Assembly mechanism of early Hsp90-Cdc37-kinase complexes

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Molecular chaperones have an essential role for the maintenance of a balanced protein homeostasis. Here, we investigate how protein kinases are recruited and loaded to the Hsp90-Cdc37 complex, the first step during Hsp90-mediated chaperoning that leads to enhanced client kinase stability and activation. We show that conformational dynamics of all partners is a critical feature of the underlying loading mechanism. The kinome co-chaperone Cdc37 exists primarily in a dynamic extended conformation but samples a low-populated, well-defined compact structure. Exchange between these two states is maintained in an assembled Hsp90- Cdc37 complex and is necessary for substrate loading. Breathing motions at the N-lobe of a free kinase domain partially expose the kinase segment trapped in the Hsp90 dimer downstream in the cycle. Thus, client dynamics poised for chaperone dependence. Hsp90 is not directly involved during loading, and Cdc37 is assigned the task of sensing clients by stabilizing the preexisting partially unfolded client state.

INTRODUCTION

Hsp90, like other broad-scope chaperones of the heat shock protein (Hsp) family, does not function independently but rather functions as the core of a complex macromolecular machinery formed with partner proteins, termed co-chaperones, that fine-tune progression through the chaperone cycle (1–5). This first tier of Hsp90 modulation includes co-chaperones that regulate its adenosine triphosphatase (ATPase) cycle (6–11), assist in substrate recruitment with various degrees of specificity (12–16), couple chaperone machineries to create intricate chaperone networks (5, 17–20), or perform other specialized functions. Reversible and chaperone cycle step-specific post-translational modification of chaperones or co-chaperones (3, 21) forms a second regulatory tier, which necessitates integration of modifying enzymes such as kinases and phosphatases (22–28), and results in the formation of higher-order complexes. Thus, efficient processing of substrates requires an evolving composition of the chaperone complex that is being altered in a sequential and directional manner, as well as a dynamic remodeling of its architecture, where both Hsp90–co-chaperone and Hsp90/co-chaperone–substrate interfaces are being reformed. This is realized owing not only to the modular nature of all partners that provide pivot points to Hsp90 from which different functionalities protrude such as phosphatase (24, 25), peptidylprolyl isomerase (29, 30), ATPase modulation (7, 31, 32), ubiquitination (33), machinery bridging (12, 16, 34, 35), and substrate specificity (30, 36–41) but also to the partially overlapping interfaces between Hsp90 and its co-chaperones, substrates, and interdomain or interprotomer interfaces (3).

A series of molecular and structural snapshots acquired on binary and higher-order Hsp90 complexes has revealed some of the mechanistic details underlying substrate loading and maturation by Hsp90 and its partner chaperones and co-chaperones (37–45). For glucocorticoid receptor (GR) (40, 41, 43), a model client steroid hormone receptor, the cycle begins with inactivation of the ligand-binding domain (LBD) through Hsp70-mediated local unfolding in the vicinity of the binding cleft. Loading to Hsp90 occurs via Hop and a second copy of Hsp70, causing further unfolding of the substrate. During maturation, the Hsp70s and Hop are dissociated, and the late co-chaperone p23 stabilizes the ligand-bound native conformation of the substrate. Both loading and maturation complexes have substrate segments trapped in the lumen structure of dimeric Hsp90, formed primarily by the two middle (M²Hsp90) and C-terminal domains (C²Hsp90). Transition between the two states then occurs via sliding of the substrate through the lumen, as Hsp90 dimer switches between to two distinct closed conformations where the N-terminal domains (N²Hsp90) are dimerized.

Chaperoning of protein kinases requires the kinome-specific co-chaperone Cdc37 and the action of amino acid–modifying enzymes. Cdc37 forms the subunit of the machinery that recognizes kinases and sorts clients from nonclients by selectively inducing partial unfolding to clients but not to nonclients (37, 39). Similarly to GR, the structure of an Hsp90-Cdc37-Cdk4 ternary complex revealed that kinase inactivation is achieved by stabilizing a partially unfolded state. Cdk4 acquires a dumbbell-like structure, where the N- and C-lobes are found separated at opposite faces of the closed dimer and the dowel, composed of the β4-β5 strands of the native state, is trapped in the lumen of Hsp90 (39). Evidently, lumen formation requires arresting the Hsp90 dimer in a closed state, commonly by using adenosine 5′-diphosphate-molybdate as a posthydrolysis transition state inhibitor, a state that affords structural analysis (46). Substrate recruitment, however, must occur in an open, V-shaped dimeric Hsp90 conformation, which is highly dynamic (6, 47–50). The molecular details that allow for the formation of these early Hsp90-recruiting complexes, their architecture, and the role of recruiting co-chaperones in kinase loading are poorly understood. In a different Hsp90-Cdc37-Cdk4 structure that may represent an unproductive loading complex, the kinase adopts an extended conformation, not in a trapped state, but associated with an exposed surface of the Hsp90-Cdc37 complex despite Hsp90 being in a closed conformation (38). This unproductive state shows the same Hsp90-Cdc37 interface as the one observed in structures obtained with protein fragments, which is distinct from the Hsp90-Cdc37 interface observed in the trapped structure. Last, despite the recent progress in understanding the molecular determinants that render a kinase
domain an Hsp90 client (37, 51, 52), the kinase conformational properties that allow for recruitment to the chaperone complex and efficient transitioning from the open to trapped state remain elusive.

Here, we investigate how client kinases are recruited to open Hsp90-Cdc37 complexes, the first step during kinase chaperoning by Hsp90. Using a combination of nuclear magnetic resonance (NMR) spectroscopy and hydrogen-deuterium exchange monitored by mass spectrometry (HDX-MS), the conformational dynamics of all partners emerges as a critical feature of the underlying loading mechanism. The modular and dynamic nature of Cdc37 co-chaperone allows it to acquire multiple conformations in solution, among which is a low-populated compact structure identified here to be required for recruitment. The conformational plasticity of Cdc37 co-chaperone is maintained in the context of an idle Hsp90-Cdc37 complex and accounts for its ability to interact with clients during loading while bridging the two lobes of a dumbbell kinase conformation when transitioning to the trapped state. Hsp90 is not directly involved in this early step, and Cdc37 is assigned the task of sensing chaperone dependent of the kinase domains by stabilizing a preexisting partially unfolded state of the c-C4 region. Thus, rather than being a sequence motif, chaperone dependence arises from breathing motions at the N-lobe that poise the kinase for Hsp90 binding by partially exposing the segment trapped in the lumen of the closed Hsp90 conformation.

**RESULTS**

**Cdc37 samples a sparsely populated closed conformation**

To understand how the Hsp90-Cdc37 complex recognizes kinase substrates during the substrate-recruiting step, we first sought to characterize the conformational properties of free Cdc37. Structural studies on full-length Cdc37 or two-domain constructs revealed that the co-chaperone exhibits an extended, open conformation, where the N-terminal (N$_{\text{Cdc37}}$), middle (M$_{\text{Cdc37}}$), and C-terminal (C$_{\text{Cdc37}}$) domains are conformationally independent (11, 37, 39, 53). To identify the presence of weak but functionally important interdomain interactions occurring between adjacent domains, we compared the $^1$H-$^15$N heteronuclear single-quantum coherence (HSQC) and $^1$H-$^1$C heteronuclear multiple-quantum coherence (HMQC) spectra of isolated Cdc37 domains to those of N-M$_{\text{Cdc37}}$ and M-C$_{\text{Cdc37}}$ two-domain fragments (fig. S1, A and B). As expected, the most prominent chemical shift changes are observed for residues at the first and last linker. In addition, M$_{\text{Cdc37}}$ residues at the M-C$_{\text{Cdc37}}$ linker helix (268 to 276) also experience chemical shift changes. When M-C$_{\text{Cdc37}}$ is considered, changes are observed for residues at the first and last helix of C$_{\text{Cdc37}}$ as well as for residues covering most of the M-C$_{\text{Cdc37}}$ linker helix (290 to 300, 333 to 342, and 265 to 276).

The regions identified above as dynamic interdomain contacts for the two domain fragments, N-M$_{\text{Cdc37}}$ and M-C$_{\text{Cdc37}}$, show partial overlap at the vicinity of M-C$_{\text{Cdc37}}$ linker helix. This suggests that full-length Cdc37 may sample a distinct, low-populated excited state that corresponds to a collapsed, closed conformation stabilized by interactions involving all three domains. To probe this type of dynamic process, we recorded the $^1$H-$^13$C HMQC spectrum of full-length Cdc37 as a function of temperature. At low temperatures (< 25°C), a set of two distinct signals is observed for a large number of methyl groups, indicating that Cdc37 samples two states in the slow exchange NMR regime (Fig. 1B and fig. S1C). These states correspond to different conformations and not to a monomer-dimer equilibrium, as free Cdc37 overexpressed without a purification tag (38) or with a fusion partner such as His–myelin basic protein (MBP) (37) and His-GB1 (protein G B1 domain) (53) exists in a monomeric form. One of the two signals corresponds to that of the isolated domains and thus originates from an open state, where the three domains behave independently. Mapping the exchanging residues on the structures of individual domains provides insights into the architecture of the closed state (Fig. 1B).

Residues at the N-terminal tail (V2, V6, I10, and I23) and coiled-coil regions (A35, V37, L50, L102, M105, and M112) of N$_{\text{Cdc37}}$, at M$_{\text{Cdc37}}$ at the base of the M-C$_{\text{Cdc37}}$ linker helix (L197, V199, and I271), and at C$_{\text{Cdc37}}$ (L287, L292, V297, L301, L305, V311, V314, L317, A320, I321, I337, and V343) are all involved in the exchange process. Thus, the closed state, which becomes highly populated at low temperatures, represents a collapsed conformation stabilized by widely distributed interdomain contacts and is a result of a global structural rearrangement of Cdc37, because neither of the two-domain fragments undergoes a two-state exchange process (fig. S1D). Moreover, the temperature behavior of the two signals is the same for all residues, independent of the domain they belong to (Fig. 1C). Signal integration and Van’t Hoff analysis of the relative populations between the two states yield comparable thermodynamic parameters for all residues ($\Delta H^\circ \sim 4.8$ kcal/mol and $\Delta S^\circ \sim 17.5$ cal mol$^{-1}$ K$^{-1}$) (Fig. 1C), providing evidence that the exchange process occurs in a cooperative manner involving two conformations and all three domains. This compact state is consistent with a low-resolution structural model obtained previously (54) using synchrotron small-angle x-ray scattering (SAXS) and acquired on a complex between Cdc37 and a destabilized FGFR3 (fibroblast growth factor receptor 3) mutant (I538F).

To gain insight into the overall architecture of the closed state, we first acquired a three-dimensional HMQC-NOESY–HMQC spectrum of full-length Cdc37 at 20°C. A small number of interdomain NOEs between the flexible N-terminal tail (1 to 26) and M$_{\text{Cdc37}}$ was unambiguously identified, including the pairs V6-A187, I10-V191, I10-1195, and I23-A274 (Fig. 1D), indicating a direct N$_{\text{Cdc37}}$-M$_{\text{Cdc37}}$ interaction through the N-terminal tail. Tagging the C terminus of Cdc37 with a flexible lanthanide tag reports on the relative position of C$_{\text{Cdc37}}$. Comparison of the diamagnetic (Lu$^{3+}$-bound) and paramagnetic (Tb$^{3+}$-bound) states (Fig. 1, E and F, and fig. S1E) reveals close proximity to the N-terminal tail (1 to 26) and the end of the coiled-coiled regions (28 to 50 and 102 to 119). In addition, titration of an N-terminal peptide encompassing the beginning of the coiled-coil (1 to 32, N32) to M$_{\text{Cdc37}}$ or C$_{\text{Cdc37}}$ causes significant chemical shift changes to C$_{\text{Cdc37}}$, demonstrating a direct contact between N32 and C$_{\text{Cdc37}}$ (fig. S1, F and G). In summary, full-length Cdc37 interconverts between two distinct states: a highly populated extended conformation with minimal interdomain association and a sparsely populated collapsed conformation stabilized by N$_{\text{Cdc37}}$-MC$_{\text{Cdc37}}$ interactions.

**The closed state is required for kinase loading**

Binary Cdc37-client complexes are stabilized through interactions of the client kinase state with N$_{\text{Cdc37}}$ and C$_{\text{Cdc37}}$ regions, identified...
here to sample two conformations (37, 54, 55). As a result of this bipartite co-chaperone association, client kinases acquire an open, partially unfolded state that facilitates loading to Hsp90 and progression through the chaperone cycle (37, 54). Therefore, we next sought to determine the functional significance of the transition between the open and closed conformations of Cdc37 during the early steps of kinase processing.

First, we tested whether uncoupling the two cores of the bipartite Hsp90-Cdc37 kinase binding module while maintaining the open-close transition affects kinase loading and stable association with Cdc37. To achieve this, we used Δ30-Cdc37, a construct lacking the first 30 amino acids of the co-chaperone. This stretch of residues interacts directly with both client and nonclient kinases in binary complexes (37) and stabilizes the partially unfolded kinase state in the context of ternary complexes with Hsp90 (39). The 1H-13C HMQC spectrum of Δ30-Cdc37 shows two signals for the same set of methyl groups observed for wild-type Cdc37 and an excellent correspondence to the chemical shifts of the wild-type closed state, indicating that it samples the same excited state as the wild type (Fig. 2A and fig. S2A). The relative population of the two states is inverted for the deletion mutant, with the closed conformation being the major species even at a high temperature (Fig. 2A). Because Δ30-Cdc37 is defective in kinase binding (Fig 2B) (37, 56), we complemented a Δ30-Cdc37–bRaf 1:1 mixture with a large excess of N32 peptide in trans. However, this did not lead to complex formation (Fig 2B), suggesting that, although free N32 interacts with client kinases, the two cores of the bipartite kinase binding module need to be covalently linked to induce kinase unfolding and thus a stable Cdc37-kinase association.

Next, we aimed to use an engineered Cdc37 that does contain all elements required for kinase binding but is impaired in sampling...
the closed conformation. To this end, we identified W342C Cdc37, a variant shown to have a detrimental effect on signaling by the sevenless receptor tyrosine kinase of Drosophila Cdc37 (57). W342 is largely solvent-exposed (58), partially contributing to the hydrophobic character of the client binding site (37), and thus the cysteine substitution does not result in folding defects (fig. S2B). The 1H-13CHMQC spectrum of W342C Cdc37 shows only one signal for each methyl group of the three Cdc37 domains, and throughout the temperature range examined (5° to 33°C), it overlays well with the signals corresponding to the open and closed states are marked as “o” and “c”, respectively. (B) The interaction of bRaf kinase domain with Δ30-Cdc37 and W342C-Cdc37 monitored by methyl-TROSY NMR. The signal of I321 reports on kinase binding for wild-type Cdc37 (left). No new signal is observed for the titration of Δ30-Cdc37 with bRaf, even in the presence of a large excess of N32 (middle) or for the titration of W342C-Cdc37 with bRaf (right).

Free client kinase samples a partially unfolded conformation

Rather than recognizing a specific sequence, the molecular basis of client kinase sorting by Cdc37 is sensing underlying physical properties, including their low thermodynamic stability and enhanced conformational dynamics (37, 52). Challenges associated with sample stability and solubility have prevented the application of NMR to reveal the conformationally labile sites in free or Cdc37-bound states of client states. To gain such insights, we performed amide HDX-MS. This approach has been applied successfully to study binary Cdc37-kinase complexes with FGFR and bRaf kinase domains (54) as well as Hsp90-Hsp70-Hsp40 complexes with receptor LBDs (43). Here, we have included short and long labeling points and performed all measurements at lower temperature and pH, aiming to capture low-populated states of mechanistic significance.

In agreement with the previous HDX-MS study (54), the vast majority of the peptides identified for free bRaf, covering ~75% of the sequence, exchange through EX2 kinetics (Fig. 3A), as anticipated for folded proteins under native conditions (59, 60). In this regime, the closing rate of a local structural fluctuation is significantly faster than the corresponding opening rate, resulting in a single isotopic distribution that progressively shifts to higher mass/charge ratio (m/z) values as a function of labeling time. The client state contains multiple peptide stretches (~29% of the sequence) that undergo rapid exchange and exhibit >50% deuterium uptake after only 30 s of labeling time (Fig. 3B). After 90 min of labeling ~70% of the sequence shows >50% deuterium uptake, providing strong evidence of enhanced local conformational
dynamics for the client state despite having a well-folded conformation, as previously observed by NMR.

In addition to the EX2 kinetics described above, the client state of bRaf undergoes HDX via a mixed EX1/EX2 (EXX) mechanism too (61–63). This is characterized by the morphing of two envelopes as a function of labeling time, where the intensity of a low m/z envelope from a protected or folded state progressively decreases, while it shifts and merges with a higher m/z envelope from an unprotected or partially unfolded state, as a result of an EX2 contribution (Fig. 3C).

**Fig. 3. The free client state of bRaf undergoes partial unfolding transitions.** (A) Representative mass spectrum of a peptide (445 to 468) that exchanges via EX2 kinetics at various time intervals. The spectrum shown at 0 s corresponds to that of the unlabeled peptide. (B) Heatmap representing percent deuterium incorporation of free bRaf over five time points, from 3 s to 90 min, color-scaled from light blue (<10% uptake) to purple (>90% uptake). The same color scale is used on the right to map % uptake on the structure of bRaf. For those peptides that display exchange through EXX kinetics, the heatmap represents deuterium uptake from the most populated state, which corresponds to the "protected state." Kinase sequence motifs are boxed in green (P loop), red (αC helix), orange (hinge), purple (catalytic loop), and blue (activation segment). The sequence of the kinase observed partially unfolded and the threaded through the Hsp90 dimer lumen in the "trapped" state is highlighted by a gray shade, while the peptide stretches exchanging through an EXX mechanism are marked with a black line. (C) Mass spectra of N-lobe peptides showing exchange via EXX kinetics, as a function of labeling time. Bimodal isotopic envelopes (gray lines) are deconvoluted to low-mass (green lines) and high-mass (pink lines) envelopes, with the corresponding centroid m/z shown at the first and last time points. The maximum mass difference (Δm_max) between the low- and high-mass envelopes of each peptide is shown at the top. (D) Mapping of the minimal polypeptide segments that exchange via an EXX mechanism highlighted in orange on the structure of bRaf. (E) Relative population of the protected, folded state (low-mass envelope) as a function of labeling time for the peptides 469 to 481 (black), 498 to 514 (green), and 515 to 536 (blue). Lines correspond to fittings into a three-parameter exponential decay.
to an unfolded state with residence time in the order of several hundreds of milliseconds (64, 65).

The peptides that were identified are mapped to the N-lobe and include 469 to 481, 498 to 514, and 515 to 536, while no C-lobe peptides showed a bimodal isotopic distribution (Fig. 3C and fig. S3A). Deconvolution of the mass isotope envelopes by fitting to a bimodal Gaussian function can be used to characterize the conformational transition for these peptides (Fig. 3C). The maximum centroid mass difference between the higher and lower mass envelopes is 5.3, 7.4, and 9.2 Da, respectively, indicating that ~50% of the amides in each of these regions undergo a concurrent unfolding transition. Because these are relatively long segments, exchange could occur partly via EXX and partly via pure EX2 kinetics. On the basis of a subtractive analysis using shorter peptides (fig. S3B), an EX1 contribution is assigned to peptides 475 to 484, 498 to 505, and 515 to 525, which cover the β2-β3 loop and the beginning of β3 strand, the C-terminal half of αC helix, and β4 strand with the β4-β5 loop (Fig. 3D). Deconvolution of the mass isotope envelopes can also be used to evaluate whether EX1 kinetics report a concerted unfolding transition of these secondary structure elements or whether each peptide region unfolds independently. To do so, we quantified the two mass populations as a function of labeling time and determined the rate of the opening (unfolding) transition ($k_{op}$) and the residence time [half-life ($t_{1/2}$)] of the folded state (Fig. 3E). The kinetic parameters obtained for the two C-terminal peptides, 498 to 514 and 515 to 536, are in perfect agreement to each other, with $k_{op}$ of 0.006 and 0.007 min$^{-1}$, respectively, indicating a coupled event. This segment encompasses partly kinase elements identified previously to become partially unfolded in the context of binary complexes with Cdc37 (37, 54) or unfolded and trapped in the lumen of Hsp90 dimer in the context of ternary complexes with Hsp90-Cdc37 (fig. S4) (39). On the other hand, the kinetic parameters for the N-terminal peptide, 469 to 481, report on a significantly faster unfolding event, with a $k_{op}$ value of 0.23 min$^{-1}$, suggesting that unfolding of this segment is not dynamically coupled to the unfolding 498 to 514 and 515 to 536 peptides.

In summary, the client state of the kinase domain of bRaf is inherently dynamic and undergoes a slow, concerted unfolding transition. As a result, the chaperone-binding interface becomes partially exposed, and thus, chaperone dependence is a result of dynamic interconversion to a preexisting, partially unfolded kinase conformation.

**Cdc37 stabilizes the partially unfolded state in early complexes with Hsp90**

During kinase chaperoneing, Hsp90 inactivates the substrate by stabilizing a partially unfolded state, where the β4-β5 segment of the N-lobe is extended and β5 strand is trapped in the lumen of a closed Hsp90 dimer (39). We sought to investigate what is the conformation of a client kinase in earlier steps of the chaperone cycle and particularly during substrate recruitment and loading to the chaperone complex. To this end, we examined the HDX properties of bRaf in ternary Hsp90-Cdc37-bRaf complexes reconstructed with unphosphorylated Cdc37 and in the absence of nucleotide and molybdate conditions under which the open Hsp90 conformation is populated (25, 39, 56).

The Hsp90-Cdc37 complex has a strong impact on the conformational properties of bRaf, causing a marked increase in the deuterium uptake of the N-lobe (448 to 531), with 93% of the sequence showing >50% deuterium uptake after 30 s of labeling, as well as at the αG helix and the flanking loops (647 to 678) of the C-lobe (Fig. 4A). The regions that account for the overall change in the dynamic properties of the N-lobe correspond largely to the peptides showing exchange via the EXX mechanism in the free state of bRaf, spanning the β2-β3 loop with a part of β3 strand, a segment of αC helix, and the entire β4 strand with the β4-β5 loop (Fig. 4A). Comparison of the mass spectra of each of these peptides, with the corresponding high-mass envelopes obtained in the free state, reveals that they exhibit the same level of deuteration (Fig. 4B). Therefore, in the context of early open complexes, Hsp90-Cdc37 stabilizes the partially unfolded state sampled in the free state of a client kinase, where the Hsp90 binding site observed in the trapped state is partially exposed (fig. S4) (39). The HDX measurements cannot provide detailed structural information on whether the kinase adopts a dumbbell-like structure, as observed for Cdk4 in the trapped complex. Still, the increased exposure detected for the C-lobe peptides 593 to 600 (Fig. 4A), at the beginning of the activation segment and the DFG motif, near the interface with the N-lobe, indicates partial opening of the bilobal structure.

To dissect the exact role of Cdc37 co-chaperone during kinase loading in modulating the conformation of the substrate, we also compared the HDX properties of bRaf in the context of binary Cdc37-bRaf complexes to those of ternary Hsp90-Cdc37-bRaf complexes described above. Cdc37 increases the deuterium uptake and extends the peptide regions that undergo rapid exchange to the same extent as the Hsp90-Cdc37 complex does (Fig. 4A). In addition, when the N-lobe peptides exchanging through the EXX mechanism in the free state are considered, there is a very good correspondence in the centroid $m/z$ values of high-mass envelopes of the free state to those obtained for the Cdc37-bound state (Fig. 4B). This suggests that Hsp90 does not have a contribution in enhancing kinase dynamics and shifting the kinase conformation to the partially unfolded state during loading. Instead, while bound to Hsp90, the co-chaperone exerts the conformational changes required for downstream processing, which is stabilizing the partially unfolded client state independently of Hsp90. In support of this finding, $^1$H-$^13$C HMQC spectra of bRaf acquired when in complex with Hsp90-Cdc37 (Fig. 4C) shows the same degree of unfolding and the same set of new signals observed for the binary Cdc37-bRaf complex, while the affinity of bRaf binding to Hsp90-Cdc37 is the same as for binding to free Cdc37 (Fig. 4D). In summary, during substrate loading to open Hsp90 complexes, Cdc37 is the component of the chaperone machinery that stabilizes a pre-existing partially unfolded state of the client kinase, thus priming the substrate to engage with Hsp90 during the inactivation step.

**Architecture of the idle Hsp90-Cdc37 complex**

Crystallographic, NMR, and other biophysical studies performed with Cdc37 and Hsp90 fragments revealed that the chaperone-co-chaperone interaction is primarily mediated by $M^{Cdc37}$ and the N-terminal ATPase domain of Hsp90 ($N^{Hsp90}$) (11, 53). However, two distinct Cdc37 poses on Hsp90 have been captured using cryo-electron microscopy (cryo-EM), when full-length proteins are used showing EXX and partly via pure EX2 kinetics. On the basis of a subtractive analysis using shorter peptides (fig. S3B), an EX1 contribution is assigned to peptides 475 to 484, 498 to 505, and 515 to 525, which cover the β2-β3 loop and the beginning of β3 strand, the C-terminal half of αC helix, and β4 strand with the β4-β5 loop (Fig. 3D). Deconvolution of the mass isotope envelopes can also be used to evaluate whether EX1 kinetics report a concerted unfolding transition of these secondary structure elements or whether each peptide region unfolds independently. To do so, we quantified the two mass populations as a function of labeling time and determined the rate of the opening (unfolding) transition ($k_{op}$) and the residence time [half-life ($t_{1/2}$)] of the folded state (Fig. 3E). The kinetic parameters obtained for the two C-terminal peptides, 498 to 514 and 515 to 536, are in perfect agreement to each other, with $k_{op}$ of 0.006 and 0.007 min$^{-1}$, respectively, indicating a coupled event. This segment encompasses partly kinase elements identified previously to become partially unfolded in the context of binary complexes with Cdc37 (37, 54) or unfolded and trapped in the lumen of Hsp90 dimer in the context of ternary complexes with Hsp90-Cdc37 (fig. S4) (39). On the other hand, the kinetic parameters for the N-terminal peptide, 469 to 481, report on a significantly faster unfolding event, with a $k_{op}$ value of 0.23 min$^{-1}$, suggesting that unfolding of this segment is not dynamically coupled to the unfolding 498 to 514 and 515 to 536 peptides.

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Fig. 4. Cdc37 stabilizes the partially unfolded state of bRaf independent of Hsp90. (A) Heatmap representing % deuterium incorporation of bRaf in the presence of Hsp90-Cdc37 (top) or Cdc37 alone (bottom) over five time points, from 3 s to 90 min. The conditions used to measure HDX are identical those of free bRaf HDX. Colors and sequence highlighting is as for Fig. 3B. (B) Mass spectra for the set of the N-lobe peptides analyzed in the free state as a function of labeling time. Bimodal isotopic envelopes (gray lines) result from incomplete saturation of bRaf kinase by Hsp90-Cdc37 or Cdc37 alone, giving rise to a low-mass envelop corresponding to free bRaf (shaded in gray). The high-mass envelopes (pink), corresponding to the bound state was obtained through deconvolution. (C) The $^1$H-$^{13}$C HMQC spectrum of VL-labeled bRaf in the free (black), Cdc37-bound (red), and Hsp90-Cdc37-bound states (blue). (D) Binding isotherms for the interaction of bRaf with Cdc37 (red) and Hsp90-Cdc37 (blue), together with the corresponding $K_d$s measured at 20°C.
we next sought to investigate the molecular details that underlie the interaction between Cdc37 and Hsp90 using 1H-13C HMQC. Addition of bRaf to methyl-labeled Cdc37 has a significant impact on M\(^{\text{Cdc37}}\), causing a strong broadening effect even to signals of residues buried in the core of the domain and chemical shifts for a small set of signals (Fig. 5A and B, and fig. S5A). The same broadening effect and identical chemical shift changes are also observed for the interaction of isolated M\(^{\text{Cdc37}}\) with full-length Hsp90 or the N-M\(^{\text{Cdc37}}\) two-domain construct (fig. S5B). Despite the conformational heterogeneity observed for M\(^{\text{Cdc37}}\), Hsp90-bound Cdc37 undergoes a global conformational transition sampling a collapsed, closed state identified above as being essential for client recruitment (Fig. 5C and fig. S5C) and interacts with client kinases through the same mode of recognition as in the context of binary Cdc37-client complexes (Fig. 5D). Still, both the open and the closed states are distinct from those observed in the free state. First, the signals corresponding to the open state for residues located at the N-terminal tail (V6, I10, I23, and A26) and at C\(^{\text{Cdc37}}\) (I271 and I337) show broadening or deviations from the free state, suggesting that secondary interactions with Hsp90 are meditated by these domains, as previously demonstrated for the nematode Hsp90-Cdc37 system (Fig. 5C and fig. S5C) and interacts with client kinases through the same mode of recognition as in the context of binary Cdc37-client complexes (Fig. 5D).

Although the profound impact of Hsp90 on M\(^{\text{Cdc37}}\) signals supports previous findings implicating this domain in stabilizing the Hsp90-Cdc37 complex, it does not unambiguously discriminate between the two different chaperone–co-chaperone interfaces observed in either the absence or presence of a client kinase. To further characterize the architecture of the Hsp90-Cdc37 complex involved in kinase recruitment, we next followed the interaction by monitoring changes in the 1H-13C HMQC spectrum of Hsp90. In contrast to Cdc37 spectra, Hsp90 signals do not experience any significant broadening in the presence of Cdc37 (Fig. 5E) but only changes in chemical shifts, allowing for a more direct determination of the interface. The most prominent perturbations upon addition of bRaf to Ile-labeled Hsp90 are observed for N\(^{\text{Hsp90}}\) residues and particularly for isoleucines I43, I104, I110, I112, I131, and I214 (Fig. 5E). In addition, no chemical shift perturbation or broadening is observed for isoleucines I129, I304, I408, and I412, which lie at the vicinity of the central M\(^{\text{Hsp90}}\) β sheet, against which Cdc37 forms a β strand in the trapped state. These data are consistent with complex formation through an N\(^{\text{Hsp90}}\)-M\(^{\text{Cdc37}}\) interface, which is compatible with the structures determined with domain fragments in the absence of client kinase. This overall Hsp90-Cdc37 complex architecture is maintained during the formation of open Hsp90-Cdc37-bRaf complexes, as no further changes are observed in the 1H-13C HMQC spectrum of Hsp90 upon bRaf addition, further supporting that, at this stage, Hsp90 is not directly involved in client binding (fig. S5D), while kinase loading does not cause a shift to the closed Hsp90 state and trapping of the substrate.

Notably, the signal of I370 at M\(^{\text{Hsp90}}\) also shows a large change in chemical shift upon Cdc37 addition (Fig. 5E). I370 is found at the N-M\(^{\text{Hsp90}}\) interface and is spatially close to R400 (yeast R380), which

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**Fig. 5. Hsp90-Cdc37 interaction during kinase loading.** (A) Val/Leu region of the 1H-13C HMQC spectrum of Cdc37 in the free (black) and Hsp90-bound (yellow) states. Assignments are shown for select M\(^{\text{Cdc37}}\) residues. (B) Mapping of the residues affected during reciprocal NMR titrations of full-length Hsp90 and Cdc37 on the NMR structure (Protein Data Bank ID: 2K5B) of the complex between N\(^{\text{Hsp90}}\) (blue) and M\(^{\text{Cdc37}}\) (gray). Isoleucine residues of Hsp90 showing changes in chemical shift upon addition of 2H-Cdc37 are highlighted in pink and VLIMA Cdc37 residues experiencing severe broadening upon addition of 2H-Hsp90 are highlighted in orange. (C) Signal of I370 after addition of bRaf to free Cdc37 (left) and to Hsp90-Cdc37 complex (right), highlighting the shift to the same position.
plays a critical role in stabilizing a N-M-Hsp90 orientation competent for efficient adenosine 5′-triphosphate (ATP) hydrolysis and acts as an arginine finger by coordinating the γ-phosphate of ATP (6, 66). The exact nature of this conformational change is partial disruption of N-M-Hsp90 contacts, as in the presence of Cdc37, the new signal observed for I370 is at the position corresponding to that of isolated M-Hsp90 (Fig. 5E and fig. S5E), which is also observed in the presence of a client kinase (fig. S5D). Thus, Cdc37 does not modulate the ATPase cycle of Hsp90 only by interfering with the catalytic Glu42 (yeast Glu33) and affecting the conformation of the lid segment (11, 53) but also by reshaping the N-M-Hsp90 interface in a way that does permit the formation of critical R400-nucleotide contacts.

In summary, the architecture of early, open Hsp90-Cdc37-kinase complexes formed during substrate loading to the chaperone complex is characterized by a M Cdc37-N Hsp90 interaction and a remodeling of the N-M-Hsp90 interface to a catalytically incompetent conformation (Fig. 5E and fig. S5E), which is also observed in the presence of a client kinase (fig. S5D). Thus, Cdc37 does not modulate the ATPase cycle of Hsp90 only by interfering with the catalytic Glu42 (yeast Glu33) and affecting the conformation of the lid segment (11, 53) but also by reshaping the N-M-Hsp90 interface in a way that does permit the formation of critical R400-nucleotide contacts.

In summary, the architecture of early, open Hsp90-Cdc37-kinase complexes formed during substrate loading to the chaperone complex is characterized by a M Cdc37-N Hsp90 interaction and a remodeling of the N-M-Hsp90 interface to a catalytically incompetent conformation. At this stage of the cycle, the substrate does interact with Hsp90 but is held in a partially unfolded state solely through its interactions with Cdc37.

**DISCUSSION**

Hsp90 processes a diverse set of client proteins such as kinases, transcription factors, E3 ubiquitin ligases, the telomerase, and tau to produce a spectrum of functional end points ranging from conformational maturation to an active form, high affinity for ligand binding, stability against degradation, or even a translocation-competent conformation. The directionality and fate of the chaperone cycle are determined by the identity of the co-chaperones associated with Hsp90, which recruit substrates in a client-selective manner and fine-tune progression between different steps of the cycle.

The chaperoning of a large portion of kinome members via the Hsp90-Cdc37 machinery is critical to the maintenance of client stability and activity (52). Here, we have elucidated the mechanism of client recruitment and revealed that the underlying dynamic properties of all partners are critical for substrate loading to Hsp90 (Fig. 6). The modular, flexible nature of Cdc37 (Fig. 6A) is instrumental to the metamorphic architecture of the Hsp90-Cdc37 complex, where distinct chaperone–co-chaperone interfaces are used during substrate trapping and loading (Fig. 6B). In addition to this heterogeneous state, Cdc37 assumes a sparsely populated collapsed conformation that enables the association of client kinase domains while bound to Hsp90 (Fig. 6, A and B). Breathing motions in the free state of clients result in partial exposure of kinase motifs that become trapped by the Hsp90 dimer during inactivation (Fig. 6A). During loading, this partially unfolded client conformation is stabilized by Cdc37 independently of Hsp90 (Fig. 6A).

Structural studies performed with binary Hsp90–co-chaperone complexes and ternary Hsp90–co-chaperone–substrate complexes, including those with Cdc37 and client kinases, have highlighted the evolving nature of partner interfaces during progression of the chaperone cycle. Here, we show that Cdc37, a modular protein comprised of three domains, can be found in two states (Fig. 6A). The first represents the average sampling of a conformational manifold strongly biased toward extended structures with minimal interdomain contacts (Fig. 1A and fig. S1, A and B) and is expected to play an important role in Cdc37’s interaction with Hsp90, while the second state, characterized by a partially unfolded conformation, traps and loads client kinases (Fig. 6A). The modular, flexible nature of Cdc37 (Fig. 6A) is instrumental to the metamorphic architecture of the Hsp90-Cdc37 complex, where distinct chaperone–co-chaperone interfaces are used during substrate trapping and loading (Fig. 6B). In addition to this heterogeneous state, Cdc37 assumes a sparsely populated collapsed conformation that enables the association of client kinase domains while bound to Hsp90 (Fig. 6, A and B). Breathing motions in the free state of clients result in partial exposure of kinase motifs that become trapped by the Hsp90 dimer during inactivation (Fig. 6A). During loading, this partially unfolded client conformation is stabilized by Cdc37 independently of Hsp90 (Fig. 6A).

**Fig. 6. Dynamic exchange between distinct conformations of the chaperone partners and the client kinase during formation of loading Hsp90-Cdc37-kinase complexes.** (A) Free Cdc37 exists as a heterogeneous conformational ensemble (open state), with N Cdc37 (represented as a coiled coil) and CCdc37 (represented as a small circle) sampling all sterically available conformations (shown in color), in equilibrium with a well-defined conformation (closed state) stabilized through extensive interdomain contacts (shown in black). The client state of a kinase domain samples a partially unfolded state where the chaperone binding site is exposed. Relevant kinase elements are highlighted in magenta (αC helix), blue (αC-β4 loop), cyan (β4 strand), black (β4-β5 loop), yellow (β5 strand), orange (hinge), and green (αE helix). Dimeric Hsp90 exists in a dynamic V-shaped open conformation when free in solution. (B) The idle Hsp90-Cdc37 complex is stabilized through the M Cdc37-N Hsp90 interaction. While Hsp90 bound, Cdc37 can sample the closed conformation and interact with client kinases to stabilize the partially unfolded kinase state of the C-Cβ4-Jβ4/β5-loop segment and expose β5 strand. Nucleotides and Cdc37 phosphorylation at S13 stabilize the N Hsp90-dimerized closed Hsp90 conformation, resulting in trapping part of the β4-β5 segment in the Hsp90 lumen, and a significant remodeling of the Hsp90-Cdc37 interface.
role in the metamorphic nature of Hsp90-Cdc37 complex along the chaperone cycle (11, 38, 39). The second represents a low-populated closed state stabilized primarily through N-Cdc37, MCdc37 interactions (Fig. 1, B to F, and fig. S1, C to F). Sampling of this well-defined but sparsely populated conformation is maintained in the context of the idle Hsp90-Cdc37 chaperone system (Fig. 5C and fig. S5, A and C) and is required for the formation of stable Cdc37-client complexes. A Cdc37 construct devoid of the closed state cannot form kinase complexes through the bipartite N-CδCdc37 kinase binding module, nor can a construct that predominantly populates the closed state, unless the N-terminal tail is present in cis (Fig. 2 and fig. S2). Although high-resolution structural information is currently lacking for this sparsely populated conformation of free Cdc37 or its binary and open ternary complexes with bRaf and Hsp90-bRaf, a SAXS-derived low-resolution model of the binary Cdc37-FGFR3 complex reveals proximity between N-Cdc37 and C-Cdc37. The closed state apparently provides a relative positioning of N32 and C-Cdc37 appropriate for a bipartite mode of binding (37, 55) and unfolding of the client kinase (37, 39) or alternatively, but not mutually exclusive, the interaction of N-Cdc37 with MCdc37 may induce the bent conformation to the flexible N-terminal tail observed previously to be competent for client binding by mimicking the kinase αC-β4 loop segment (39).

Hsp90 dependence of protein kinases shows a strong correlation to their corresponding melting temperature (Tm) (52), a global molecular quantity measuring protein thermal stability, and the Cdc37-mediated sorting activity of the machinery is based on sensing this property and leading to partial unfolding of client states only (37, 54). The EXX mechanism of HDX observed in this study for the free client state of bRaf suggests that the N-lobe of the kinase domain undergoes slow unfolding transitions (Figs. 3C and 6A). For the 475 to 484 segments, encompassing the β2-β3 loop and the beginning of β3 strand, the rate of unfolding is fast and uncoupled from other regions. However, for the peptides 498 to 505 and 515 to 525, which span the αC helix, together with the β4 strand and the β4-β5 loop, the unfolding transition is significantly slower but occurs in a concerted fashion (Fig. 3, D and E). Although the half-life of the native state for this latter region is relatively long, the breathing motions revealed by HDX-M5 partially expose the Hsp90 binding site observed in the trapped state of the ternary complex, which comprises αC-β4-β5 (39). This suggests that client states are conformationally poised to interact with Hsp90 by sampling accessible microstates with exposed hydrophobic side chains (67) in a mechanism that resembles a conformational selection mode of recognition (68). Thus, as with other chaperone machineries (69, 70), the Hsp90-Cdc37 complex uses conformational dynamics near the active site (71) to sort kinases within the client-nonclient continuum rather than a global property such as thermodynamic stability (52). This is in accordance with the cleft metastability hypothesis proposed on the basis of interaction of nuclear receptors with Hsp90, where particular sequences at the rim of binding clefts open more readily, allowing access to the chaperones (72). Our data, therefore, reconcile previous observations on the differential association of closely related homologs with Hsp90 and the underlying role of the αC-helix/β4 strand conformational dynamics in this process. The G778 point mutant in the ErbB2 αC-β4 loop to the corresponding aspartate of ErbB1 sequence is expected to suppress dynamics in this region and compromises association with the chaperone system (73, 74). Evidently, the extensive allosteric coupling identified between residues throughout the catalytic domain of protein kinases accounts for the differential dependence and association to Hsp90-Cdc37 caused by perturbations occurring at positions other than the αC-β4 loop (75), presumably by inducing analogous breathing motions identified in here to expose the chaperone binding site. Similarly, sampling of a wide array of either active or inactive kinase states (76) provides a diverse reservoir of native conformations from which breathing motions can lead to chaperone association. Last, our data show that in the context of the assembled Hsp90-Cdc37 machinery, kinase sorting and loading is facilitated by Cdc37 in a manner that is independent of Hsp90. Cdc37 alone stabilizes the partially unfolded state of the αC-β4-β5-loop segment observed in the free kinase state in exactly the same way as the Hsp90-Cdc37 complex does (Fig. 4B). Although the β5 strand shows a significant deuterium uptake in the chaperone-bound state (~75% at 30s) (Fig. 4A), this cannot be unambiguously attributed to an unfolded state of this region, but it could be the result of greater exposure upon αC-β4-β5-loop partial unfolding. Still, further expansion of the substrate and β5 strand unfolding could occur upon transition to the trapped N-Hsp90-dimerized state.

The transition of the chaperone complex from the open to the closed conformation is characterized by a major remodeling of the chaperone-co-chaperone interface (11, 38, 39, 53). Our data show that the cycle begins with an open Hsp90-Cdc37 complex, where Cdc37 is bound to N-Hsp90 through its M-Cdc37, as observed in the structures acquired with Hsp90 and Cdc37 fragments or the unproductive Hsp90-Cdc37-Cdk4 ternary complex (Figs. 6B and 5) (11, 38, 53). In this state, Hsp90 has no effect on the conformational properties of the client kinase domain, and Cdc37 is charged with presenting a partially unfolded substrate while reshaping the N-Hsp90-M-Cdc37 interface and inhibiting its ATPase activity (Fig. 5 and fig S5). Therefore, during loading, Hsp90 is blind as to what is the nature of the bound substrate, and the fate of the chaperone cycle is determined by the identity of the bound co-chaperone. Progression to the next step of the chaperone cycle and trapping of the expanded kinase domain, split between the two faces of N-Hsp90-dimerized Hsp90, would then occur in the presence of phosphorylated Cdc37 (S13) and ATP combined with the dynamic closing transition of dimeric Hsp90 (47, 48, 77).

In summary, kinase loading to open Hsp90-Cdc37 complexes is facilitated by dynamic sampling of low-populated conformational states of all partners. Although the ensemble of extended Cdc37 conformations can account for the metamorphic nature of the Hsp90-Cdc37 interface, a unique low-populated closed state of the co-chaperone leads to stable complexes with protein kinases. Similarly, client protein kinases in their native states visit partially unfolded conformations at regions lining the catalytic cleft, which poise them for chaperone binding and dependence. This early step of kinase recruitment and loading occurs independently of Hsp90 through complexes with distinct architecture from those found in subsequent steps of the cycle. Still, to obtain a complete picture of the loading mechanism, a high-resolution structure of a binary Cdc37-kinase complex or of an open ternary Hsp90-Cdc37-kinase complex is required. We expect that solution-state NMR will assist in determining these structures and reveal the details of how Cdc37 stabilizes the preexisting partially unfolded kinase.

**MATERIALS AND METHODS**

**Sample preparations**

A lanthanide tag was engineered at the C terminus of Cdc37 using a shorter construct (amino acids 1 to 350) to reduce the inherent
dynamic properties of this region. The sequence of lanthanide tag used is YIDTNNDGWYGDELLA and starts at position 351 (78). All proteins were expressed in BL21(DE3). Cdc37 constructs were expressed from pDB.His.MBP or pDB.His.GST vectors at 30°C for 5 hours [0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG)] and purified over two steps of Ni²⁺ affinity [50 mM tris (pH 8.0), 400 mM NaCl, 20 mM imidazole, 1.5 mM tris(2-carboxyethyl) phosphine (TCEP), and 1 mM phenylmethylsulfonyl fluoride (PMSF)], followed by size exclusion chromatography [50 mM tris (pH 8.0), 150 mM NaCl, and 1.5 mM TCEP] and ion exchange chromatography [20 mM tris (pH 8.0), 1 M NaCl, 0.5 mM EDTA, and 3 mM DTT].

B Raf was expressed overnight from a pDB.His.MBP vector at 16°C for 20 hours (0.15 mM IPTG) and purified over two steps of Ni²⁺ affinity [50 mM tris (pH 8.0), 150 mM NaCl, 20 mM imidazole, 10 mM MgCl₂, 5 mM 2-mercaptoethanol, 5% glycerol, and 1 mM PMSF], followed by size exclusion chromatography [50 mM tris (pH 8.0), 150 mM NaCl, 5 mM MgCl₂, 5 mM 2-mercaptoethanol, 1 M EDTA, and 5% glycerol]. His-tagged Hsp90αε was expressed initiated by diluting these stock solutions 11-fold into 20 mM tris base (pH 7.5), 100 mM NaCl, 5 mM MgSO₄, 2 mM DTT, and 0.5 mM EDTA prepared in H₂O. HDX stocks were prepared at a final 2:1 (Hsp90:Cdc37:bRaf) or 1:1 (Cdc37:bRaf) molar ratios and incubated at 3°C. HDX was initiated by diluting these stock solutions 11-fold into 20 mM tris base (pH 7.1) (uncorrected), 100 mM NaCl, 5 mM MgSO₄, 2 mM DTT, and 0.5 mM EDTA prepared in D₂O at 3°C. Such a low temperature was used to capture earlier exchange kinetics of bRaf. At a designated exchange time (0.05, 0.5, 5, 50, and 90 min), the exchange was quenched by diluting the samples with an equal volume solution of 0.4% formic acid in H₂O, yielding pH 2.6. All subsequent steps were performed in an ice bath using a setup described elsewhere (82). The quenched sample was digested online using Enzymate BEH Pepsin column (Waters). The digestion was performed at a flow rate of 0.15 ml/min using 0.15% formic acid/3% acetonitrile as the mobile phase. The resulting peptides were collected and desalted with an inline 4-µl C8-Opti-lynx II trap cartridge (Optimize Technologies) and then eluted through a C-18 column (Thermo Fisher Scientific, 50 × 1 mm Hypersil Gold C-18) using a rapid gradient from 2 to 90% acetonitrile containing 0.15% formic acid and a flow rate of 0.04 ml/min, leading directly into a maxIS-II ETD-ESI-QqTOF mass spectrometer. The total time for the digest and desalting was 3 min, and all peptides had eluted from the C-18 column by 15 min. To avoid cross-contamination from carry-over peptides, comprehensive pepsin and C-18 column wash steps were included after each run.

The peptide fragments were identified using Bruker Compass and Biotools software packages. The level of deuterium incorporation was assessed using the commercial software HDExaminer-3 (Trajan Scientific). SIs for deuterium incorporation were calculated from triplicate technical measurements at a single representative time point.

NMR spectroscopy
Backbone and methyl group resonance assignment of Cdc37 and Hsp90 was reported previously (37, 58, 79, 80). NMR experiments were performed on Varian direct drive 600- and 800-MHz instruments equipped with a cryoprobe. Spectra were processed using NMRpipe (81) and analyzed using Sparky (University of California, San Francisco).

H-N¹ combined chemical shifts are reported as Δδ = \sqrt{\Delta δ_H^2 + (\frac{\Delta δ_N}{5})^2}. ¹H-¹⁵N HSQC spectra of Cdc37 constructs were acquired in 20 mM tris (pH 7.5), 100 mM NaCl, 0.5 mM EDTA, and 2 mM DTT at 30°C. ¹H-¹³C HMBC spectra were acquired to monitor protein-protein interactions during the formation of open Hsp90-Cdc37-bRaf complexes, on binary or ternary complexes. All spectra were acquired at 20°C in 20 mM tris-de₆ (pH 7.2), 100 mM NaCl, 5 mM MgCl₂, 0.5 mM EDTA, and 2 mM DTT, made in 100% D₂O. For binary complexes, the protein partner titrated in the methyl-labeled sample was perdeuterated, while for ternary complexes, both partners were perdeuterated. Complexes of bRaf were assembled using protonated bRaf, except for the case when bRaf was methyl-labeled when a partially deuterated form was used. Assembly of complexes with Hsp90 and Cdc37 was performed with either perdeuterated protein or methyl-labeled protein depending on which partner was observed in the spectrum. For binary or ternary complexes with at either Hsp90 or Cdc37, methyl-labeled proteins were mixed at a final concentration of [Hsp90] = 140 µM, [Cdc37] = 70 µM, and [bRaf] = 70 µM. For binary or ternary complexes with methyl-labeled bRaf, proteins were mixed at a final concentration of [Hsp90] ~ 100 µM, [Cdc37] = 50 µM, and [bRaf] = 50 µM.

Hydrogen/deuterium exchange
Proteins for HDX experiments were mixed in 20 mM tris base (pH 7.5), 100 mM NaCl, 5 mM MgSO₄, 2 mM DTT, and 0.5 mM EDTA prepared in H₂O. HDX stocks were prepared at a final 2:1 (Hsp90:Cdc37:bRaf) or 1:1 (Cdc37:bRaf) molar ratios and incubated at 3°C. HDX was initiated by diluting these stock solutions 11-fold into 20 mM tris base (pH 7.1) (uncorrected), 100 mM NaCl, 5 mM MgSO₄, 2 mM DTT, and 0.5 mM EDTA prepared in D₂O at 3°C. Such a low temperature was used to capture earlier exchange kinetics of bRaf. At a designated exchange time (0.05, 0.5, 5, 50, and 90 min), the exchange was quenched by diluting the samples with an equal volume solution of 0.4% formic acid in H₂O, yielding pH 2.6. All subsequent steps were performed in an ice bath using a setup described elsewhere (82). The quenched sample was digested online using Enzymate BEH Pepsin column (Waters). The digestion was performed at a flow rate of 0.15 ml/min using 0.15% formic acid/3% acetonitrile as the mobile phase. The resulting peptides were collected and desalted with an inline 4-µl C8-Opti-lynx II trap cartridge (Optimize Technologies) and then eluted through a C-18 column (Thermo Fisher Scientific, 50 × 1 mm Hypersil Gold C-18) using a rapid gradient from 2 to 90% acetonitrile containing 0.15% formic acid and a flow rate of 0.04 ml/min, leading directly into a maxIS-II ETD-ESI-QqTOF mass spectrometer. The total time for the digest and desalting was 3 min, and all peptides had eluted from the C-18 column by 15 min. To avoid cross-contamination from carry-over peptides, comprehensive pepsin and C-18 column wash steps were included after each run.

The peptide fragments were identified using Bruker Compass and Biotools software packages. The level of deuterium incorporation was assessed using the commercial software HDExaminer-3 (Trajan Scientific). SIs for deuterium incorporation were calculated from triplicate technical measurements at a single representative time point.

Gaussian deconvolution of bimodal isotopic distributions was performed in Origin 7 using the Levenberg-Marquardt algorithm. The rate of the unfolding transition (k_{eq}) and the half-life (t_{1/2}) of the folded state for free bRaf were determined by plotting the fraction of the folded state as a function of deuterium labeling and fitting the data to a three-parameter exponential decay function y = y₀ + A_{1}e^{-\frac{t}{t_{1/2}}}. For peptides 498 to 514 and 515 to 536, the asymptote, y₀, was set to 0.

Isothermal titration calorimetry
Isothermal titration calorimetry (ITC) was used to measure the K_d (dissociation constant) for the interaction between Hsp90-Cdc37 and bRaf. Experiments were performed using a PEAK-ITC calorimeter (Malvern Scientific) at 20°C in 20 mM tris base (pH 7.5), 100 mM NaCl, 5.0 mM MgSO₄, 0.5 mM TCEP, and 0.5 mM EDTA. Hsp90 and Cdc37 were placed in the cell at a molar ratio of 2:1 (30:15 µM),
and the complex was titrated with bRaf (140 μM) in 13 injections, including an initial 0.2-μl injection that was excluded from the analysis. The reported error was determined from three technical replicates.

SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.abm9294

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Acknowledgments: NMR experiments were carried out at the USF's Florida Center of Excellence for Drug Discovery and Innovation. Funding: This work is supported by the National Institute of General Medical Sciences, grant GM115854 to I.G. Author contributions: D.K. and I.G. conceived and designed the study. D.K. and V.K.M.V. prepared the samples. D.K. and V.K.M.V. performed and analyzed the NMR experiments. R.R.A. and I.G. wrote the manuscript.

Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Raw HDX-MS data have been deposited to the MassIVE data repository at the University of California, San Diego Center for Computational Mass Spectrometry with the dataset identifier MSV000088635.