

Phosphorylation of Serine 13 Is Required for the Proper Function of the Hsp90 Co-chaperone, Cdc37*

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The Hsp90 co-chaperone Cdc37 provides an essential function for the biogenesis and support of numerous protein kinases. In this report, we demonstrate that mammalian Cdc37 is phosphorylated on Ser¹³ *in situ* in rabbit reticulocyte lysate and in cultured K562 cells and that casein kinase II is capable of quantitatively phosphorylating recombinant Cdc37 at this site. Mutation of Ser¹³ to either Ala or Glu compromises the recruitment of Cdc37 to Hsp90-kinase complexes but has only modest effects on its basal (client-free) binding to Hsp90. Furthermore, Cdc37 containing the complementing Ser to Glu mutation showed altered interactions with Hsp90-kinase complexes consistent with compromised Cdc37 modulation of the Hsp90 ATP-driven reaction cycle. Thus, the data indicate that phosphorylation of Cdc37 on Ser¹³ is critical for its ability to coordinate Hsp90 nucleotide-mediated conformational switching and kinase binding.

Cdc37 is an Hsp90¹ co-chaperone that interacts with and is required for the function of a wide array of eukaryotic tyrosine and Ser/Thr protein kinases involved in regulating multiple facets of cell physiology (reviewed in Refs. 1–5). In its role as an Hsp90 co-chaperone, Cdc37 interacts with both Hsp90 and kinase client, and this interaction is required to stimulate Hsp90 nucleotide-modulated conformational switching, thus capturing the client kinase in high affinity salt-stable Hsp90-Cdc37 heterocomplexes (6, 7).

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¹ The abbreviations used are: Hsp90, 90-kDa heat shock protein; Cdc37, generically refers to the protein product of CDC37 gene homologues regardless of source of organism; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; MS, mass spectrometry; CKII, casein kinase II; HRI, heme-regulated eIF2 α kinase; eIF, eukaryotic initiation factor; eIF2 α , α -subunit of eukaryotic initiation factor 2; PIPES, piperazine-*N,N'*-bis[2-ethanesulfonic acid]; RRL, rabbit reticulocyte lysate; ACN, acetonitrile.

The most conserved residues among Cdc37 homologues are located within the N-terminal 30 or so amino acids (8, 9). The N-terminal half of Cdc37 binds kinase, and mutation of the first 8 amino acids of Cdc37 inhibits its ability to bind kinase (7). The highly conserved N-terminal region of Cdc37 contains an absolutely conserved Ser residue (Ser¹³ in human, Ser¹⁴ in yeast) present within a consensus casein kinase II (CKII) phosphorylation motif; this sequence motif is present in all known Cdc37 gene products (8, 9). A S14L mutation within yeast *cdc37* yields a gene product that is nonfunctional and having expression levels that are greatly reduced, suggesting that its structural stability is compromised (9). Recent genetic experiments indicate that a Cdc37/S14A,S17A double mutation compromises Cdc37 function in yeast, whereas a Cdc37/S14E,S17E mutation compromises Cdc37 function to a lesser degree (8). This compromised function prompted the proposal that Ser¹⁴ and Ser¹⁷ were targets for CKII phosphorylation, but no direct physical evidence for such phosphorylation has yet been presented to support this hypothesis (8).

In this report, we demonstrate that Cdc37 is phosphorylated on Ser¹³. This phosphorylation is required for the biochemical function of Cdc37, namely for its kinase binding activity and its ability to stimulate the nucleotide-modulated conformational switching of Hsp90.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis of Cdc37—Point mutations at the N terminus of Cdc37 were introduced using standard PCR-based methodologies utilizing primers corresponding to the N terminus of human Cdc37 and encoding the desired Ser to Ala or the Ser to Glu mutations (10–12). PCR products were ligated into a modified pSP64T vector (13). The fidelity of each mutation was confirmed by DNA sequencing.

Analysis of the Interaction of Wild Type and Mutant Cdc37 Constructs with Hsp90 and Kinase—His-tagged wild type Cdc37, Cdc37/S13A, and Cdc37/S13E were synthesized and radiolabeled via coupled transcription/translation in rabbit reticulocyte lysate (RRL) for 40 min at 30 °C as described previously (6, 7, 11, 14). Cdc37 gene products were either directly immunoadsorbed with agarose-bound anti-(His₆) antibody (Qiagen) or with the 8D3 monoclonal anti-Hsp90 antibody (7, 15, 16). To examine kinase binding activities of each Cdc37 gene product, non-His-tagged versions of Cdc37 gene products were mixed with RRL containing newly synthesized His-tagged kinase client, namely the heme-regulated eIF2 α kinase (HRI) (6, 7, 11, 14), and His-tagged HRI was adsorbed with anti-His antibody. Unless otherwise indicated in the figure legends, immunoresins were washed four times with 10 mM PIPES buffer (pH 7.2) containing 150 mM NaCl and 0.5% Tween 20. Samples were assessed by SDS-PAGE, Western blotting, and/or autoradiography.

Purification of Recombinant Cdc37 Protein Constructs from Escherichia coli—His-tagged full-length Cdc37 and His-tagged Cdc37/ Δ C were purified as described previously (7). His-tagged Cdc37/ Δ C (~2 mg/ml) was incubated *in vitro* in chromatography elution buffer supplemented with 2.5 mM ATP and 10 mM MgCl₂ with or without 50,000 units of CKII (Calbiochem) for 5 h at 30 °C. Unphosphorylated and phosphorylated His-tagged Cdc37/ Δ C (~5 μ g) were digested with chymotrypsin and analyzed by MALDI-TOF MS.

Analysis of the Phosphorylation Status of Cdc37 at Ser¹³—His-tagged wild type human Cdc37 or Cdc37/S13A mutant were synthesized in RRL and immunoadsorbed with anti-His tag antibody (7). The wild type Cdc37 samples were incubated at 37 °C for 3 h in either the presence or absence of alkaline phosphatase (calf intestine, Calbiochem). For the *in vivo* analysis, Cdc37 was immunoadsorbed with mouse polyclonal anti-Cdc37 antibodies (7) from ~7 \times 10⁶ K562 cells that were grown to 80% saturation at 37 °C in RPMI 1640 medium containing 10% fetal calf serum, washed with Hanks's salts containing 20 mM NaF and 10 mM sodium vanadate, and lysed in 1 ml of 20 mM Hepes buffer (pH 7.5) containing 100 mM NaCl, 1 mM sodium vanadate, 2 mM EGTA, 1 mM

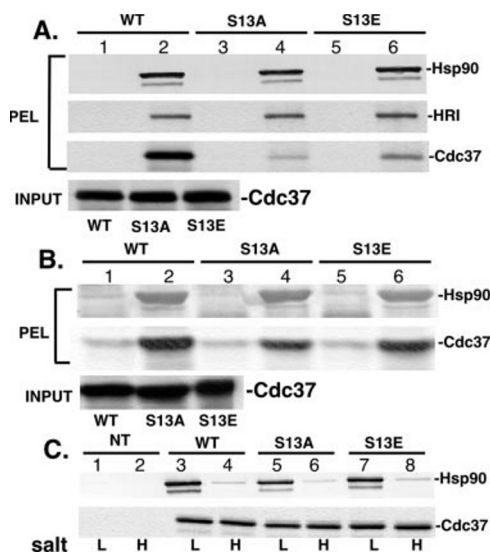


FIG. 1. Effect of mutation of Ser¹³ on the ability of Cdc37 to bind HRI and Hsp90. A, ³⁵S-labeled wild type, S13A, or S13E Cdc37 gene products were synthesized in RRL and then mixed with lysate containing (lanes 2, 4, and 6) or lacking (lanes 1, 3, and 5) ³⁵S-labeled His-tagged HRI. Subsequently, His-tagged HRI was immunoadsorbed, and immunopellets (PEL, upper panel) or equivalent aliquots of input sample (INPUT) were analyzed by SDS-PAGE and Western blotting (Hsp90) or autoradiography (HRI, Cdc37). B and C, ³⁵S-labeled His-tagged wild type (WT), S13A, or S13E Cdc37 gene products were synthesized in RRL and were subsequently immunoadsorbed with anti-Hsp90 (lanes 2, 4, and 6) or control (lanes 1, 3, and 5) antibodies (B) or with anti-His tag antibodies (C). Immunopellets (PEL (B)) and equivalent amounts of unfractionated input lysate (INPUT (B)) were analyzed by staining with Coomassie Blue (Hsp90 (B)), Western blotting (Hsp90 (C)), or autoradiography (Cdc37). C, immune resins were washed with buffer containing high (H) (500 mM NaCl) or low (L) (150 mM NaCl) salt. NT, no template control.

dithiothreitol, 0.5% Nonidet P-40, 10% glycerol, and 20 μ l of phosphatase inhibitor mixture (Sigma, 8540). Immunopellets were washed and then incubated in the presence or absence of alkaline phosphatase. Samples were separated by SDS-PAGE, excised from the gel, and analyzed for phosphorylation.

To identify Cdc37 phosphopeptides, gel pieces were washed with 50% acetonitrile (ACN), dehydrated in 100% ACN, rehydrated with 50 mM NH₄HCO₃, washed with 50% ACN, 25 mM NH₄HCO₃, and then dried under vacuum and rehydrated/infiltrated with 1 gel volume of 12.5 ng/ μ l chymotrypsin (Promega, sequencing grade) prepared in 50 mM NH₄HCO₃, 5 mM CaCl₂. Digestions were incubated at 37 $^{\circ}$ C for ~16 h. The digestion fluid was removed, and the gel pieces were extracted with 50 μ l of 50% acetonitrile, 0.1% trifluoroacetic acid. The extracts and digestion fluid were combined, concentrated to a volume of 10–20 μ l, and purified with Zip-tips (Millipore) according to the manufacturer's recommended procedure.

Aliquots (1 μ l) of the peptide mixtures were spotted onto a MALDI plate and were immediately covered by the same volume of saturated α -cyano-4-hydroxycinnamic acid (matrix) prepared in 50% acetonitrile, 0.1% trifluoroacetic acid. After drying, samples were analyzed using a Voyager DE-PRO MALDI-TOF mass spectrometer (Applied Biosystems) operated in the linear, negative-ion mode. Mass spectra were compared with the chymotryptic peptide mass fingerprints predicted for Cdc37 by the MS-Digest program from the Protein Prospector suite (17).

RESULTS AND DISCUSSION

Mutation of Ser¹³ of Cdc37 Inhibits Its Kinase Binding Capacity—To examine the role of Ser¹³ in modulating the function of Cdc37, the ability of wild type Cdc37, and the Cdc37/S13A or Cdc37/S13E mutants to bind to newly synthesized kinase client, HRI, in RRL was assessed. The binding of Cdc37/S13A and Cdc37/S13E to HRI client was compromised by 98 and 85%, respectively, relative to wild type Cdc37 (Fig. 1A). This reduction was not simply due to a lack of interaction with Hsp90; the binding of the S13A and S13E mutants to immunoadsorbed

Hsp90 was decreased by only ~40 and ~10%, respectively (Fig. 1B). A similar reduction in the binding of Hsp90 to immunoadsorbed Cdc37 mutants was observed relative to wild type Cdc37 (Fig. 1C). Similar to wild type Cdc37 (6), the binding of the mutant proteins to Hsp90 was sensitive to washing with high salt (Fig. 1C). The ability of the S13E mutant to bind Hsp90 at near wild type levels compared with the S13A mutant likely contributed to its greater capacity to bind HRI. The differences in the kinase binding capacities of the Cdc37/S13E and S13A mutants is consistent with the extent to which the growth of yeast strains carrying the double Ser to Glu or Ser to Ala mutations in the analogous Ser¹⁴,Ser¹⁷ motif were compromised (8).

Cdc37 Is Phosphorylated on Ser¹³ in Situ and in Vivo—The phosphorylation status of Cdc37 was analyzed by MALDI-TOF MS performed in linear negative-ion mode. Chymotrypsinolysis of recombinant Cdc37 generated a peptide anion with an average m/z of 2497 (Fig. 2A) that corresponded well with that predicted for the theoretical peptide anion containing Cdc37 amino acids 8–29 (DHIEVS¹³DDEDETHPNIDTASLF; average m/z 2498), with the difference between the observed and the theoretical m/z lying well within the mass accuracy of the instrument in this mode of operation (500 ppm mass accuracy in linear mode). However, this peptide was absent from the mass spectra of wild type Cdc37 synthesized in RRL. Instead, a peptide with an m/z of 2577 was observed, which corresponds to that predicted for the phosphorylated peptide-(8–29) (2498 + 80) (Fig. 2B). Treatment of this Cdc37 with alkaline phosphatase yielded spectra containing a peptide peak with the average m/z of 2497 (Fig. 2C), corresponding to that predicted for the unphosphorylated peptide-(8–29).

To confirm that the apparent phosphorylation was present on Ser¹³, Cdc37/S13A was synthesized in RRL and analyzed. For this mutant, a peptide anion with the average m/z of 2480 was observed (Fig. 2D), corresponding within error to that predicted for the unphosphorylated S13A 8–29 peptide (m/z 2481). The mass of this mutant peptide and the absence of phosphorylation thereon demonstrated that Ser¹³ was the site phosphorylated on Cdc37 produced in RRL.

Because peptide-(8–29) of Cdc37 contains a consensus CKII phosphorylation motif at Ser¹³, we determined whether CKII could phosphorylate this site *in vitro*. These assays utilized purified recombinant Cdc37 lacking its C-terminal domain, because of its availability due to ongoing structural characterizations.² The spectra of chymotryptic peptides from Cdc37/ Δ C included a peak with an average m/z (2497) corresponding to that of the unphosphorylated peptide-(8–29) (Fig. 2H). This peak was absent from spectra of peptides derived from recombinant protein treated with CKII (Fig. 2G). Instead, a peak corresponding to the phosphorylated peptide-(8–29) (m/z 2477) was observed (Fig. 2G). Additionally, Coomassie Blue staining of the gel indicated that treatment of Cdc37/ Δ C with CKII quantitatively slowed its electrophoretic mobility (not shown). Thus CKII had the capacity to quantitatively phosphorylate Cdc37/ Δ C *in vitro*.

To determine whether Cdc37 was phosphorylated *in vivo*, Cdc37 was immunoadsorbed from extracts prepared from K562 cells, and immunoadsorptions were or were not treated with alkaline phosphatase. MALDI-TOF analysis detected a chymotryptic peptide peak with an average anionic m/z of 2578, consistent with the predicted average mass of the phosphorylated form of the 8–29 peptide (Fig. 2E): the peak corresponding to the predicted average m/z of the unphosphorylated 8–29 peptide was absent. Treatment of these Cdc37 immunoadsorptions

² R. L. Matts, T. Prince, and S. D. Hartson, unpublished data.

FIG. 2. MALDI-TOF analysis of the phosphorylation state of Ser¹³ in Cdc37. MALDI-TOF mass spectra of chymotryptic peptides generated from: A, recombinant Cdc37 purified from *E. coli*; B, His-tagged Cdc37 synthesized in RRL; C, His-tagged Cdc37 synthesized in RRL and treated with alkaline phosphatase prior to analysis; D, S13A mutant of Cdc37 synthesized in RRL; E, Cdc37 immunoprecipitated from extracts of K562 cells; F, Cdc37 immunoprecipitated from extracts of K562 cells and treated with alkaline phosphatase prior to analysis; G, purified recombinant Cdc37/ΔC protein incubated with CKII prior to analysis; H, purified recombinant Cdc37/ΔC protein incubated in the absence of CKII prior to analysis. Only the regions of the spectra containing the peptides of interest are shown.

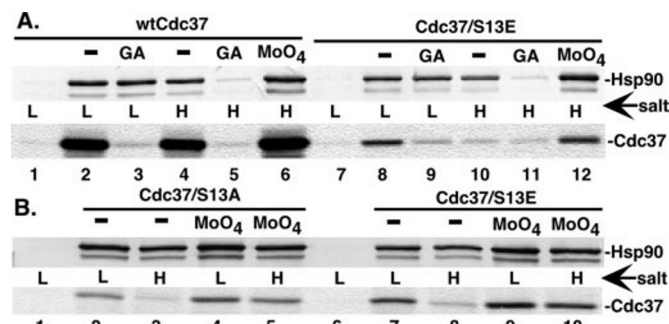
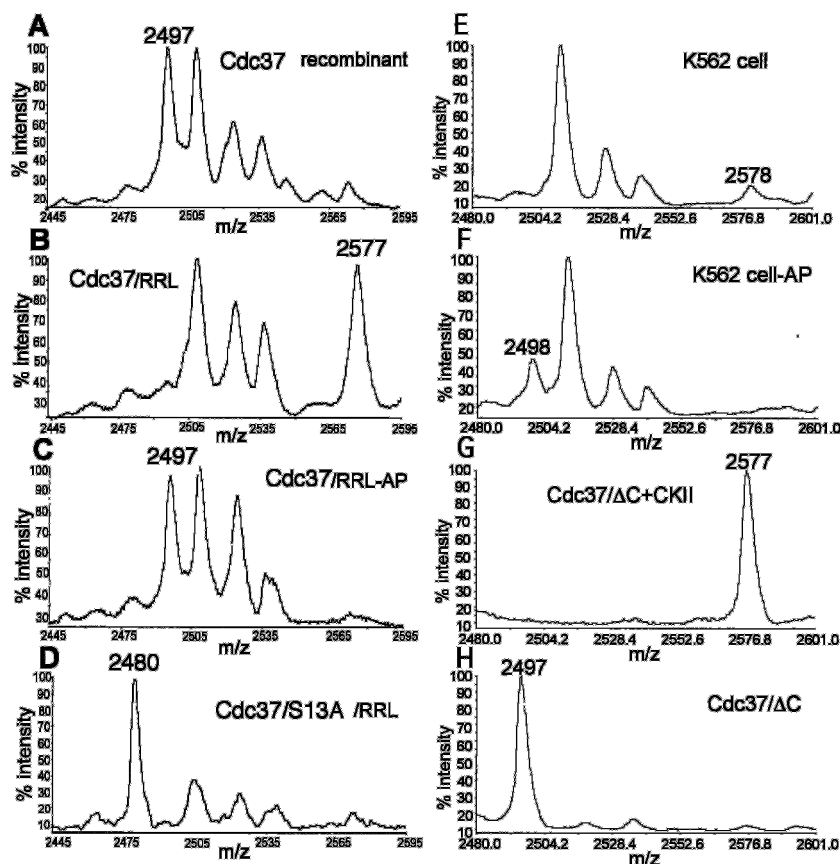


FIG. 3. Characterization of the properties of chaperone complexes formed between the Cdc37/S13E protein and kinase client. ³⁵S-labeled wild type or Cdc37/S13E (A), and Cdc37/S13A or S13E (B) were synthesized in RRL and then mixed and incubated with lysate containing (A, lanes 2–6 and 8–12; B, lanes 2–5 and 7–10) or lacking (A, lanes 1 and 7; B, lanes 1 and 6) His-tagged HRI kinase. A, samples were then incubated for 20 min with 10 μg/ml geldanamycin (lanes 3, 5, 9, and 11) or with Me₂SO vehicle (lanes 2, 4, 6, 8, 10, and 12) and subsequently supplemented with 20 mM sodium molybdate (lanes 6 and 12) or buffer (lanes 1–5 and 7–11). B, samples were incubated for 20 min and subsequently supplemented with 20 mM sodium molybdate (lanes 4, 5, 9, and 10) or buffer (lanes 1–3 and 6–8). After 5 min, His-tagged HRI heterocomplexes were immunoadsorbed and washed with buffers containing low salt (L) or high salt (H) as described previously (6, 18). Samples were analyzed by Western blotting (Hsp90) and autoradiography (Cdc37).

tions with alkaline phosphatase led to the loss of the peak at *m/z* 2578, while a new peak at *m/z* 2498 was now detected (Fig. 2F). The mass difference between these peaks (80 Da) was consistent with phosphorylation and its loss upon phosphatase treatment. Thus, Cdc37 appears to be heavily phosphorylated on Ser¹³ in K562 cells, consistent with the apparent quantitative phosphorylation of Cdc37 observed in RRL (Fig. 2B). However, our data do not rule out the possibility that a small pool of unphosphorylated Cdc37 exists *in vivo* but that representa-

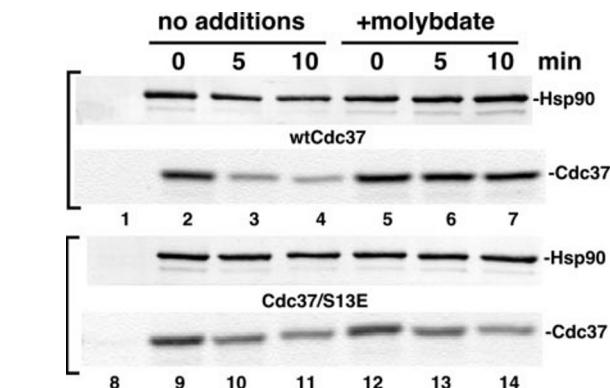


FIG. 4. Effect of molybdate on the dynamic turnover of complexes formed between protein kinase client and wild type Cdc37 or Cdc37/S13E. ³⁵S-labeled wild type Cdc37 (lanes 1–7) and Cdc37/S13E (lanes 8–14) were synthesized in RRL and subsequently mixed with lysate containing (lanes 2–7 and 9–14) or lacking (lanes 1 and 8) His-tagged HRI. Samples were then chased into 5 volumes of normal RRL containing (+molybdate: lanes 5–7 and 12–14) or lacking (no additions: lanes 1–4 and 8–11) 20 mM sodium molybdate. His-tagged HRI was subsequently immunoadsorbed after 0 (lanes 2, 5, 9, and 12), 5 (lanes 3, 6, 10, and 13), and 10 (lanes 1, 4, 7, 8, 11, and 14) min of chase incubation. HRI heterocomplexes were washed and analyzed by SDS-PAGE and Western blotting (Hsp90) or autoradiography (wtCdc37 and Cdc37/S13E).

tive peptides were not detectable in our MALDI-TOF mass spectra.

Mutation of Ser¹³ Compromises the Ability of Cdc37 to Stimulate Nucleotide-modulated Conformational Switching of Hsp90—Cdc37 interacts with both kinase clients and with Hsp90 (6, 7). This coincident binding allows Cdc37 to stimulate Hsp90 nucleotide-modulated conformational switching, resulting in the formation of salt-stable heterocomplexes containing Hsp90, Cdc37, and kinase clients (6, 7). The formation of these

meta-stable complexes is inhibited by the Hsp90 antagonist, geldanamycin, and is enhanced by the stabilizing agent, molybdate (6, 7). To investigate the role of phosphorylated Ser¹³ on the Cdc37 stimulation of Hsp90 conformational switching, we examined the ability of the S13E and S13A mutants to form salt-stable complexes with HRI. The S13E mutant was constructed in anticipation that the Ser to Glu mutation would wholly or partially mimic phosphorylated Cdc37.

Wild type Cdc37 and Cdc37/S13E were synthesized in RRL and mixed with RRL containing His-tagged molecules of the client kinase, HRI (Fig. 3A). As previously demonstrated, wild type Cdc37 formed salt-stable complexes with newly synthesized HRI in the absence of molybdate, and the Hsp90 inhibitor geldanamycin inhibited Cdc37 binding to HRI but not the weak salt-labile binding of Hsp90 to HRI (6, 7). In contrast, Cdc37/S13E formed salt-labile complexes with kinase client and Hsp90; high salt washes stripped ~80% of the bound Cdc37/S13E from the kinase client. Furthermore, although geldanamycin nearly quantitatively blocked the interaction of wild type Cdc37 with HRI, the interaction of Cdc37/S13E with HRI was reduced by only 70%. The cause and relevance of residual salt- and geldanamycin-resistant binding remains uncertain. The addition of molybdate restored the salt-stability of Cdc37/S13E complexes with Hsp90 and client kinase, consistent with the documented ability of molybdate to stabilize Hsp90 hetero-complexes (6, 7). Similarly, Cdc37/S13A was not capable of forming salt-stable complexes with newly synthesized HRI, and the addition of molybdate was required to restore the salt stability of Hsp90-kinase complexes containing the S13A protein (Fig. 3B). Thus, although the S13E and S13A mutations diminished Cdc37 recruitment to kinase complexes, it also compromised the normal function of Cdc37 in stimulating Hsp90 conformational switching to form high affinity salt-stable complexes with kinase client.

To further investigate the effect of the S13E mutation on the ability of Cdc37 to modulate conformational switching of Hsp90, we characterized the impact of molybdate on the dynamic interactions of Cdc37/S13E with Hsp90-kinase hetero-complexes. Normally, the interaction of Cdc37 with its clients is reiterative, but molybdate "freezes" Cdc37 binding and thus prevents the turnover of these complexes (6). Consistent with our previous results (6), inhibition by molybdate of the Hsp90 ATP-driven reaction cycle blocked the chase of [³⁵S]Cdc37 from Hsp90-kinase complexes (Fig. 4). Surprisingly, however, at 30 °C [³⁵S]Cdc37/S13E chased from Hsp90-kinase complexes even in the presence of molybdate. Therefore, the S13E mutation compromised the ability of Cdc37 to coordinate the sensing and communication of the protein-protein interactions that regulate Hsp90 nucleotide-modulated conformational switching. Thus, although the carboxylate anion of Glu is able to maintain a near wild type level of binding of Cdc37/S13E to

Hsp90, its trigonal planar geometry cannot mimic the tetrahedral geometry of a phosphate anion and maintain normal Cdc37 function.

In summary, our data indicate that Ser¹³ of Cdc37 is phosphorylated *in vivo*. This phosphorylation is functionally relevant, since mutation of S¹³ disrupts Cdc37 recruitment to and retention within Hsp90-kinase hetero-complexes and disrupts the Cdc37 role in regulating Hsp90 nucleotide-mediated conformational switching (6, 7). Whether the diminished capacity of the mutant proteins to bind kinase is caused by the mutations altering the structural integrity of the Cdc37 N-terminal kinase binding domain, directly diminishing their capacity to bind kinase and thus to coordinate kinase binding with Hsp90 conformational switching, or is a result of the inability of the mutants to stimulate Hsp90 conformational switching, which is required to generate high affinity complexes among Hsp90, Cdc37, and kinase client, remains to be determined. Regardless of the mechanism, the results suggest that CKII regulates Cdc37 function *in vivo* by phosphorylating S¹³ of Cdc37, enabling Cdc37 modulation of Hsp90-mediated kinase folding. These findings further invite the speculation that reversible phosphorylation of Cdc37 may regulate the ATP-driven reaction cycle of Hsp90 and Cdc37 with protein kinases and that Cdc37 activity may be regulated physiologically by fluctuations in CKII activity.

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