pH 7.0, 150 mM KCl, 5 mM glycine, 2 mM EDTA, 3.5 mM DTT and stored at −80°C.

TPR fragment. The TPR fragment was purified similar to the full-length Ppt1, except for the use of a ResourceS cation exchange column (Amersham Biosciences, Freiburg, Germany).

Phosphatase fragment. The phosphatase fragment was expressed in inclusion bodies. These were solubilized as described elsewhere (Rudolph *et al*, 1997). Denatured protein (6 M GdmHCl) was applied on an Ni-IDA column (Amersham Biosciences, Freiburg, Germany). For renaturation, the phosphatase fragment was diluted in 700 mM Tris (pH 7.5), 250 mM L-arginine, 5 mM DTT. Afterwards, it was dialyzed against 50 mM HEPES, pH 7.5, 150 mM KCl and 5 mM DTT.

yHsp90, Ssa1 and Sti1 were purified as described elsewhere (Buchner *et al*, 1998; Prodromou *et al*, 1999; Panaretou *et al*, 2002; Wegele *et al*, 2003).

Hsp90 from ppt1Δ and the respective wt yeast strain. The cleared lysate was applied on a 5 ml Ni^{2+}-loaded IDA-IMAC column (Amersham Biosciences, Freiburg, Germany). After extensive washing, the protein eluted without visible contaminants.

Spectroscopic phosphatase assay

The phosphatase activity of Ppt1 was assayed by monitoring hydrolysis of *p*NPP similar to that described by Ramsey and Chinkers (2002) at 410 nm in 300 mM Tris–Cl, pH 7.8, 150 mM KCl, 25 mM $MgCl_2$, 5 mM glycine and 1 mM DTT. Reaction velocities at various *c*(*p*NPP) were used for the determination of the Michaelis–Menten parameters (Sigmaplot, SPSS Software, Chicago, IL).

Radioactive phosphatase assay

Substrate proteins were labeled with [γ-^{32}P]ATP using 0.1 mU CKII at 37°C for 2 h. The remaining [γ-^{32}P]ATP was hydrolyzed by treatment with apyrase for 45 min at 30°C. Dephosphorylation with Ppt1 was carried out at 30°C for up to 120 min. For competition experiments, competitors of Ppt1 were incubated with yHsp90 prior to the addition of Ppt1. Dephosphorylation was stopped by the immediate addition of Laemmli buffer (Laemmli, 1970) and by boiling at 95°C for 5 min. Proteins were separated from free radioactive phosphate on SDS–PAGE. The degree of protein phosphorylation was determined by phosphoimaging on a Typhoon 9200 Phosphoimager (Amersham Biosciences, Freiburg, Germany).

Determination of differences in free phosphorylation sites between Hsp90 derived from ppt1Δ and wt yeast cells: The Hsp90 batches were phosphorylated with identical aliquots of a CKII and [γ-^{32}P]ATP-containing solution for 2 h. Equal quantities of Hsp90 were used. Protein concentrations were monitored by Coomassie-stained SDS–PAGE. This experiment was repeated multiple times to assure reproducibility. Nonradioactive analysis of protein phosphorylation was performed by phosphoprotein staining using the ProQ Diamond phosphostain (Invitrogen, Karlsruhe, Germany).

Isothermal titration calorimetry

The binding constant and stoichiometry of the Ppt1–yHsp90 complex were determined by ITC as described elsewhere (Richter *et al*, 2003). The buffer was 40 mM Tris–Cl (pH 7.0), 150 mM KCl, 5 mM glycine, 2 mM EDTA and 3.5 mM DTT.

Interaction of Ppt1 and fragments with yHsp90

To analyze the binding of Ppt1 and its fragments, we adopted an ELISA-based interaction assay (Muller *et al*, 2004). In brief, ELISA plates (Greiner-BioOne, Kremesmuenster, Austria) were coated with Ppt1, the TPR- or the phosphatase fragment (5 μM). After blocking and washing, 5 μM FITC-labeled yHsp90 or the C-terminal yHsp90 fragment 530C were added (provided by Lin Mueller) for 90 min (4°C). After a further washing step, FITC-labeled protein was visualized with a Typhoon 9200 Imager.

Assay for GR, v-Src and luciferase activity in ppt1Δ yeast cells

GR and the v-Src assays were performed as described elsewhere (Nathan and Lindquist, 1995). Luciferase activity was determined as described elsewhere (Nathan *et al*, 1997).

Assay for α-mating factor induced growth arrest

Growth arrest by α-mating factor was induced as described elsewhere (Abbas-Terki *et al*, 2000). For analysis of the effect of the α-mating factor upon Ste11-mediated cell cycle arrest, 10^6 cells/ml of the *ppt1Δ* or wt yeast (both Mat-a) were diluted stepwise and spotted on YPD plates with and without supplementation of 5 μM α-factor and grown at 30°C.

Expression and analysis of eGFP in yeast cells

Yeast cells expressing eGFP were lysed in the presence of a protease inhibitor. To allow complete oxidation of the eGFP chromophore, the cleared lysate was agitated at 4°C over night. After centrifugation, fluorescence spectra were recorded (excitation at 395 nm, emission 430–560 nm) and normalized for the total protein concentration as determined by a Bradford assay (Bradford, 1976).

Scanning electron microscopy

Scanning electron microscopy was performed as described elsewhere (Haslbeck *et al*, 2004).

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