Thr<sup>90</sup> phosphorylation of Hsp90<sub>α</sub> by protein kinase A regulates its chaperone machinery

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INTRODUCTION

Hsp90 (heat-shock protein 90) is one of the most important molecular chaperones in eukaryotes. Hsp90 facilitates the maturation, activation or degradation of its client proteins. It is now well accepted that both ATP binding and co-chaperone association are involved in regulating the Hsp90 chaperone machinery. However, other factors such as post-translational modifications are becoming increasingly recognized as being involved in this process. Recent studies have reported that phosphorylation of Hsp90 plays an unanticipated role in this process. In the present study, we systematically investigated the impact of phosphorylation of a single residue (Thr<sup>90</sup>) of Hsp90α (pThr<sup>90</sup>-Hsp90α) on its chaperone machinery. We demonstrate that protein kinase A specifically phosphorylates Hsp90α at Thr<sup>90</sup> and that the pThr<sup>90α</sup>-Hsp90<sub>α</sub> level is significantly elevated in proliferating cells. Thr<sup>90</sup> phosphorylation affects the binding affinity of Hsp90α to ATP. Subsequent examination of the interactions of Hsp90α with co-chaperones reveals that Thr<sup>90</sup> phosphorylation specifically regulates the association of a subset of co-chaperones with Hsp90α. The Hsp90α T90E phosphor-mimic mutant exhibits increased association with Aha1 (activator of Hsp90 ATPase homologue 1), p23, PP5 (protein phosphatase 5) and CHIP (C-terminus of Hsp70-interacting protein), and decreased binding affinity with Hsp70, Cdc37 (cell division cycle 37) and Hop [Hsc70 (heat-shock cognate protein 70)/Hsp90-organizing protein], whereas its interaction with FKBP52 (FK506-binding protein 4) is only moderately affected. Moreover, we find that the ability of the T90E mutant to form complexes with its clients, such as Src, Akt or PKCγ (protein kinase Cy), is dramatically impaired, suggesting that phosphorylation affects its chaperoning activity. Taken together, the results of the present study demonstrate that Thr<sup>90</sup> phosphorylation is actively engaged in the regulation of the Hsp90α chaperone machinery and should be a generic determinant for the cycling of Hsp90α chaperone function.

Key words: chaperone, heat-shock protein 90α, phosphorylation, protein kinase A (PKA).

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Abbreviations used: Hsp, heat-shock protein; Aha1, activator of Hsp90 ATPase homologue 1; Cdc37, cell division cycle 37; CHIP, C-terminus of Hsp70-interacting protein; c-Src, cellular Src; FKBP52, FK506-binding protein 4; GFP, green fluorescent protein; HA, haemagglutinin; HRP, horseradish peroxidase; Hsc70, heat-shock cognate protein 70; Hop, Hsc70/Hsp90-organizing protein; PKA, protein kinase A; PKB, protein kinase B; PKCy, protein kinase Cy; PP5, protein phosphatase 5; R.R., relative ratio; siRNA, small interfering RNA; TBST, Tris-buffered saline/Tween 20; TPR, tetratricopeptide repeat; WT, wild-type.

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as acetylation [11] and S-nitrosylation [19] have also been found to play a regulatory role in Hsp90 chaperone function, such that a conserved acetylation site in the middle domain of Hsp90 could regulate its chaperone activity [11].

As more and more evidence is accumulating to support the regulatory function of the post-translational modification of Hsp90, a detailed and thorough understanding of the chaperoning requirement of these modifications starts to be revealed [2,4,5]. Hsp90 could be phosphorylated at multiple sites; yet how much the individual phosphorylation site contributes to Hsp90 chaperone function, whether these sites are coupled or act independently, or how these phosphorylation sites are spatiotemporally controlled remain poorly understood. Although it is well accepted that co-chaperones play dominant roles in the cycling of Hsp90 chaperone machinery [23], it is still unclear how these distinct co-chaperones preferentially associate with Hsp90 at different stages of the chaperone cycle. Thus it is likely that post-translational modifications such as phosphorylation may assist the association of co-chaperones to the Hsp90 complex. We and others have previously shown that Hsp90α could be phosphorylated at Thr90 by PKA (protein kinase A), which correlates with its secretion in tumour cells [16,18]. Still, whether Thr90 phosphorylation is coupled with Hsp90α chaperone function remains unexplored.

In the present study, we investigated the functional correlation of Hsp90α Thr90 phosphorylation with its chaperone machinery. In the present study, we show that phosphorylation of Thr90, a residue conserved in Hsp90 in higher eukaryotes, not only affects the intrinsic ATPase activity of Hsp90α, but also regulates differential association abilities of distinct co-chaperones to Hsp90α, and consequently results in the loading/releasing of a subset of Hsp90α client proteins. These results demonstrate that Thr90 phosphorylation is a key regulator for Hsp90α chaperone machinery in vivo.

**EXPERIMENTAL**

**Cell culture and reagents**

HeLa cells (A.T.C.C.) were cultured in DMEM (Dulbecco’s modified Eagle’s medium; Gibco) supplemented with 10 % (v/v) fetal bovine serum (Gibco), 100 units/ml penicillin (Sigma–Aldrich) and 100 μg/ml streptomycin (Sigma–Aldrich). Cells were maintained at 37 °C in an atmosphere of 95 % air and 5 % CO2.

The anti-Hsp90α mouse monoclonal antibody for immunoblotting was from a laboratory stock, and the following antibodies were obtained from commercial sources: anti-[phospho-serine/threonine] (PKA) substrate antibody and anti-Src antibody (Cell Signaling Technology); anti-(PKA catalytic subunit) rabbit polyclonal antibody (Santa Cruz Biotechnology); anti-HA (haemagglutinin) and anti-Myc mouse monoclonal antibodies, polyclonal antibody (Santa Cruz Biotechnology); anti-HA (Cell Signaling Technology); anti-(PKA catalytic subunit) rabbit [serine/threonine] PKA] substrate antibody and anti-Src antibody were obtained from commercial sources: anti-[phospho-]

**Cell transfection and siRNA (small interfering RNA)**

For plasmid transfections, HeLa cells were plated in six-well plates 24 h before transfection with a confluence of 50–60 %. For single transient transfections, 2 μg of plasmid per well was used with TurboFect™ in vitro transfection reagent (Thermo Fisher Scientific). For co-transfections, 3 μg of plasmid (1.5 μg of each) was used per well. Cells were harvested for analysis 36 h post-transfection.

For siRNA transfections, HeLa cells were plated in six-well plates 24 h before transfection with a confluence of 40–50 %. siRNA against the human PKA catalytic subunit and control scrambled siRNA were from Santa Cruz Biotechnology. The siRNA assay was performed according to the manufacturer’s instructions using Lipofectamine™ 2000 (Invitrogen). Cells were harvested for analysis 48 h post-transfection.
Co-immunoprecipitation

Cells harvested for immunoprecipitation were processed as follows: whole cell lysate was prepared using cell lysis buffer consisting of 20 mM Tris/HCl (pH 7.5, 25 °C), 150 mM NaCl, 1% Nonidet P40 and 1 mM dithiothreitol with protease and phosphatase inhibitor cocktails, then the lysates were centrifuged for 15 min at 13000 g at 4 °C. The pellet was discarded while the soluble fraction was used as the whole cell lysate for immunoprecipitation. Protein concentrations were measured using the BCA protein assay kit (Thermo Fisher Scientific). Immunoprecipitation was performed in a 1.5 ml Eppendorf tube at 4 °C using 1 μg of antibody per mg of cell lysate. The cell lysate was pre-incubated with Protein A–agarose to reduce non-specific binding. Antibody was first incubated with the cleared cell lysate for 1 h with rotation, then 20 μl of suspended Protein A–agarose was added to each tube and incubation was continued with rotation at 4 °C overnight. After incubation, the resin was pelleted with a brief centrifugation and the supernatant was discarded. The remaining resin was repeatedly washed with ice-cold lysis buffer three times and finally resuspended in reducing SDS/PAGE loading buffer for further analysis. For anti-HA affinity matrix immunoprecipitation, 20 μl of suspended resin was directly added to the cell lysate and incubated overnight at 4 °C, then washed with lysis buffer. The results are representative images of several co-immunoprecipitation experiments. Quantification of blots was performed using Gel-Pro Analyzer.

Immunoblotting

Samples from whole cell lysates or immunoprecipitated resin were mixed with reducing SDS/PAGE loading buffer, boiled at 100 °C for 5 min, separated by SDS/PAGE (10% or 12% gels) and transferred on to a PVDF membrane (Millipore). The membrane was blocked in TBST (Tris-buffered saline/ Tween 20; 20 mM Tris/HCl, pH 7.5, 150 mM NaCl and 0.1% Tween 20) plus 5% (w/v) non-fat dried skimmed milk for 60 min at room temperature (25 °C). The membrane was incubated with the indicated primary antibodies in PBST (PBS/0.1% Tween 20) plus 1% (w/v) non-fat dried skimmed milk overnight at 4 °C. The membrane was then washed with TBST, immunoreactive bands were detected by enhanced chemiluminescence (Pierce).

In vitro PKA phosphorylation assay

The in vitro phosphorylation of Hsp90α by PKA (Promega) was performed as described previously [18] with slight modifications. Myc-tagged WT (wild-type) Hsp90α was transiently expressed in HeLa cells, immunoprecipitated with anti-Myc antibody and treated with γ-protein phosphatase (NEB). Recovered Hsp90α was incubated with PKA at 30 °C for 30 min, then the sample was mixed with reducing SDS/PAGE loading buffer for further analysis.

ATP-binding assay

Proteins used for these assays were ectopically expressed in HeLa cells, then immunopurified with anti-Myc antibodies. Proteins were first washed with cell lysis buffer three times, and eluted with buffer consisting of 20 mM glycine/HCl (pH 2.2). The eluted samples were immediately neutralized with Tris/HCl buffer to a pH of 7.5 in preparation for the next step of the assay.

For the ATP-binding assay, proteins in Tris/HCl buffer (pH 7.5) containing 10 mM MgCl₂ and 100 mM NaCl were incubated with High-Affinity ATP Agarose (Innova) at 4 °C for 1 h, then washed extensively with the same buffer three times. The pelleted resin was mixed with reducing SDS/PAGE loading buffer for immunoblotting.

RESULTS

Thr⁹⁰ of Hsp90α is phosphorylated by PKA in vitro and in vivo

We and others have previously shown that Hsp90α can be phosphorylated at Thr⁹⁰ by PKA [16,18]. To further confirm and validate that Hsp90α is specifically phosphorylated at Thr⁹⁰ by PKA, we produced Hsp90α by immunoprecipitation from HeLa cells, which was then treated with λ-protein phosphatase. Recovered Hsp90α was then co-incubated with PKA in the absence or presence of ATP for 15 min and subjected to Western blotting. The membrane was probed with a phospho-serine/threonine) PKA substrate antibody that can detect proteins containing a phospho-serine/threonine residue with an arginine residue at the -3 position. As expected, PKA could efficiently phosphorylate Hsp90α at the Thr⁹⁰ residue in vitro (Figure 1A), as sequence R⁸⁷L⁹⁰T (the RXXT motif) is the only substrate motif of PKA in Hsp90α. To further confirm this effect in vivo, we first overexpressed the catalytic subunit of murine PKA in HeLa cells. Hsp90α was then immunoprecipitated from cells transfected with control vector or PKA, and examined by Western blotting. As shown in Figure 1B (left-hand panel), transient overexpression of PKA could significantly enhance the Thr⁹⁰ phosphorylation of Hsp90α. Next, we used siRNA to specifically knock down the expression of the endogenous catalytic subunit of PKA. As shown in Figure 1B (right-hand panel), siRNA-mediated knockdown of PKA resulted in almost complete abolishment of Thr⁹⁰ phosphorylation of Hsp90α by two independent siRNA duplexes, confirming the specificity of PKA-mediated Hsp90α Thr⁹⁰ phosphorylation. To further demonstrate that PKA specifically phosphorylates Hsp90α at the Thr⁹⁰ residue, we constructed two mutants: T90A (non-phospho mutant) and T90E (phosphor-mimic mutant) of Hsp90α, then transiently overexpressed them respectively in HeLa cells, together with the WT Hsp90α. The ectopically expressed WT or mutant Hsp90α was immunoprecipitated with the anti-Myc antibody. As shown in Figure 1C, only WT Hsp90α showed intensive Thr⁹⁰ phosphorylation, whereas both mutants (T90A and T90E) lost this specific phosphorylation. This result strongly demonstrates that PKA specifically phosphorylates Hsp90α at the single Thr⁹⁰ residue, which is consistent with the analysis that R⁸⁷L⁹⁰T (the RXXT motif) is the only substrate motif of PKA in Hsp90α. Remarkably, we analysed this motif and the neighbouring sequences on Hsp90 from various species and isoforms and found that this motif is highly conserved in higher eukaryotes (Figure 1D), which suggests that phosphorylation of Thr⁹⁰ is a conserved event during evolution and may play a fundamental role in regulating the chaperone activity of Hsp90α.

PKA, also known as cAMP-dependent protein kinase, has been implicated in a wide range of cellular processes, including transcription, metabolism, cell-cycle progression and other signal-transduction pathways [24]. Specially, PKA regulates cell proliferation in response to growth factors and/or nutrients [25,26]. We therefore examined whether Hsp90α Thr⁹⁰ phosphorylation is linked to the proliferative status of cells. As expected, starvation of HeLa cells significantly reduced the Hsp90α Thr⁹⁰ phosphorylation level compared with that of the proliferative one, and forced ectopic expression of the...
catalytic subunit of PKA could rescue this effect (Figure 1E). Taken together, these results demonstrate that PKA specifically phosphorylates Hsp90α at the single Thr90 residue, and this is a positively regulated process closely linked with the activation status of PKA, i.e. the proliferative condition. Considering that this site is conserved in higher eukaryotes, we speculate that phosphorylation of Thr90 may play some functional roles in regulating its chaperone activity.

Thr90 phosphorylation affects the binding affinity of Hsp90α to ATP

It is well accepted that the intrinsic ATPase activity of Hsp90α is essential for the cycling of chaperone machinery [5,9]. Hsp90α binds and hydrolyzes ATP, acting as one of the major driving forces for the conformational change of the chaperone complex [9]. The ATPase activity of Hsp90α is located at its N-terminal domain, thus we postulated that Thr90 phosphorylation would have some potential effect on the binding affinity of Hsp90α with ATP. We used ATP–agarose, in which ATP is attached to agarose beads via its γ-phosphate, to examine the ATP-binding affinity of Hsp90α. Immunoprecipitated Hsp90α from HeLa cells was incubated with ATP–agarose in the absence or presence of ATP for 30 min. As expected, the results demonstrate the specificity of this assay (Figure 2A). Then, we immunoprecipitated endogenous Hsp90α from HeLa cells transfected with control vector or the catalytic subunit of PKA. As expected, immunoprecipitated Hsp90α from PKA-overexpressing cells was highly phosphorylated at Thr90 compared with that from the control cells (Figure 2B). We then incubated these two forms of Hsp90α with ATP–agarose and collected the supernatant. The remaining pellet (resin) was extensively washed to eliminate any non-specific binding. Interestingly, the result showed that Thr90 phosphorylation of Hsp90α impaired its ATP-binding affinity, suggesting that phosphorylation at this residue may regulate the intrinsic ATPase activity of Hsp90α. To further confirm this result, WT Hsp90α, T90A and T90E mutants were ectopically expressed in HeLa cells and immunoprecipitated with anti-Myc antibodies. Immunoprecipitated proteins were then incubated with ATP–agarose. Similarly, the phosphor-mimic mutant T90E exhibited decreased ATP-binding affinity, whereas the non-phospho mutant T90A showed comparable binding ability compared with that of WT Hsp90α (Figure 2C). This indicates that phosphorylation of Thr90 could result in a local conformational change that in turn affects the ATP-binding affinity of Hsp90α. Taken together, these results demonstrate that Thr90 phosphorylation is a functional event on Hsp90α which directly affects the ATP-binding affinity of Hsp90α. We then propose that Thr90 phosphorylation actively participates in the regulation of Hsp90α chaperone machinery.

Thr90 phosphorylation regulates the Hsp90α co-chaperone machinery

Hsp90α, as one of the most important intracellular chaperone proteins, does not act alone. Instead, it associates with other chaperones or co-chaperones to form a series of dynamic complexes to fulfill its function [1,4,5]. Hsp70 is another essential molecular chaperone that can form a large complex with Hsp90 to regulate the maturation, stabilization, activation or degradation of the client proteins [27]. Therefore we initially asked whether Thr90 phosphorylation could pose some effect on Hsp70–Hsp90 complex formation. We transfected HeLa cells with control vector, WT, and T90A and T90E mutants of Hsp90α respectively, then used the anti-Myc antibody to specifically immunoprecipitate the ectopically expressed proteins and analyze the co-immunoprecipitated endogenous Hsp70. Quite surprisingly, whereas WT Hsp90α could efficiently pull down endogenous Hsp70, indicating the formation of the complex, the T90A Hsp90α mutant showed a slightly decreased amount of co-immunoprecipitated Hsp70, and more strikingly, the T90E Hsp90α mutant exhibited a dramatically reduced interaction with Hsp70 (Figure 3A). This immediately implies that Thr90 phosphorylation can regulate the Hsp90α chaperone machinery.

As mentioned earlier, Hsp90α can interact with several co-chaperones, which consist of proteins with diversified structures as well as functions [1,2,4,5]. Through differential interactions with respective co-chaperones, the Hsp90α chaperone machinery can work smoothly and efficiently [5]. Since Thr90 phosphorylation affects the interaction of Hsp90α with Hsp70, indicating its functional relevance in the regulation
of the Hsp90α chaperone complex, we wondered how it would affect the dynamic interaction of Hsp90α with these co-chaperone proteins. To facilitate the investigation, we chose seven of these co-chaperones, each of which represents a unique structure or function regarding their roles in the Hsp90α chaperone cycle (Table 1) [5,28]. We cloned these co-chaperones and constructed them with a HA tag (Figure 3B). Next, we co-transfected these co-chaperones with control vector, WT, and T90A and T90E mutants of Hsp90α in HeLa cells respectively and performed co-immunoprecipitation to examine their interactions.

Initially, we analysed the interaction of WT, non-phospho and phosphor-mimic mutants of Hsp90α with Aha1. The co-chaperone Aha1 enhances the ATPase activity of Hsp90 in biochemical assays, thus promoting the chaperone cycle [29]. We used reciprocal co-immunoprecipitation (with an anti-Myc antibody to pull down ectopically expressed Hsp90α and with an anti-HA antibody to pull down ectopically expressed Aha1, then we analysed the co-precipitated proteins) to validate the fidelity of this assay. Interestingly and unexpectedly, we found that whereas WT and non-phospho Hsp90α showed a basal-level interaction with Aha1, the Hsp90α phosphor-mimic T90E mutant exhibited a remarkably increased affinity in binding with Aha1 (Figure 3C). This suggests that Thr90 phosphorylation would promote complex formation between Hsp90α and Aha1.

One key feature of Hsp90 chaperone machinery is to activate an important subgroup of its clients [4,5], i.e. the kinases, the recruitment of which are mediated mainly by Cdc37 (also known as p50cdc37) [30,31]. Therefore we next examined the interaction of Cdc37 with these Hsp90α constructs. We first used anti-HA antibody affinity matrix to immunoprecipitate the HA-tagged ectopic Cdc37 in HeLa cells and analysed the co-immunoprecipitated variants of Hsp90α. As expected, co-immunoprecipitation of ectopic (exogenous) Hsp90α with Cdc37 was observed in the WT group, confirming the efficiency of the protocol (Figure 3D). Surprisingly, in the phosphor-mimic mutant T90E-transfected group, the amount of the co-immunoprecipitated ectopic T90E mutant was significantly reduced, indicating the impaired binding affinity of this mutant to Cdc37 (Figure 3D). Instead, the amount of non-phospho mutant T90A co-immunoprecipitated was comparably similar to that of the WT group, or marginally decreased, if at all (Figure 3D). Consistently, using an anti-Myc antibody to precipitate the ectopic Hsp90α variants, we also obtained similar results in that the T90E mutant indeed showed an apparently decreased binding with Cdc37 (Figure 3D). This result indicates that Thr90 phosphorylation may lead to the disassociation of Cdc37 with the Hsp90α chaperone complex.

p23, a small acidic protein, has been shown to bind with Hsp90α as a control factor to stabilize the interaction of client proteins with Hsp90α, presumably through inhibiting the ATPase activity [32]. Owing to the similar molecular mass of p23 and the light chain of IgG (results not shown), we used anti-HA antibody affinity matrix to immunoprecipitate the HA-tagged ectopic p23 in HeLa cells and analysed the co-immunoprecipitated variants of Hsp90α. Quite opposite to Cdc37, whereas endogenous Hsp90α was equally co-immunoprecipitated in all three groups, the exogenous T90E mutant was more effectively co-immunoprecipitated with HA–p23 compared with Hsp90α from WT and T90A groups (Figure 3E), suggesting that Thr90 phosphorylation facilitates the association of p23 with Hsp90α. To further confirm this result, we used another construct and demonstrated that GFP does not affect the interaction of p23 with Hsp90α (results not shown). Next, we performed the co-immunoprecipitation using the anti-HA antibody. Consistently, we found that the T90E mutant shows a similarly increased association with p23–GFP (Figure 3E), further demonstrating that phosphorylation of Thr90 enhances the association of p23 with Hsp90α.

The TPR (tetratricopeptide repeat) domain-containing proteins consist of a unique family of co-chaperones [33]. The TPR domain can directly interact with the C-terminal EEVD motif of Hsp90 to form different complexes with distinct functions, which are major regulators of the Hsp90α chaperone machinery [33,34]. Therefore we examined the interactions between Hsp90α and four of these TPR-containing proteins with different functions.

PP5 is a unique Hsp90α co-chaperone that was reported to be responsible for Hsp90α dephosphorylation in yeast [22]. We have previously demonstrated that PP5 can specifically dephosphorylate pThr50-Hsp90α in MCF-7 cells [18]. More importantly, PP5 shows a preferential binding to Thr90-phosphorylated Hsp90α [18]. Consistently, we found that the Hsp90α phosphor-mimic T90E mutant exhibited increased association with PP5, as demonstrated by the reciprocal co-immunoprecipitation in HeLa cells co-transfected with HA–PP5 and variants of Hsp90α (Figure 3F), whereas T90A showed a slightly decreased interaction with PP5 (Figure 3F). This result further confirms that PP5 not only functions as a co-chaperone of Hsp90α to dephosphorylate other client proteins, but also directly targets Hsp90α itself to regulate the chaperone cycle.

The multiple-TPR-domain-containing co-chaperone Hop [also called Stip (stress-induced phospho-protein 1)] plays a critical role in mediating interactions between Hsp70 and Hsp90 as part of the cellular assembly machinery, thus facilitating their client proteins to transfer between them [35]. Interestingly, but within expectation, the Hsp90α phosphor-mimic T90E mutant showed a remarkably diminished binding affinity with Hop, and even the Hsp90α non-phospho T90A mutant showed a significantly reduced interaction.
Figure 3  Thr<sup>90</sup> phosphorylation regulates the Hsp90α co-chaperone machinery

(A) HeLa cells transfected with control vector, WT Hsp90α, or the T90A or T90E mutant were lysed and immunoprecipitated (IP) with anti-Myc antibody. Co-immunoprecipitated endogenous Hsp70 was then detected by immunoblotting. (B) Ectopic expression profiling of constructed HA-tagged co-chaperones in HeLa cells. Molecular mass (MW) in kDa is indicated on the right-hand side.

(C–I) Interaction of various co-chaperones with WT Hsp90α, or the T90A or T90E mutant. HeLa cells were co-transfected with Aha1 (C), Cdc37 (D), p23 or p23–GFP (E), PP5 (F), Hop (G), FKBP52 (H) or CHIP (I) and control vector, WT Hsp90α, or the T90A or T90E mutant respectively as indicated. Cells were lysed and immunoprecipitated using an anti-Myc antibody or incubated with anti-HA affinity matrix as indicated, and co-immunoprecipitated proteins were detected by immunoblotting. The results are representative images of several co-immunoprecipitation experiments (n = 3). Endo., endogenous Hsp90α; Exo., exogenous form of ectopically expressed Hsp90α; H.C., heavy chain. The IgG H.C. signal is due to the non-specific interaction of secondary antibody during blotting with the antibody used in immunoprecipitation.

with Hop, whereas WT Hsp90α exhibited a basal-level interaction with Hop (Figure 3G). This is consistent with the above observation that the T90E mutant lost its association with Hsp70 (Figure 3A), which is presumably mediated by Hop. This result strongly suggests that Thr<sup>90</sup> phosphorylation could impellingly affect the dynamic complex formation of Hsp90α with Hop.

FKBP52 is a member of the immunophilin protein family, which contains the TPR domain. It also associates with Hsp90 and Hsp70 to play a role in the intracellular trafficking of hetero-oligomeric forms of the steroid hormone receptors [36]. Surprisingly, the interaction of the Hsp90α phosphor-mimic T90E mutant with FKBP52 was only moderately affected, whereas the T90A mutant exhibited similar binding compared with WT Hsp90α (Figure 3H). We then detected the interaction of WT, T90A and T90E with the glucocorticoid receptor and found that there were no significant differences among the three groups (results not shown). These results suggest that Hsp90α Thr<sup>90</sup> phosphorylation may be spatiotemporal or pathway specific, at least in our present system. It seems not to be involved in the FKBP52-mediated signalling pathway or chaperone cycle.

Besides its function in assisting the maturation and activation of the client proteins, another important role for Hsp90α chaperone machinery is to mediate the degradation of client proteins [2,5]. CHIP is a TPR-containing co-chaperone [37] as well as an E3 ubiquitin ligase that integrates the client proteins for degradation via the Hsp90α chaperone machinery [38]. Co-immunoprecipitation experiments showed a very low basal-level interaction of WT Hsp90α with CHIP in HeLa cells (Figure 3I). However, the Hsp90α non-phospho T90A mutant showed a dramatically enhanced association with CHIP, whereas
the phosphor-mimic T90E mutant exhibited a slight increment (Figure 3I). We then wondered whether increased association of T90A with CHIP could result in enhanced ubiquitination of Hsp90α itself. However, no such effect was observed in all three groups (results not shown). These results suggest that, under normal conditions, CHIP is weakly associated with Hsp90α [39] and that Thr90 phosphorylation could slightly promote the association. Still, it remains unclear why the T90A mutant has a dramatically enhanced association with CHIP, possibly because CHIP is more sensitive to the conformational change resulting from the T90A mutation.

We quantified the co-immunoprecipitation results, shown in Figure 3 as R.R. (relative ratio), and summarize the different binding patterns of WT, non-phospho T90A and phosphor-mimic T90E mutants of Hsp90α with different co-chaperones in Table 1. From these results, we find that different co-chaperones exhibit variable binding affinities towards the different forms of Hsp90α (non-phospho and phosphor-mimic), suggesting that this is determined by the nature of the protein itself. On the basis of the discovery that a single-site phosphorylation can influence such a wide spectrum of co-chaperones, we can also deduce that Thr90 phosphorylation plays a key and functional role in mediating the dynamic complex formation of Hsp90α with these co-chaperones to initiate or turn off downstream events.

As the above experiments are all performed using Hsp90α mutants, we next wondered whether the interaction of these co-chaperones with endogenous wild-type Hsp90α would also change under in vivo PKA overexpression or knockdown. Cells were co-transfected with the indicated co-chaperones with PKA-expressing vector or control empty vector, or with siRNA of PKA or non-silencing scRNA (Supplementary Figures S1 and S2 at http://www.BiochemJ.org/bj/441/bj4410387add.htm). Co-immunoprecipitation was performed using an anti-Hsp90α antibody. We unexpectedly found that upon PKA overexpression, some co-chaperones, such as Aha1, Cdc37, Hop and p23, showed marginally or slightly increased interaction, and other co-chaperones, such as PP5, FKBP52 and CHIP, showed apparently enhanced interaction (Supplementary Figure S1). On the other hand, upon PKA silencing, some co-chaperones, such as Aha1, Cdc37 and p23, showed slightly decreased interaction, and other co-chaperones, such as Hop, PP5, FKBP52 and CHIP, showed significantly decreased interaction with Hsp90α (Supplementary Figure S2). We propose that these effects on the regulation of co-chaperone interaction with Hsp90α can be both direct and indirect (see the Discussion section).

**Thr90 phosphorylation affects the affinity of Hsp90α with its client proteins**

The above results demonstrate that Thr90 phosphorylation plays a fundamental role in regulating the Hsp90α chaperone machinery. The observation that the phosphor-mimic T90E mutant showed a reduced association with Cdc37 and Hop prompted us to speculate whether Thr90 phosphorylation would affect Hsp90α interacting with the kinases, a major subgroup of the client proteins, because previous studies have shown that Cdc37 functions to specifically integrate kinases into the Hsp90α chaperone complex [30,31].

Among the many substrate kinases of Hsp90α, the non-receptor tyrosine kinase Src, the maturation and activation of which is facilitated by Hsp90, has been studied extensively [40,41]. We transfected HeLa cells with control vector, WT, T90A and T90E Hsp90α and performed co-immunoprecipitation to examine their interactions with the endogenous c-Src (cellular Src) kinase [42]. Consistently, although both WT Hsp90α and the T90A mutant could efficiently co-immunoprecipitate endogenous c-Src (Figure 4A), the phosphor-mimic mutant of Hsp90α almost completely lost its interaction with c-Src (Figure 4A). This suggests that Thr90 phosphorylation indeed regulates the Hsp90α–client complex formation, which is probably achieved by the dynamic association with co-chaperones such as Cdc37.

The serine/threonine kinase Akt (also called PKB) is another well-studied client protein of Hsp90α. Binding of Akt to Hsp90α facilitates its maturation and activation, leading to the constitutive activation of the downstream signalling pathways involved in cell survival and proliferation [43]. We then cloned human full-length Akt and constructed it with an HA tag. The HA–Akt construct was then co-transfected with control vector, WT, T90A or T90E Hsp90α respectively in HeLa cells. Co-immunoprecipitation was then performed using an anti-Myc antibody to pull down the ectopic Hsp90α, and the bound HA–Akt was then analysed using anti-HA antibodies. Consistently, both WT and T90A mutant Hsp90α could co-precipitate Akt efficiently (Figure 4B).

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**Table 1** Summary of various co-chaperone interactions with the Hsp90α non-phospho T90A and phosphor-mimic T90E mutants

<table>
<thead>
<tr>
<th>Co-chaperone</th>
<th>Hsp90-binding site</th>
<th>Characteristics</th>
<th>Interaction with T90A mimic</th>
<th>Interaction with T90E mimic</th>
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<td>Hsp70</td>
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<td>Stabilizes pre-existing proteins against aggregation and mediates the folding of newly translated polypeptides</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>Aha1</td>
<td>Middle domain</td>
<td>Associates with Hsp90, acting as a potent simulator of Hsp90 ATPase activity</td>
<td>NC</td>
<td>++</td>
</tr>
<tr>
<td>p23</td>
<td>N-terminus</td>
<td>Cytosolic prostaglandin E2 synthase; stabilizes the closed formation of Hsp90</td>
<td>NC</td>
<td>++</td>
</tr>
<tr>
<td>Cdc37</td>
<td>N-terminus</td>
<td>Binds to kinases and promotes their interaction with the Hsp90 complex, resulting in stabilization and promotion of their activity</td>
<td>NC</td>
<td>++</td>
</tr>
<tr>
<td>Hop</td>
<td>C-terminus</td>
<td>TPR domain; mediates the association of Hsp70</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>PP5</td>
<td>C-terminus</td>
<td>TPR domain, serine/threonine PP5</td>
<td>NC</td>
<td>++</td>
</tr>
<tr>
<td>FKBP52</td>
<td>C-terminus</td>
<td>TPR domain, FK506i-binding protein, peptidyl-prolyl cis–trans isomerase</td>
<td>NC</td>
<td>NC</td>
</tr>
<tr>
<td>CHIP</td>
<td>C-terminus</td>
<td>TPR domain, E3 ubiquitin protein ligase; modulates the activity of Hsp90</td>
<td>+ +</td>
<td>+</td>
</tr>
</tbody>
</table>
However, similarly, the phosphor-mimic T90E mutant Hsp90α showed dramatically impaired association with Akt (Figure 4B), further demonstrating that Thr<sup>90</sup> phosphorylation plays a critical role in mediating the Hsp90α–kinase complex formation.

To further confirm the above phenomenon that Thr<sup>90</sup> phosphorylation regulating client protein loading to, and release from, Hsp90α is generic, we extended our investigation to another less-studied protein, PKCγ. PKCγ has been reported to be one of the clients for Hsp90α. Binding of PKCγ to the Hsp90α–Cdc37 complex is necessary for its maturation and activation [44]. Similarly, we cloned the human full-length PKCγ and constructed it with an HA tag, which was then co-transfected with control vector, WT and the T90A or T90E mutants of Hsp90α respectively. As expected, both WT Hsp90α and the T90A mutant were indeed associated with PKCγ. In contrast, the Hsp90α phosphor-mimic T90E mutant showed an almost completely abolished interaction with PKCγ compared with WT and the T90A mutant of Hsp90α (Figure 4C). The above results that reduced interactions of the T90E mutant are obtained with endogenous Src and ectopic Akt and PKCγ leads to the conclusion that Thr<sup>90</sup> phosphorylation indeed plays a fundamental role in regulating the dynamic cycle of Hsp90α chaperone machinery. This is probably mediated by distinct preferential affinities with distinct co-chaperones, which consequently affects Hsp90α chaperoning activity towards a subgroup of the clientele, the protein kinases.

Finally, we wondered how Hsp90α would respond to cell proliferation by altered binding to the co-chaperones as well as to Src, Akt and PKC, since we have shown that Thr<sup>90</sup> phosphorylation is enhanced under proliferative status. As shown in Supplementary Figures S3 and S4 (at http://www.BiochemJ.org/bj/441/bj4410387add.htm), we observed that most of the co-chaperones examined showed similar binding to Hsp90α, as well as to Src, Akt and PKC (Supplementary Figures S3 and S4), under these two conditions. Interestingly, we noticed that both Hop and Hsp70 show decreased association with Hsp90α under starved conditions (Supplementary Figure S3).

DISCUSSION

In the present study we show that phosphorylation of Hsp90α at Thr<sup>90</sup>, a conserved residue in higher eukaryotes, plays a fundamental role in regulating Hsp90α chaperone machinery, which is achieved through affecting the intrinsic ATPase activity of Hsp90α as well as the association of distinct co-chaperones that leads to the change of Hsp90α–client interactions. More importantly, the conserved Thr<sup>90</sup> partly reflects the requirements for its sensitivity in regulating the chaperone functions of Hsp90 upon phosphorylation. We therefore propose a working model based on our present results as shown in Figure 5: Hsp90α Thr<sup>90</sup> phosphorylation, mediated by PKA, results in a conformational change that (i) leads to reduced ATP binding, and (ii) switches between different subsets of co-chaperones with Hsp90α, consequently affecting the chaperoning of a subset of client proteins, especially the kinases, such as Src, Akt and PKC. Different stages of Hsp90α (in complexes with ATP/ADP or co-chaperones) might be interchangeable upon Thr<sup>90</sup> phosphorylation (Figure 5), which means that the equilibrium is dynamic but not fixed.

Thr<sup>90</sup> phosphorylation by PKA

In the present study, we have confirmed that PKA can phosphorylate Hsp90α at the single Thr<sup>90</sup> residue. We also tried
to use two forms of Hsp90α for LC (liquid chromatography)-MS analysis: Hsp90α phosphorylated by in vitro co-incubation with PKA, and Hsp90α immunoprecipitated from cells after overexpressing PKA. However, owing to current technical issues, we have not obtained MS data to show direct Thr90 phosphorylation. Still, our present results as well as previous reports, namely that the T90A mutation resulted in the inability of PKA to phosphorylate Hsp90α, and that phosphorylated Thr90 of Hsp90α could be recognized by the specific antibody (Figures 1A–1C) [16,18], firmly prove that PKA can indeed phosphorylate Hsp90α at Thr90. In many cases, it is speculated that the spatial and temporal micro-compartmentation of protein kinase or phosphatase activities is required to achieve specific or optimized modulation, especially for a highly regulated signal-transduction pathway [45]. PKA is such a protein kinase that is ubiquitously presented and widely involved in numerous parallel signalling cascades [45]. It is reported that the specificity of PKA is achieved, in part, through the compartmentation of PKA at discrete subcellular locations that recruits PKA, along with its anchor proteins, close to its target substrates, thereby affecting their biological activities [46,47]. Compartmentation of other kinases, phosphatases and substrates is widely conjectured to be a key determinant in the specificity of other signalling pathways, although the molecular basis and cellular consequences of such compartmentation are less well understood [46,47]. The catalysis of Hsp90α Thr90 phosphorylation by PKA should be a highly regulated process. This may be achieved by Hsp90α itself or other cofactors, or by the micro-compartmentation of Hsp90α. Therefore it is most likely that Thr90 phosphorylation of Hsp90α only occurs for a subpool of Hsp90α. Currently, it is unclear how the specificity of this particular phosphorylation is regulated. On the basis of the observation that the pThr90-Hsp90α level is significantly elevated in proliferating cells in which the PKA signalling is constantly active, it is reasonable to deduce that Hsp90α Thr90 phosphorylation may be a fast and dynamic process. Besides, it is also unclear whether other phosphorylation sites sites are coupled with Thr90 to regulate Hsp90α chaperone function, or whether these distinct sites are regulated at discrete micro-compartmentations within the cells triggered by different stimuli, thus independently affecting Hsp90α chaperone machinery. Further investigations on the above issues will improve our current understanding of post-translational-modification-regulated Hsp90α chaperone function.

**Thr90 phosphorylation with ATP binding and co-chaperone interaction**

It is now widely accepted that ATP binding and hydrolysis is critical for the cycling of Hsp90α chaperone machinery. Hsp90α in solution does not have a single ‘relaxed’ conformation, but exists as a continuum of conformations [5]; nucleotide binding to the N-terminal pocket results in the conformational change of Hsp90α, which is believed to define distinct, non-overlapping subsets of co-chaperones, forming a so-called ‘super-chaperone machine’ that cycles between at least two conformations, resulting in the opening and closing of a ‘molecular clamp’. The ATPase activity domain locates at the N-terminus of Hsp90α, and it is noteworthy that Thr90 phosphorylation may cause a similar structural change (either locally or extending to the whole protein complex) such that pThr90-Hsp90α may adopt a unique transient conformation in which the ATP binding or hydrolysis activity is affected, as evident in our present study (Figures 2B and 2C). In addition, Thr90 phosphorylation does not affect the dimer formation of Hsp90α, as dimerization of T90A and T90E mutants is similar to that of WT Hsp90α (results not shown).

More importantly, our observation that the Hsp90α phosphorymimic T90E mutant exhibits varied association abilities with distinct co-chaperones examined in the present study indicates that Thr90 phosphorylation may confer a dramatic impact on the switch of Hsp90α between super-chaperone complexes. Specifically, the fact that Thr90 is highly conserved in higher eukaryotes partly reflects the requirements for its sensitivity in regulating the chaperone functions of Hsp90 upon phosphorylation. The ATP-binding-and-hydrolysis-induced conformational change of Hsp90α is traditionally considered as a key determinant for co-chaperone binding to Hsp90α, even though a full understanding of the spatiotemporal regulation of Hsp90α–co-chaperone super-complex formation remains lacking [2,5]. Interestingly, using a single-molecule assay to examine Hsp90 dimers in real time under various nucleotide conditions, Mickler et al. [48] found that the large conformational changes of Hsp90 were only weakly coupled with ATP hydrolysis, suggesting that other factors should be involved. Our present finding adds another determinant, Thr90 phosphorylation, to this dynamic process: we find that Thr90 phosphorylation confers differential binding affinities of Hsp90α towards a subset of co-chaperones. Our explanation is that phosphorylation of Thr90 causes a conformational change, which, in turn endorses a complementary shape with its co-chaperone proteins, resulting in an increased affinity, or impairs the previously complementary shape, thus decreasing the interaction. Co-chaperones that have been studied are known to modulate Hsp90 function mainly in four ways: co-ordinate the interplay between Hsp90 and other chaperone systems, such as Hsp70; stimulate or inhibit the ATPase activity of Hsp90; recruit specific classes of clients to Hsp90; and contribute to various aspects of the chaperone cycle through their enzymatic activities [5]. The discovery that Thr90 phosphorylation distinguishes the association of co-chaperones to the Hsp90α complex provides a novel mechanistic explanation as to how distinct co-chaperones are recognized or modulated by a single post-translational modification, reflecting the dynamic Hsp90α chaperone machinery with possible positive and negative feedbacks [12,13]. Still, similar questions remain as to how much this pThr90-Hsp90α subpool contributes to the full-pool chaperone activity of cytoplasmic Hsp90α, and how other known phosphorylation sites are separately or collectively involved in regulating Hsp90α chaperone machinery [12,20,21]. In an effort of trying to study co-chaperone interaction with Hsp90α upon PKA overexpression or silencing, we unexpectedly found that most of the co-chaperones studied showed enhanced association with Hsp90α when PKA is forcedly overexpressed, whereas they exhibit decreased interaction with Hsp90α upon siRNA-mediated PKA silencing (Supplementary Figures S1 and S2). This cannot be attributed solely to the effect of Thr90 phosphorylation status, but rather due to the overall effect of PKA-signalling pathways. Overactivation or silencing of PKA-transduction pathways may impose great effects on cellular events, considering the complicated intersecting pathways of PKA with other key transducers. This further suggests that PKA might also be indirectly involved in the regulation of Hsp90α chaperone machinery via other mechanisms in addition to direct Thr90 phosphorylation of Hsp90α. Nevertheless, on the basis of our present study as well as other reports that phosphorylation of other residues regulates Hsp90α function [20,21], we can draw the conclusion that the dynamics of Hsp90α chaperone machinery is significantly influenced by other factors, including unique post-translational modifications.
Thr90 phosphorylation and client protein interaction

One key feature of the Hsp90α chaperone machinery is its clientele consisting of >100 proteins dominated by kinases, hormone receptors and transcription factors [1,2,4,5]. Binding of these client proteins to Hsp90α chaperone machinery facilitates their maturation and activation [1,2,4,5]. Chaperoning of these clients by Hsp90α is achieved via cycling of the machinery itself driven by an ATP hydrolysis-induced conformational switch, which is also modulated by loading and release of functionally distinct co-chaperones. Our finding that Thr90 phosphorylation specifically affects the association of a subset of kinases to Hsp90α defines its regulatory role in the Hsp90α chaperone machinery. Although how Hsp90α recognizes and distinguishes its large clientele remains poorly understood [5], it is beginning to be appreciated that co-chaperone-mediated client loading may play a dominant role. Among these, p50<sup>α</sup>-mediated kinase interaction with Hsp90α is perhaps the most studied [30]. We find that Hsp90α Thr90 phosphorylation dramatically affects its association with Cdc37 and consequently affects kinase interaction with Hsp90α, which strengthens the point that this modification is functionally correlated with Hsp90α chaperone machinery in vivo. Importantly, the interplay of functionally distinct co-chaperones has a central role in client loading and release, so the varied interactions of the examined co-chaperones with pThr<sup>α</sup>-Hsp90α may therefore co-ordinately modulate the dynamics of the Hsp90α–co-chaperone–client complex. Although we found the pThr<sup>α</sup>-Hsp90α level is increased under proliferation conditions, we did not observe any apparent differences in client or co-chaperone interaction with Hsp90α under these two conditions. This could be because the contributing effect of pThr<sup>α</sup>-Hsp90α fraction could be very small and masked by the majority ‘normal’ or ‘WT’ Hsp90α, or different co-chaperones have differences in their sensitivities to Hsp90α binding. It is clear that Hop and Hsp70 show enhanced binding with Hsp90α under a proliferation status, whereas other co-chaperones or clients tested in the present study are not very sensitive (Supplementary Figure S3).

Taken together, the results of the present study demonstrate that PKA specifically phosphorylates Hsp90α at a conserved residue, Thr<sup>α</sup>, which is tightly linked with altered ATPase activity of Hsp90α. More importantly, Thr<sup>α</sup> phosphorylation switches the Hsp90α chaperone machinery between distinct co-chaperones to regulate its association with client proteins, such as protein kinases. These findings prove the concept that post-translational modifications (phosphorylation in the present study) can serve as an important regulator of the Hsp90α chaperone machinery, reflecting the complex and dynamic nature of this machinery. Our present study promotes further studies on the co-ordination of different modifications on Hsp90α, which will provide insight into its chaperone machinery.

AUTHOR CONTRIBUTION

Yonghao Luo designed the project, interpreted the data and wrote the paper. Xiaofeng Wang designed and performed all experiments, interpreted the data and wrote the paper. Xin-an Lu, Xiaomin Song, Wei Zhuo, Lin Jia and Yushan Jiang performed experiments and discussed the data.

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SUPPLEMENTARY ONLINE DATA

Thr90 phosphorylation of Hsp90α by protein kinase A regulates its chaperone machinery

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**Figure S1** Hsp90α–co-chaperone interaction upon PKA overexpression

HeLa cells were co-transfected with Aha1, Cdc37, p23–GFP, PP5, Hop, FKBP52 or CHIP, and control vector or PKA respectively as indicated. Cells were lysed and immunoprecipitated (IP) using an anti-Hsp90α antibody as indicated, and co-immunoprecipitated proteins were detected by immunoblotting. * indicates the immunoglobulin heavy chain (for Cdc37 and p23–GFP).

**Figure S2** Hsp90α–co-chaperone interaction upon PKA silencing

HeLa cells were co-transfected with Aha1, Cdc37, p23–GFP, PP5, Hop, FKBP52 or CHIP, and control non-silencing scRNA (scrambled RNA) or PKA siRNA respectively as indicated. Cells were lysed and immunoprecipitated (IP) by anti-Hsp90α antibody as indicated, and co-immunoprecipitated proteins were detected by immunoblotting. * indicates the immunoglobulin heavy chain (for Cdc37 and p23–GFP).
Figure S3  Hsp90α–co-chaperone interaction under starved or proliferating conditions

HeLa cells were transfected with Aha1, Cdc37, p23–GFP, PP5, Hop, FKBP52 or CHIP respectively as indicated. Cells were cultured in FBS (fetal bovine serum)-free medium (S, starved) or 10% (v/v) FBS medium (P, proliferation) and then lysed and immunoprecipitated (IP) using an anti-Hsp90α antibody as indicated, and co-immunoprecipitated proteins were detected by immunoblotting. * indicates the immunoglobulin heavy chain (for Cdc37 and p23–GFP).

Figure S4  Hsp90α–client interaction under starved or proliferating conditions

HeLa cells were transfected with Akt or PKCγ as indicated. Cells were cultured in FBS-free medium (S, starved) or 10% (v/v) FBS medium (P, proliferation) and then lysed and immunoprecipitated (IP) using an anti-Hsp90α antibody as indicated, and co-immunoprecipitated proteins were detected by immunoblotting.

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