Dynamics of the regulation of Hsp90 by the co-chaperone Sti1

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In eukaryotic cells, Hsp90 chaperones assist late folding steps of many regulatory protein clients by a complex ATPase cycle. Binding of clients to Hsp90 requires prior interaction with Hsp70 and a transfer reaction that is mediated by the co-chaperone Sti1/Hop. Sti1 furthers client transfer by inhibiting Hsp90's ATPase activity. To better understand how Sti1 prepares Hsp90 for client acceptance, we characterized the interacting domains and analysed how Hsp90 and Sti1 mutually influence their conformational dynamics using hydrogen exchange mass spectrometry. Sti1 stabilizes several regions in all three domains of Hsp90 and slows down dissociation of the Hsp90 dimer. Our data suggest that Sti1 inhibits Hsp90's ATPase activity by preventing N-terminal dimerization and docking of the N-terminal domain with the middle domain. Using crosslinking and mass spectrometry we identified Sti1 segments, which are in close vicinity of the N-terminal domain of Hsp90. We found that the length of the linker between C-terminal dimerization domain and the C-terminal MEEVD motif is important for Sti1 association rates and propose a kinetic model for Sti1 binding to Hsp90.

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Introduction

The highly conserved Hsp90 chaperones control maturation, stability and activity of many client proteins, among them numerous signalling kinases and transcription factors (Picard, 2002; summarized in <http://www.picard.ch/downloads/Hsp90interactors.pdf>). Since for many of these clients interaction with Hsp90 chaperones is essential, Hsp90's plays a key role in cell homeostasis, proliferation, differentiation and programmed cell death.

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Hsp90 proteins consist of an N-terminal nucleotide binding domain (NBD), a middle domain (MD) involved in client binding and a C-terminal dimerization domain (DD) (Ali *et al*, 2006; Pearl and Prodromou, 2006; Shiao *et al*, 2006). It is believed that most clients interact with Hsp90 in a similar chaperone cycle, originally proposed for progesterone receptor (Smith, 1993). Clients first interact with Hsp40 and Hsp70 chaperones. Through an intermediate Hsp70–Hsp90–client complex the mature Hsp90–client complex is formed. This complex dissociates with a certain half-life (ca. 5 min for progesterone receptor) and the client can reenter the cycle by binding to Hsp40 and Hsp70. Progression through the chaperone cycle requires ATP hydrolysis by Hsp90 and involves substantial conformational changes including N-terminal dimerization and docking of the NBD with the MD (Obermann *et al*, 1998; Panaretou *et al*, 1998; Wegele *et al*, 2003; Ali *et al*, 2006; Shiao *et al*, 2006; Southworth and Agard, 2008; Hessling *et al*, 2009; Mickler *et al*, 2009; reviewed in Richter and Buchner, 2006; Mayer, 2010). The Hsp90 chaperone cycle is regulated by a number of co-chaperones including Sti1 (Hop), peptidyl-prolyl-cis/trans-isomerases (e.g., FKBP52 in mammals and Cpr6 in yeast), p23/Sba1 and Aha1, which regulate the ATPase activity of Hsp90 and progression through the cycle (Wegele *et al*, 2004).

Sti1 plays a key role in the Hsp90 cycle since it promotes transfer of the client from Hsp70 to Hsp90 by simultaneously binding to Hsp70 and Hsp90 thereby stabilizing the intermediate Hsp70–Hsp90–client complex (Wegele *et al*, 2006). Sti1 consists of three tetratricopeptide repeat (TPR) domains and two aspartate-proline motif (DP) regions (Figure 1A). TPR1 specifically interacts with the C-terminal GPTIEVD motif of Hsp70 and TPR2a with the C-terminal MEEVD motif of Hsp90 (Scheufler *et al*, 2000; Odunuga *et al*, 2003). The function of TPR2b, DP1 and DP2 is currently unknown. Yeast Sti1 inhibits the basal ATPase activity of yeast Hsp90 the molecular basis for which is unclear. In this study, we employed truncation mutants, chemical crosslinking and fluorescence spectroscopy to better characterize the interaction of Sti1 with Hsp90 and we analysed changes in conformational dynamics of Hsp90 and Sti1 upon complex formation using hydrogen exchange mass spectrometry (HX-MS) to elucidate the molecular mechanism of Sti1-mediated inhibition of Hsp90's ATPase activity.

Results

Sti1 domains interacting with Hsp90

Previous work has shown that TPR2a of Sti1/Hop interacts with the C-terminal MEEVD motif of Hsp90 and that this interaction is essential for the binding of Sti1 to Hsp90 (Scheufler *et al*, 2000; Richter *et al*, 2003; Johnson *et al*, 2007). To determine which additional domains of Sti1 are necessary for the inhibition of Hsp90's ATPase activity, we constructed different deletion variants of Sti1 and determined the k_{cat} in steady-state ATPase assays. As shown in Figure 1,

deletion of TPR1, DP1 or DP2 did not affect the ability of Sti1 to inhibit the ATPase activity of Hsp90. Only the deletion of TPR2b-DP2 significantly reduced this ability suggesting that TPR2b contributes to the inhibitory effect of Sti1. Since Hsp90's ATPase activity in the presence of Sti1 Δ (TPR2b-DP2) was still lower than in the absence of Sti1, other parts

of Sti1 also add to the inhibition. In the absence of TPR2a, TPR2b-DP2 was not able to inhibit the ATPase activity of Hsp90 even at a 10-fold excess over Hsp90 (Figure 1D). Although TPR2a is known to interact with the C-terminal MEEVD motif of Hsp90, at high enough concentrations, Sti1 is able to inhibit an Hsp90 variant lacking the MEEVD motif (Hsp90 Δ MEEVD; Figure 1D). Together, these data demonstrate that TPR2a and TPR2b together inhibit the ATPase activity of Hsp90 and that the interaction with the MEEVD motif is not essential but increases efficacy of the inhibition (see below).

Using HX-MS, we analysed the conformational changes in Sti1 induced by binding of full-length Hsp90 and Hsp90 N-terminal deletion variants missing the NBD or NBD and MD. In the absence of Hsp90, Sti1 rapidly incorporated deuterons into most parts of the protein (Supplementary Figure S2; original spectra are shown in Supplementary Figure S1). Notably, the first and last helix of each TPR domain exchanged >80% of its amide hydrogens within 30 s. The presence of Hsp90 had no influence on deuterium incorporation into TPR1 but led to a strong reduction of deuterium incorporation into TPR2a and TPR2b (Figure 2A; Supplementary Figure S2). The reduced hydrogen exchange in TPR2a is not unexpected, since the C-terminal MEEVD segment of Hsp90 forms hydrogen bonds and hydrophobic interactions with side chains of TPR2a thereby stabilizing the helices of this domain (Figure 2B). This protection was also observed when NBD or NBD and MD were deleted in Hsp90 (Figure 2A, middle and right panels). Interestingly, in the presence of full-length Hsp90, regions N-terminal of TPR2a, TPR2b, the linker between TPR2a and TPR2b, and a segment C-terminal of TPR2b showed also a strong protection, indicating that Hsp90 interacts also with these regions of Sti1 (Figure 2). The protection observed in the linker between TPR2a and TPR2b suggests that the TPR2a-TPR2b core of the Sti1 protein becomes rigid when Sti1 binds to Hsp90 and that movement of both domains relative to each other is prevented. Consistent with this hypothesis is the observation that the protection in the linker is missing like most of the protection in TPR2b when Sti1 is incubated with Hsp90 lacking NBD or NBD and MD, suggesting that the NBD is important for stable binding of TPR2b to Hsp90 and the stabilization of TPR2b relative to TPR2a (Figure 2A, middle panel).

To elucidate the contribution of the MEEVD motif to the change in conformational dynamics in Sti1, we performed HX-MS experiments with Sti1 in the presence of the Hsp90 Δ MEEVD variant. To compensate for the reduced affinity, these experiments were performed at higher concentrations of Hsp90 and Sti1. In the absence of the MEEVD motif the protection in TPR2a was strongly reduced, while

Figure 1 TPR2a and TPR2b are essential for Sti1's ability to inhibit Hsp90's ATPase activity. (A) Domain structure of Sti1 and different deletion constructs used in this study. (B) Michaelis-Menten kinetic of the ATPase activity of yeast Hsp90 (2.5 μ M; diamonds) and inhibition of ATPase activity by Sti1 (4 μ M; squares). (C) ATPase rate k_{cat} of Hsp90 in the absence of Sti1 and in the presence of full-length Sti1 and different deletion variants. Error bars are standard error of mean of three or four independent experiments. (D) Relative ATPase rate of Hsp90 and Hsp90 with deleted C-terminal MEEVD motif (Hsp90 Δ MEEVD) (2 μ M) in the absence of Sti1 and in the presence of full-length Sti1 and Sti1-TPR2b-DP2 (20 μ M) at 500 μ M ATP. Error bars are standard error of mean of three independent experiments.

Figure 2 Stabilization of the conformation of Sti1 in the presence of Hsp90. (A) Difference plots of deuterium incorporation into Sti1 segments in the presence of Hsp90 (left panel), Hsp90 (262–709) (middle panel) or Hsp90 (525–709) (right panel) minus deuterium incorporation into Sti1 in the absence of Hsp90 full-length or truncation proteins. Domain structure of Sti1 indicated to the left. The reaction mixtures were incubated for 30 s in D₂O. Error bars are standard error of mean of three independent experiments. (B) Cartoon representation of a homology model of TPR2a and TPR2b onto the crystal structure of human Hop TPR2a in complex with the MEEVD peptide (PDB entry code 1ELR; Peitsch, 1995; Scheufler *et al*, 2000; Arnold *et al*, 2006; Kiefer *et al*, 2009) coloured according to the change in deuterium incorporation as indicated. (Note that due to the resolution of the method, which is limited by the size of the proteolytic fragments, the coloured region and the actually protected region are not necessarily identical and that it cannot be distinguished between the case in which all amide hydrogens within the region are partially protected and the case in which only a subset of the indicated region is fully protected within the incubation time while other parts are not affected.) For TPR2a, the MEEVD peptide is shown and possible hydrogen bonds to side chains are indicated by dashed lines. The first and the last residues of each TPR domain and the residues of the MEEVD peptide are labelled.

significant protection was still observed in the linker between DP1 and TPR2a and in TPR2b (Supplementary Figure S3A and B). The remaining protection in TPR2a was almost completely lost when only the DD with deleted MEEVD motif was used. These data indicate that most of the protection of TPR2a originates from the interaction of the MEEVD motif with this domain and that interaction with the MD of Hsp90 contributes to the protection in TPR2a. The protection in the C-terminal part of the DP1-motif domain and in TPR2b seems to be independent of the MEEVD motif and the DD of Hsp90.

Together, these data suggest that TPR2a together with TPR2b inhibit the ATPase activity of Hsp90 by direct stabilization of Hsp90's MD and NBD in a defined conformation.

Influence of Sti1 on the dynamics of Hsp90

To analyse how Sti1 inhibits Hsp90's ATPase activity, we analysed the conformational dynamics of Hsp90 in the presence of Sti1, Sti1ΔTPR1 and Sti1Δ(TPR2b-DP2) (original spectra are shown in Supplementary Figure S4; kinetics of exchange for all peptic fragments is shown in Supplementary Figure S5). In the presence of full-length Sti1, a protection from deuterium incorporation was observed in all three domains of Hsp90 (Figure 3A, left panel). These data indicate an overall reduction of conformational flexibility of Hsp90 when Sti1 is bound. In particular, protection from hydrogen exchange was observed in the dimerization interface (regions 665–671; compare Figure 3B, right panel and Supplementary

Figure 3 Stabilization of the conformation of Hsp90 in the presence of Sti1. **(A)** Difference plots of deuterium incorporation into Hsp90 segments in the presence of Sti1 (left panel), Sti1ΔTPR1 (middle panel) or Sti1Δ(TPR2B-DP2) (right panel) minus deuterium incorporation into Hsp90 in the absence of Sti1 full-length or truncation proteins. Domain structure of Hsp90 is indicated to the left. The reaction mixtures were incubated for 30 s in D₂O. Error bars are standard error of mean of three independent experiments. **(B)** Cartoon representation of Hsp90 (PDB entry code 2CG9) coloured according to the difference in hydrogen exchange as indicated. Right panel rotated by 90° relative to left panel as indicated. **(C)** Subunit exchange kinetics in the absence and presence of Sti1 measured by FRET using ATTO488- and ATTO550-labelled Hsp90-E57C. The exchange was fitted by a single exponential decay function to yield the rates of 0.0315 s⁻¹ and 0.0105 s⁻¹ for absence and presence of Sti1, respectively.

Figure S5A peptides 652–664 and 652–671), in the hinge region between MD and DD (segments 480–496), and in several structural elements of the NBD. The increased protection in the dimerization interface suggests that Sti1 prevents C-terminal opening and may slow down subunit exchange in the Hsp90 dimer (Ratzke *et al*, 2010). This hypothesis was confirmed by a fluorescence resonance energy transfer (FRET) assay (Hessling *et al*, 2009; Figure 3C).

When comparing the difference in HX in the absence and presence of Sti1 at 30, 100 and 1000 s in D₂O, three different

types of segments can be distinguished: (1) The degree of protection stays roughly the same throughout the incubation time (e.g., 8–15, 480–496, 652–671; Supplementary Figure S5), revealing stabilization of a highly flexible structural element, which exchanges protons within 30 s in the absence of Sti1 but not within 1000 s in its presence. (2) The degree of protection increases with time (e.g., 183–200, 322–332, 358–364; Supplementary Figure S5), indicating that an existing slow exchanging secondary structure element was further stabilized by Sti1. (3) The degree of protection

decreases with time (e.g., 43–62, 114–124; Supplementary Figure S5), suggesting either a lower degree of stabilization or a region of dynamic interactions with frequent dissociation and reassociation.

To distinguish conformational changes induced by direct contact of Sti1 with Hsp90 and allosterically induced changes, we compared the effect of Sti1 on Hsp90 with the previously studied effect of ATP on the *Escherichia coli* Hsp90 HtpG (Graf *et al*, 2009) representing the conformational changes of Hsp90 upon transition into the closed, ATP hydrolysis-competent state (Supplementary Figure S6). In HtpG within regions 21–31, 192–206 and 319–334 corresponding to regions 20–30, 189–203 and 363–378 in yeast Hsp90, a strong protection was observed upon addition of ATP at 10–100 s exchange time. The protection within regions 21–31 is most likely due to N-terminal dimerization and the protection within regions 192–206 and 319–334 to docking of NBD and MD (Graf *et al*, 2009). The corresponding regions in yeast Hsp90 do not exhibit significant change in deuterium incorporation in the presence of Sti1 at 30 and 100 s, indicating that regions, which are flexible in the absence of Sti1 remained flexible in its presence. These data indicate that Sti1 inhibits ATP hydrolysis by preventing N-terminal dimerization and docking of NBD with the MD, which is a prerequisite to allow access of the catalytic arginine 380 to the γ -phosphate of ATP.

In contrast, protection is observed within the exposed regions 43–62 in yeast Hsp90 upon addition of Sti1 but not in HtpG upon addition of ATP (Supplementary Figure S6). This region might be a direct contact site for Sti1 (see below). Additional contact sites for Sti1 might be within regions 270–283, 333–343, 454–459 and 480–496, which also exhibit protection in the presence of Sti1. When the isolated MD of Hsp90 was incubated with Sti1 most protection was strongly reduced. This may be due to the missing interactions of Sti1 and/or the MD with the NBD and DD. Instead, a strong protection was observed within segments 455–472, which, for technical reasons, could not be detected in experiments with the full-length protein (Supplementary Figure S7).

When Hsp90 was analysed in the presence of the deletion variant of Sti1 lacking TPR1 the observed protection was very similar, in some parts even stronger than in the presence of full-length Sti1 (Figure 3A, middle panel). When instead TPR2b and DP2 were missing, all protection from hydrogen exchange was lost and even more exchange was observed in some segments of Hsp90, in particular around the nucleotide binding pocket (residues 63–145) and the charged linker (residues 250–266), indicating an increase in flexibility in the presence of Sti1 Δ (TPR2b-DP2). (Note: peptides covering the C-terminal region of Hsp90, residues 672–709 including the MEEVD motif, were not detected in our experiments.) Binding of Sti1 Δ (TPR2b-DP2) to Hsp90 was verified by HX-MS (Supplementary Figure S3C).

Together, these data suggest that Sti1 stabilizes Hsp90 in an open conformation by preventing N-terminal dimerization and docking of the NBD with the MD and that TPR2b is essential for this stabilizing effect.

Sti1 can be crosslinked to position 57 of Hsp90

To get independent evidence for a close proximity of Sti1 and the NBD of Hsp90, we introduced a cysteine into the exposed

region in the NBD, which is stabilized in the presence of Sti1 by replacing glutamate 57 (Hsp90-E57C). We labelled this cysteine with the thiol-specific UV-activatable crosslinker benzophenone-4-iodoacetamide (BPIA), which bridges 10 Å, formed the complex with Sti1, and induced crosslinking by UV irradiation. The reaction products were resolved by SDS-PAGE and detected by immunoblotting using Sti1-specific antisera. As shown in Figure 4A only in the presence of Sti1, labelled Hsp90 and UV a crosslinking product was visible. To test whether this crosslink is specific for Hsp90-bound Sti1, we added the non-hydrolysable ATP analogue AMPPNP and the co-chaperone Sba1, known to displace Sti1 under these conditions (Richter *et al*, 2004). In the presence of Sba1, the crosslinking product between Hsp90 and Sti1 was significantly reduced, demonstrating that the formation of the crosslinking product requires a functional Hsp90–Sti1 complex. It is unlikely that Sba1 binding to Hsp90 blocks access to the crosslinker, since Glu57 is completely accessible in the co-crystal structure of Hsp90 with Sba1 (Ali *et al*, 2006; Supplementary Figure S8) and this cysteine remains accessible to a maleimide in the presence of Sba1 (C-TL and MPM, unpublished data). These data indicate that parts of Sti1 are <10 Å away from residue 57 of Hsp90 in the functional Hsp90–Sti1 complex. TPR1 of Sti1 is not necessary to form the crosslink (Figure 4C and data not shown).

To identify the crosslinked site in Sti1, we used an N-terminally STREP-tagged Hsp90-E57C and labelled it with the cleavable thiol-specific UV-activatable crosslinker benzophenone-4-carboxamidocysteine methanethiosulphonate, which bridges 13 Å. We crosslinked this Hsp90 variant with N-terminally His-tagged Sti1 or Sti1 Δ TPR1 and isolated the crosslinking product by successive StreptactinTM and denaturing Ni²⁺-affinity chromatography. The purified crosslinking product was cleaved by addition of dithiothreitol, urea denatured and digested by trypsin. The desalted sample was then subjected to nano reversed-phase chromatography and Matrix-Assisted/Laser Desorption Ionization (MALDI) tandem mass spectrometry. Surprisingly, we detected two different peptides, one from TPR2a (VISK, residues 334–337) and one from TPR2b (EIDQLYYKASQQR, residues 505–517), each modified with the crosslinker alternatively at different residues (Supplementary Figures S9 and S10), indicating that in the Hsp90–Sti1 complex both regions seem to approach a distance of 13 Å or less to position 57 in Hsp90. Both peptides were independently detected in the sample with Sti1 Δ TPR1 (data not shown). For TPR2b, this might have been expected but not for TPR2a. The question arises how TPR2a can bind to the C-terminal MEEVD motif and reach at the same time so close to the NBD of Hsp90, though the distance between position 57 and the last residue visible in the crystal structure of yeast Hsp90 (PDB entry code 2CG9; Ali *et al*, 2006) is about 110 Å. Interestingly, residues 678–709, which contain the C-terminal MEEVD motif, are disordered in the structure. These residues could extend to about 100 Å, which would be sufficient to allow TPR2a within the reach of a 10 Å crosslinker at position 57.

To verify our findings with an independent experiment, we constructed a Sti1 variant consisting only of TPR2a and TPR2b with a cleavage site for the TEV protease introduced between the two domains and an N-terminal FLAG tag and a C-terminal HA tag (Sti1-FTH; Figure 4B). This construct was crosslinked to BPIA-labelled Hsp90. The crosslinking pro-

ducts were digested with TEV protease and analysed by SDS-PAGE and immunoblotting with FLAG and HA-specific monoclonal antibodies. With both specific antibodies we detect before and after TEV cleavage a crosslink band at the expected size (Figure 4C, lanes 6 and 7). Comparing band intensities before and after TEV cleavage reveals that crosslinking with TPR2b is more efficient than with TPR2a. As a control, we used a variant of Hsp90-E57C with a deletion of the MEEVD motif to exclude that crosslinks occur by a mechanism that is independent of TPR2a binding to the MEEVD motif of Hsp90. For Hsp90-E57C-ΔMEEVD, no

crosslinking products could be observed (Supplementary Figure S11). Our results demonstrate that position 57 in Hsp90 crosslinks to TPR2a as well as to TPR2b, indicating that both TPR domains can approach the NBD of Hsp90 at least transiently up to 10 Å. The crosslinked segments identified by mass spectrometry are shown in homology models in Figure 4D.

The flexible C-terminal linker accelerates Sti1 binding

Since simultaneous binding of TPR2a to the MEEVD motif and a region in the MD close enough to the NBD to allow the observed crosslink requires an extended linker between the DD and the MEEVD motif, we hypothesized that the linker with the MEEVD motif might be important for Sti1 binding to Hsp90. To test this hypothesis, we deleted 20 residues (aa 695–704) between the DD and the MEEVD motif (Hsp90Δ20) and measured association of Sti1 with Hsp90 using FRET with ATTO488 maleimide-labelled Hsp90-E57C and Hsp90Δ (695–704)-E57C (Hsp90-488 and Hsp90Δ20-488) as donor and ATTO550 maleimide-labelled Sti1 (Sti1-550) as acceptor. Sti1 has three cysteines, two in TPR1 and one in TPR2b. We took care that Sti1 was labelled at an average ratio of ~1:1 with the dye. Rapid mixing of Sti1-550 with Hsp90-488 resulted in an association curve with at least three phases, indicating a multistep binding process (Figure 5A). Rates and amplitudes of all three phases are given in Table I. Binding of Sti1 to Hsp90Δ20 was significantly slower than to the wild-type protein demonstrating that the linker between the C-terminal domain and the MEEVD motif accelerates Sti1 binding.

To assess the functional relevance, we determined the ability of Sti1 to inhibit the ATPase activity of wild-type Hsp90 and the linker-deletion variant. As a control, we included the Hsp90 variant with deleted MEEVD motif. The basal ATPase activity of Hsp90Δ20 was 27% higher than the activity of the wild-type protein. Hsp90ΔMEEVD had even a

Figure 4 Sti1 TPR2a and TPR2b can be crosslinked to the NBD of Hsp90. **(A)** Western blot using Sti1-specific antisera of the SDS-PAGE separated crosslinking reactions. Sti1 was incubated in the absence of Hsp90 (lanes 1, 2, 7 and 8) or in the presence of BPIA-labelled Hsp90 (lanes 3, 4, 9, and 10) and in the absence of any other additives (lanes 1–4) or in the presence of Sba1 and AMPPNP (lanes 7–10). In lanes 5 and 6, BPIA-labelled Hsp90 was incubated in the absence of Sti1, in the presence of Sba1. UV irradiation was applied as indicated. **(B)** Cartoon of the Sti1-FTH construct consisting of TPR2a and TPR2b of Sti1 with an N-terminal FLAG tag, a C-terminal HA tag and a TEV-protease cleavage site between the two TPR domains. **(C)** α-FLAG (upper panel) and α-HA western blot (lower panel) of SDS-PAGE separated crosslinking reactions containing the component as indicated above the panels. Samples of lanes 7–12 were treated with TEV protease after UV irradiation. 90 x Sti1-FTH, crosslink of Hsp90 to the Sti1 construct (lane 6); 90 x 2a, crosslink of Hsp90 to TPR2a (lane 7, upper panel); 90 x 2b, crosslink of Hsp90 to TPR2b (lane 7, lower panel); 90, unspecific detection of Hsp90; Sti1-FTH, Sti1 construct; Sti1-F, FLAG tag-TPR2a after TEV cleavage of Sti1-FTH; Sti1-H; TPR2b-HA after TEV cleavage of Sti1-FTH; additional bands visible in lanes 2–6 and 7–10 are contaminants and not crosslinking products since they appear even in the absence of crosslinker (Hsp90-E57C-BPA, lanes 3 and 4; lanes 9 and 10) or UV (lanes 3 and 5; lanes 8 and 10) **(D)** Cartoon representation of homology models of Sti1-TPR2a (left panel) and Sti1-TPR2b (right panel) coloured according to HX as in Figure 2 with the residues identified by MS to be labelled with the crosslinker BPC shown in magenta.

57% higher basal ATPase rate. Increasing Sti1 concentrations were still able to inhibit Hsp90Δ20, but compared with wild-type Hsp90 higher Sti1 concentrations were necessary to reach the same level of inhibition (Figure 5B). Sti1 was also able to inhibit the ATPase activity of Hsp90ΔMEEVD but very high concentrations were needed (see also Figure 1D). We

conclude that the linker is not essential for Sti1-mediated inhibition of Hsp90's ATPase activity but increases efficacy.

Discussion

This study yielded several insights into the regulation of Hsp90 proteins by the co-chaperones Sti1/Hop. The TPR2a-TPR2b core of Sti1 is mainly responsible for the inhibition of Hsp90's ATPase activity consistent with *ex-vivo* pull-down experiments (Flom *et al*, 2007). Hsp90-induced protection in Sti1 is mainly observed in TPR2a and TPR2b and the NBD of Hsp90 is necessary for most of the protection in TPR2b. Sti1 reduces the conformational dynamics of Hsp90 in all three domains of the protein and thus stabilizes Hsp90 in a conformation, in which the NBDs of the Hsp90 dimers are separated and not docked onto the MDs, consistent with earlier FRET data and a recent negative stain electron microscopy study (Hessling *et al*, 2009; Southworth and Agard, 2011). For this stabilization, TPR2a and TPR2b are necessary. Both TPR2a and TPR2b crosslink to position 57 in the NBD of Hsp90, demonstrating that they can approach this position at least transiently as close as 10 Å. We identified the cross-linked residues in TPR2a and TPR2b using mass spectrometry. Finally, we found that shortening of the linker between the C-terminal domain and the MEEVD motif reduces the rate of Sti1 association with Hsp90 and Sti1's ability to inhibit the ATPase activity of Hsp90. Our data support a model in which Sti1-TPR2a first binds to the MEEVD motif and subsequently aligns on the MD of Hsp90 consistent with a recent study (Schmid *et al*, 2012). Thereby, the NBD of Hsp90 contributes to a stable interaction between TPR2b and MD and contact between the MEEVD motif and TPR2a is maintained (Figure 6).

In our ATPase assays TPR2b-DP2 alone was not able to inhibit the ATPase activity of Hsp90 (Figure 1D). The reason for this could be that the affinity of TPR2b is too low for sufficient binding to Hsp90 at the concentrations tested. Alternatively, TPR2b bound to Hsp90 may not be able to prevent N-terminal dimerization and NBD-MD docking and thus ATP hydrolysis. In contrast, interaction with the MEEVD motif is not essential for the inhibition of the ATPase activity since Sti1 was able to inhibit ATP hydrolysis of the Hsp90ΔMEEVD variant, albeit much higher concentrations were needed for significant inhibition (Figures 1D and 5B).

HX-MS experiments revealed that Sti1 and Hsp90 influence each other's conformation and that this influence depends on Hsp90's NBD and Sti1's TPR2b. Deletion of Hsp90's NBD abolished most of the stabilization observed in TPR2b in the presence of Hsp90 (Figure 2) and deletion of TPR2b-DP2 of

Figure 5 Deletion of the linker between C-terminal domain and MEEVD motif of Hsp90 reduces association rate of Sti1 and its ability to inhibit Hsp90's ATPase activity. (A) ATTO550-labelled Sti1 was rapidly mixed with ATTO488-labelled Hsp90-E57C or Hsp90Δ20-E57C in a stopped-flow device. Excitation wavelength was 480 nm and a cutoff filter of 590 nm was used. The curve through the data points is a fit of the equations

$$y = A_0 + A_1 \cdot (1 - e^{-k_1 t}) + A_2 \cdot (1 - e^{-k_2 t}) + A_3 \cdot e^{-k_3 t}$$

(Hsp90wt) and

$$y = A_0 + A_1 \cdot (1 - e^{-k_1 t}) + A_2 \cdot (1 - e^{-k_2 t}) + A_3 \cdot (1 - e^{-k_3 t})$$

(Hsp90Δ20) to the data. (B) ATPase rates of Hsp90 wild-type (Hsp90wt), linker-deletion variant (Hsp90Δ20), and MEEVD-deletion variant (Hsp90ΔMEEVD) (1 μM) at increasing Sti1 concentrations (0–2 μM) and saturating ATP concentrations (2 mM). Error bars represent standard error of mean of four independent determinations. The curves are fits of the equation $y = IC_{50}/(IC_{50} + [Sti1])$ to the data. The IC_{50} values are given in Table I.

Table I The linker between C-terminal domain and MEEVD motif reduces Sti1 association rates and its ability to inhibit Hsp90's ATPase activity

	Sti1 association						ATPase inhibition
	k_1 (s ⁻¹) ^a	k_2 (s ⁻¹)	k_3 (s ⁻¹)	A_1 ^b	A_2	A_3	IC_{50}
Hsp90wt	35.9 ± 4.8	3.55 ± 0.23	0.35 ± 0.04	36%	58%	7%	0.23 ± 0.02
Hsp90Δ20	25.5 ± 2.3	1.97 ± 0.18	0.19 ± 0.01	34%	27%	39%	0.30 ± 0.02*
Hsp90ΔMEEVD	—	—	—	—	—	—	4.9 ± 0.9

^a k_1 , k_2 , k_3 , rates of association of Sti1 with Hsp90 proteins, mean and standard error of mean of two experiments.

^b A_1 , A_2 , A_3 , relative amplitudes of the three phases of association.

* $P < 0.05$ as compared with value for Hsp90wt.

Figure 6 Model for Sti1 binding to Hsp90. The MEEVD motif is connected to the C-terminal domain of Hsp90 by a long flexible linker (26 residues corresponding to ca. 100 Å if extended), providing a high degree of diffusional freedom to the MEEVD motif. This results in high association rates for binding of the MEEVD motif to the TPR2a domain of Sti1. After this first interaction the tethered Sti1 has limited diffusional freedom and association of TPR2a and TPR2b of Sti1 with the MD of Hsp90 is favoured. HX-MS experiments suggest additional interaction with DD and also NBD, which is possible due to the flexible linker between NBD and MD. It cannot be excluded that Sti1 contacts both protomers of an Hsp90 dimer. Sti1 fixes Hsp90 in an open conformation. At high enough concentrations of Sti1, a second molecule of Sti1 could bind in a similar manner to form a symmetric complex. MEEVD motif and C-terminal linker act as hook-and-fishing line to accelerate binding of Sti1 and possibly other TPR domain-containing co-chaperones.

Sti1 abrogated the protection observed in Hsp90 in the presence of Sti1 (Figure 3). The difference in deuterium incorporation observed in Hsp90 in the presence of Sti1 allows several conclusions. (1) The protection in the C-terminal dimerization interface indicated that Sti1 stabilizes the Hsp90 dimer and prevents opening of the C-terminus that has been observed recently by single molecule FRET experiments (Ratzke *et al*, 2010). The stabilization of the Hsp90 dimer by Sti1 was confirmed by FRET experiments (Figure 3C). (2) The protection in the hinge region between MD and DD suggests that Sti1 arrests the continuous opening-closing movement of the V-shaped Hsp90 dimer in a defined conformation. (3) The unchanged flexibility in segments involved in N-terminal dimerization and NBD–MD docking in the crystal structure of yeast Hsp90 in the closed conformation, which is on the verge of ATP hydrolysis (PDB entry code 2CG9; Ali *et al*, 2006), suggests that Sti1 inhibits ATP hydrolysis by preventing N-terminal dimerization and NBD–MD docking. This observation is consistent with the fact that single amino-acid replacement mutants and an N-terminal

deletion variant of Hsp90 that show an increased tendency to N-terminal dimerization and increased ATP hydrolysis rates, exhibit reduced binding to Sti1 (Richter *et al*, 2003; Johnson *et al*, 2007). They are also inline with more recent FRET and electron microscopy studies (Hessling *et al*, 2009; Southworth and Agard, 2011). (4) Sti1 influences conformational flexibility in three segments of the NBD. The protection in segments 8–15 is consistent with the previous deletion analysis, which implicated the first 24 residues of Hsp90 in Sti1 binding (Richter *et al*, 2003). The reduction of the flexibility of the ATP lid (residues 94–124 (Prodromou *et al*, 1997) covered by segments 90–105 and 114–124) would be expected to have an impact on nucleotide binding. Indeed, Buchner and coworkers observed increased ATP association and dissociation rates in the presence of Sti1 (Richter *et al*, 2003). No immediate explanation is apparent for the other protected site in the NBD. Protection within this site might be caused by direct contact with Sti1 or, alternatively, by a Sti1-induced interaction with the MD of the same protomer or with MD and/or NBD of the other protomer in the Hsp90 dimer in an ATP hydrolysis incompetent conformation, requiring extensive rotation of the NBDs. We recently found that the NBD of Hsp90 rotates by some 180° as compared with the crystal structure of yeast Hsp90 (C-TL and MPM, unpublished results). (5) In the MD, three sites of significant protection were observed (within segments 270–283, 322–343 and 480–496). The segment close to the NBD might be protected by Sti1-induced contact to the NBD. Alternatively or in addition, this region together with the other regions of the MD could be interaction sites for Sti1. Thereby, TPR2b most likely binds to the N-terminal half of the MD, while TPR2a interacts with the C-terminal half of MD and maybe the DD. However, when we performed HX experiments with the isolated MD of Hsp90 in the presence of Sti1, little protection was observed in these regions, which may be due to the missing interactions of Sti1 and/or the MD with the NBD and DD. Instead, we observed a strong protection in the peptic fragments 455–472, which, for technical reasons, could not be detected in experiments with the full-length protein (Supplementary Figure S7). In a recent NMR study, Buchner and coworkers detected chemical shift perturbations in residue 456 of the MD when it was incubated in the presence of TPR2a–TPR2b of Sti1 (Schmid *et al*, 2012). In our experiments with full-length Hsp90 this region was covered by two other peptides (443–459 and 458–472), which also showed some protection in the presence of Sti1. The differences observed in this region in the two experiments are not necessarily conflicting since proteolytic cleavage leads to the conversion of a backbone amide group into an amino group, which rapidly exchanges any incorporated deuterium back to a proton during desalting. In addition, the first amide group in a peptide also has a high exchange rate under quench conditions and thus does not retain the incorporated deuterium. It should also be noted that the observed difference in deuterium incorporation within one peptide is an average over the entire peptide and a segment, which exchange less protons in the presence of Sti1 than in its absence, could be hidden by another segment, which exchange more protons.

Sti1 also seems to interact with the DD. When the isolated DD was incubated with Sti1 HX was reduced in TPR2a and in the linker between DP1 and TPR2a (segments 199–218;

Figure 2A, right panel). The protection in TPR2a can be explained with the interaction with the MEEVD motif but not the protection in the linker. If a truncated DD without MEEVD motif was used most of the protection was lost including the protection in segments 199–218, most likely due to the much-reduced affinity of TPR2a to the DD, which prevented a stable interaction during the HX experiment.

As mentioned above, the linker between TPR2b and DP2 also exhibited a protection in the presence of Hsp90 indicating an interaction. Other parts of DP2 do not appear to interact with Hsp90 since no protection was observed in DP2 in the presence of Hsp90.

Recently, it was shown that one Sti1 per Hsp90 dimer is sufficient to inhibit the ATPase activity (Li *et al*, 2011). The HX-MS experiments were therefore repeated with a stoichiometry of one Sti1 per Hsp90 dimer. We observed a general reduction of the Sti1-induced protection but no bimodal distribution of isotope peaks, indicating that the two Hsp90 molecules in the dimer could not be distinguished in this situation. This result suggests that both protomers of the Hsp90 dimer are in the same conformation, either because Sti1 binding to one protomer induces similar conformational changes in both subunits of the dimer, or Sti1 dissociates and reassociates several times within the 10-s time frame of this experiment.

In our HX experiments, we found a protection within regions 43–62, which decreased with time. This observation would be consistent with a direct but dynamic interaction of this region in the NBD with Sti1. Our crosslinking experiments revealed that position 57 within this region of Hsp90 can be crosslinked to TPR2a and TPR2b of Sti1, demonstrating that both TPR domains can approach a distance of 10 Å to this position. In the available crystal structures of Hsp90 proteins, the residue equivalent to E57 in yeast Hsp90 is at different distances from the last in the structure resolved residue. These distances vary from 80 to 110 Å. The linker between the C-terminal domain and the MEEVD motif, which was disordered in the crystal structure, could extend to about 100 Å. The linker plus the TPR2a domain itself (distance between the methionine of the MEEVD and the crosslinked residues) plus the crosslinker would bridge a total distance of 128 Å, which is more than sufficient to accommodate simultaneous MEEVD binding and approaching of E57. Shortening of the linker reduced the association rate for Sti1, indicating that the flexible linker contributes to the binding process of Sti1. We propose that the MEEVD motif with the flexible linker acts as a hook-and-fishing line for loading of Sti1 and other TPR-containing co-chaperones (Figure 6). Such an arrangement increases the capture cross-section of Hsp90 for TPR-containing co-chaperones. The three different phases of association of Sti1 in such a scenario would most likely correspond to the initial binding of TPR2a to the MEEVD motif, a conformational change that brings TPR2a and TPR2b in contact with the MD, and a subsequent conformational change that might be caused by Hsp90 arresting in an open conformation. During the swinging-up of Sti1 onto the MD, TPR2a might come close enough to the NBD to allow cross-linking to position 57. Phases two and three of the association of Sti1 seemed to differ for Hsp90 wild type and Hsp90Δ20 since the amplitudes of fluorescence change of the second and third phase were markedly different suggesting distinct conformational changes. However, the final conformation of

the Hsp90Δ20-Sti1 complex still prevents ATP hydrolysis by Hsp90.

Although the interaction surface between TPR2a and TPR2b and the MD could be much larger than the interaction surface between the MEEVD motif and TPR2a, the affinity for MEEVD interaction to TPR2a seems to be higher than the affinity of TPR2a and TPR2b to the MD. The K_d for binding of a MEEVD-containing peptide was determined to 300 nM (Schmid *et al*, 2012). When we determined the efficacy of Sti1 to inhibit the ATPase activity of Hsp90ΔMEEVD we found an IC_{50} of around 5 μM (Figure 5B; Table I), suggesting that the K_d of Sti1 binding to Hsp90 in the absence of the MEEVD–TPR2a interaction is in the μM range. The reason for the higher affinity of the MEEVD motif to TPR2a as compared with TPR2a–TPR2b binding to MD could be the high diffusional freedom of the MEEVD motif, resulting in high association rates for the MEEVD–TPR2a complex. An initial interaction of the MEEVD motif with TPR2a would tether Sti1 and thus favour binding of TPR2a and TPR2b to the MD. It also seems possible that Sti1 dissociates from the MD and reassociates with the same MD or the MD of the second protomer in the Hsp90 dimer without losing contact between TPR2a and the MEEVD motif. If such dissociation and reassociation happens several times within the time frame of our HX experiments, the protection we observed would be an underestimation of the actual effect of Sti1 on the conformational flexibility of Hsp90. Such a scenario would explain why we did not observe asymmetric protection but only a general reduction of protection when we performed the HX experiments at a 1:1 ratio of Hsp90 dimer to Sti1 monomer.

Our hook-and-fishing line hypothesis is consistent with a recent electron microscopy study, which detected two types Hsp90–Sti1 complexes, one with Sti1 visible as separate entity loosely attached to Hsp90 and a second with both proteins within a single electron density envelope (Southworth and Agard, 2011). Our kinetic evidence indicates that both complexes are different stages of the loading process of Sti1.

Materials and methods

Reagents

Fine chemicals were purchased from SIGMA-Aldrich (St. Louis, MO), ATP and AMPNP were obtained from Roche Applied Science, Mannheim, Germany. Radicol (RA) was obtained from IRIS Biotech GmbH, Marktredwitz, Germany. Deuterium oxide was purchased from Euriso-top, Gif-sur-Yvette, France. His-tagged Ulp1 was prepared in-house.

Protein expression and purification

Genes encoding yeast Hsp90, Hsp90(262–709), Hsp90(525–709), Hsp90-E57C, Hsp90Δ20, yeast Sti1, Sti1ΔTPR1, Sti1Δ(TPR1-DP1), Sti1ΔTPR2B-DP2, Sti1ADP2 and Sti1-FTH were cloned into the bacterial expression vector pCA528 encoding an N-terminal His₆-Smt3 tag (Andreasson *et al*, 2008). The fusion proteins were overexpressed in the *E. coli* strain BL21(DE3)Star/pCodonPlus (Invitrogen). The cultures were grown to $OD_{600} = 0.7–0.8$ and expression was induced with 0.5 mM IPTG for 5 h at 30°C. Cells were lysed by a microfluidizer (Avestin EmulsiFlex-C5) in lysis buffer A (20 mM HEPES/KOH pH 7.5, 100 mM KCl, 5 mM MgCl₂, 10% glycerol, 4 mM β-mercaptoethanol) and 5 mM PMSF, 1 mM Pepstatin A, 1 mM Leupeptin and 1 mM Aprotinin. The lysate was clarified by centrifugation (15 000 r.p.m. for 30 min) and incubated with Ni-IDA matrix (Protino, Macherey-Nagel) for 30 min. After incubation, the matrix was washed with buffer A and bound protein eluted with buffer A containing 250 mM imidazole. The eluted

fusion proteins were supplemented with Ulp1 protease, which cleaved the His₆-Smt3 tag and the mixture was dialysed overnight against buffer A containing 20 mM KCl. Cleaved recombinant proteins were recovered in the flow-through fractions after a second incubation with Ni-IDA matrix whereas the N-terminal His₆-Smt3 tag and Ulp1 remained on the column. Proteins were further purified by anion-exchange chromatography (ReSourceQ; GE Healthcare) with a linear gradient of 0.02–1 M KCl, and finally dialysed against storage buffer (40 mM HEPES/KOH, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 10% glycerol, 4 mM β -mercaptoethanol). The purity and molecular mass were verified by SDS-PAGE and HPLC-electrospray mass spectrometry, confirming the correct primary sequence containing only the N-terminal start-methionine. The purified Hsp90 proteins were checked to be nucleotide free by anion-exchange chromatography (ReSourceQ) and UV detection by 254 nm.

Hydrogen-exchange experiments, mass spectrometry and data processing

For experiments of the effects of Sti1 on Hsp90, nucleotide-free Hsp90 proteins (20 μ M) were preincubated with 2–3-fold excess (40–60 μ M) of Sti1, Sti1 Δ TPR1 or Sti1 Δ (TPR2B-DP2) for 10 min at 30°C to reach equilibrium. For experiments of the effects of Hsp90 on Sti1, 20 μ M of Sti1 was preincubated with 40–60 μ M of Hsp90, Hsp90MC, or Hsp90C for the same length of time at the same temperature. Hydrogen-deuterium exchange reaction was initiated by adding nine volumes of D₂O buffer, quenched after a 30-s duration by mixing with a chilled quench buffer (400 mM H₃PO₄/KH₂PO₄, pH 2.2, 0°C), and injected into an HPLC-MS set-up. Mass spectrometry analysis and data processing were performed based on the procedures described earlier (Rist *et al*, 2003, 2006; Graf *et al*, 2009).

Fluorescence measurement

For the determination of subunit exchange rates, Hsp90-E57C was labelled with ATTO488 maleimide (ATTO-TEC GmbH) and in a separate sample with ATTO550 maleimide. The two labelled proteins were mixed in storage buffer at a concentration of 400 nM each and incubated for at least 30 min. The Hsp90-488/Hsp90-550 sample was rapidly mixed with unlabelled Hsp90 (4.3 μ M) in a stopped-flow device (Applied Photophysics) and fluorescence monitored with excitation at 480 nm and a cutoff filter of 590 nm.

For the determination of the Sti1 association rate, Hsp90-E57C and Hsp90 Δ (695–704)-E57C (Hsp90 Δ 20-E57C) were labelled with ATTO488 maleimide and Sti1 was labelled with ATTO550 maleimide. In all, 400 nM of Hsp90-E57C-488 or Hsp90 Δ 20-E57C-488 labelled proteins was rapidly mixed 1:1 with 400 nM Sti1-550 in a stopped-flow device (Applied Photophysics) with 480 nm excitation and 590 nm cutoff filter.

ATPase assays

Steady-state ATPase rates were determined as described earlier (Ali *et al*, 1993; Graf *et al*, 2009) except that 40 mM HEPES/KOH pH 7.5, 50 mM KCl, 5 mM MgCl₂, 4 mM DTT, 10% glycerol was used as buffer.

UV-specific crosslinking

Hsp90-E57C and Hsp90 Δ 20-E57C were labelled with BPIA (Santa Cruz Biotechnology, Heidelberg, Germany). Hsp90-E57C-BPIA or Hsp90 Δ 20-E57C-BPIA (2–3 μ M) was preincubated with Sti1 or Sti1-FTH (8–9 μ M) for 10 min at 30°C. For determining the specificity of the crosslinking, Sba1 (20 μ M) and AMPPNP (4 mM) were preincubated with Hsp90-E57C-BPIA for 10 min at 30°C, then Sti1 was added and the reaction mixture incubated again for 10 min at 30°C before crosslinking induced by UV irradiation at 365 nm for 20 min. The efficiency of the crosslinking was evaluated by western analysis using a purified Sti1 antibody. For the identification of the crosslinked site in Sti1, N-terminally Strep-tagged Hsp90-E57C was labelled with benzophenone-4-carboxamidocysteine methanethio-sulphonate (Toronto Research Chemicals Inc. North York, Ontario, Canada). Crosslinking products were purified by successive denaturing Ni²⁺-IDA affinity chromatography and native Streptactin chromatography. The products were cleaved by addition of dithiothreitol (10 mM) and digested with trypsin overnight at 30°C in the presence of 1 M Urea. Peptides were separated by nanoHPLC, spotted on a MALDI target and analysed by a UltrafleXtreme™ MALDI-TOF/TOF mass spectrometer (Bruker Daltonik, Bremen, Germany).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: C-TL constructed and purified wild-type and mutant proteins; performed experiments and analysed data for Figures 1, 2, 3, 4A, 5 and Supplementary Figures S1–S7 and S11; contributed to manuscript writing. CG constructed and purified wild-type and mutant proteins; performed experiments and analysed data for Figures 2 and 3, and Supplementary Figure S2. FJM performed experiments and analysed data for and Supplementary Figures S9 and S10. SMR constructed and purified mutant proteins; performed experiments for Figure 4b. MPM designed and supervised experiments, analysed data, prepared figures and wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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