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

Jieya Shao, Thomas Prince, Steven D. Hartson and Robert L. Matts‡

RUNNING TITLE: Ser13 Phosphorylation and Cdc37 Function

Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK 74078-3035

* This work was supported by the American Heart Association (0250556N to R.L.M.) and by the Oklahoma Agricultural Experiment Station (Project 1975).

‡For correspondence: Robert L. Matts, 246 NRC, Department of Biochemistry and Molecular Biology, Oklahoma State University, Stillwater OK 74078-3035
Tel.: (405)744-6200, FAX: (405)744-7799,
Email: rmatts@biochem.okstate.edu
ABSTRACT:

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 \textit{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 Hsp90’s ATP-driven reaction cycle. Thus, the data indicate that phosphorylation of Cdc37 on Ser13 is critical for its ability to coordinate Hsp90’s nucleotide-mediated conformational switching and kinase binding.
Cdc37 is an Hsp90 co-chaperone that interacts with and is required for the function of a wide array of eukaryotic tyrosine and Ser/Thr protein kinases that are involved in regulating multiple facets of cell physiology [reviewed in (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’s nucleotide-modulated conformational switching, thus capturing the client kinase in high-affinity salt-stable Hsp90/Cdc37 heterocomplexes (6,7).

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 (S13 in human, S14 in yeast) present within a consensus casein kinase II (CKII) phosphorylation motif, and 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 non-functional and whose expression levels are greatly reduced, suggesting that its structural stability is compromised (9). Recent genetic experiments indicate that a Cdc37/S14,17A double mutation compromises Cdc37 function in yeast, while a Cdc37/SS14,17 E mutation compromises Cdc37 function to a lesser degree (8). This compromised function prompted the proposal that S14 and 17 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 S13. This
phosphorylation is required for Cdc37’s biochemical function, namely for its kinase binding activity and for its ability to stimulate 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 S to A or the S to E 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 previously described (6,7,11,14). Cdc37 gene products were either directly immunoabsorbed with agarose-bound anti-(His5) 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 eIF2α 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 4 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 E. coli-  His-tagged full length Cdc37 and His-tagged Cdc37/ΔC were purified as previously described (7). His-tagged Cdc37/ΔC (~ 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 º C. Unphosphorylated and phosphorylated His-tagged Cdc37/ΔC (~5 µg) were digested with chymotrypsin, and analyzed by MALDI-TOF MS.

Analysis of the phosphorylation status of Cdc37 at Ser-13S-  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 º 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 x 10⁶ K562 cells that were grown to 80% saturation at 37 º C in RPMI 1640 medium containing 10% fetal calf serum, washed with HANKS 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 dithiothreitol, 0.5% Nonident P-40, 10 % glycerol and 20 µl of phosphatase inhibitor cocktail (Sigma P8540). 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/25mM NH₄HCO₃, then dried under vacuum and rehydrated/infiltrated with one gel volume of 12.5 ng/µl chymotrypsin (Promega, sequencing grade) prepared in 50 mM NH₄HCO₃, 5 mM CaCl₂. Digestions were incubated at 37 ºC for ~16 h. The digestion fluid was removed and the gel pieces were extracted with 50 µl of 50% acetonitrile/0.1% TFA. The extracts and digestion fluid were combined, concentrated to a volume of 10-20 µl, and purified with Zip-tips (MILLIPORE) according to the manufacturers recommended procedure.

Aliquots (1 µl) of the peptide mixtures were spotted onto a MALDI plate, and immediately covered by the same volume of saturated α-cyano-4-hydroxycinnamic acid (matrix) prepared in 50% acetonitrile/0.1% TFA. 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 to 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 S13 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, the heme-regulated eIF2α
kinase (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 due to a lack of interaction with Hsp90: the binding of the S13A and S13E mutants to immunadsorbed Hsp90 were decreased by ~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 to 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 S to E or S to A mutations in the analogous S14/S17 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 observed and theoretical m/z’s lying well within the instrument’s mass accuracy 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 corresponded 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 (2481). The mass of this mutant peptide, and the absence of phosphorylation thereon, demonstrated that S13 was the site phosphorylated on Cdc37 produced in RRL.

Since peptide 8-29 of Cdc37 contains a consensus CKII phosphorylation motif at S13, we determined if 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 (Matts et al., unpublished). The spectra of chymotryptic peptides from Cdc37/ΔC included a peak whose average m/z (2497) corresponded 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 (2477) was observed (Fig 3G). Additionally, Coomassie blue staining of the gel indicated that treatment of Cdc37/ΔC with CKII quantitatively slowed its
electrophoretic mobility (not shown). Thus CKII had the capacity to quantitatively phosphorylate Cdc37/ΔC 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 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 representative peptides were not detectable in our MALDI-TOF mass spectra.

**Mutation of S13 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’s 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 Cdc37’s stimulation of Hsp90’s 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 S to E 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, while geldanamycin nearly quantitatively blocked the interaction of wild type Cdc37 with HRI, the interaction of Cdc37/S13E with HRI was only reduced by 70%. The cause and relevance of residual salt- and geldanamycin-resistant binding remains uncertain. Addition of molybdate restored the salt-stability of Cdc37/S13E complexes with Hsp90 and client kinase, consistent with molybdate’s documented ability to stabilize of Hsp90 heterocomplexes (6,7). Similarly, Cdc37/S13A was not capable of forming salt-stable complexes with newly synthesized HRI, and addition of molybdate was required to restore the salt-stability of Hsp90-kinase complexes containing the S13A protein (Fig 3B). Thus, while the S13E and S13A mutations diminished Cdc37’s
recruitment to kinase complexes, it also compromised Cdc37’s normal function in stimulating Hsp90’s 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 Cdc37/S13E’s dynamic interactions with Hsp90/kinase heterocomplexes. Normally, Cdc37’s interaction 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), molybdate inhibition of Hsp90’s ATP-driven reaction cycle blocked the chase of $^{35}$S[Cdc37 from Hsp90-kinase complexes (Fig. 4). Surprisingly, however, at 30 °C $^{35}$S[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’s nucleotide-modulated conformational switching.

Thus, while the carboxylate anion of E is able to maintain near wild type 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’s recruitment to, and retention within Hsp90-kinase heterocomplexes, and disrupts Cdc37’s role in regulating
Hsp90’s 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 Cdc37’s N-terminal kinase binding domain, directly diminishing their capacity to bind kinase, and thus 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 between 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’s 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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ACKNOWLEDGEMENTS: Geldanamycin was provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, NIH. We would like to thank the Sarkey’s Biotechnology Research Laboratory (OSU) for oligonucleotide synthesis, DNA sequencing and training in MALDI-TOF MS analysis.

Abbreviations: 90 kDa heat shock protein, Hsp90; Cdc37, generically used to refer 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 eIF2α kinase; eIF, eukaryotic initiation factor; eIF2 α, α-subunit of eukaryotic initiation factor 2; EGTA, ethyleneglycol tetraacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PIPES, piperazine-N,N-bis[2-ethanesulfonic acid]; DMSO; dimethylsulfoxide; PCR, polymerase chain reaction; rabbit reticulocyte lysate, RRL.
Figure Legends:

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 & 6) or lacking (lanes 1, 3 & 5) [^35S]His-tagged HRI. Subsequently, His-tagged HRI was immunoadsorbed and immune resins (PEL: upper panel) or equivalent aliquots of input sample (INPUT) were analyzed by SDS-PAGE and Western blotting (Hsp90) or autoradiography (HRI, Cdc37).  [^35S]-Labeled His-tagged wild-type, S13A or S13E Cdc37 gene products were synthesized in RRL and were subsequently immunoadsorbed with (B) anti-Hsp90 (lanes 2, 4 & 6) or control (lanes 1, 3 & 5) antibodies, or (C) anti-His tag antibodies.  Immunoresins (B: PEL) and equivalent amounts of unfractionated input lysate (B: INPUT) were analyzed by staining with Coomassie Blue (B; Hsp90), Western blotting (C: Hsp90) 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.

Fig. 2  **MALDI-TOF analysis of the phosphorylation state of Ser23 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.

Figure 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 & 8-12; B: lanes 2-5 & 7-10) or lacking (A: lanes 1 & 7; B: lanes 1 & 6) His-tagged HRI kinase. A) Samples were then incubated for 20 min with 10 µg/ml geldanamycin (lanes 3, 5, 9, &11) or with DMSO vehicle (lanes lanes 2, 4, 6, 8, 10 & 12) and subsequently supplemented with 20 mM sodium molybdate (lanes 6 & 12) or buffer (lanes 1-5 & 7-11). B) Samples were incubated for 20 min and subsequently supplemented with 20 mM sodium molybdate (lanes 4, 5, 9 & 10) or buffer (lanes 1-3 & 6-8). After 5 min, His-tagged HRI heterocomplexes were immunoadsorbed, and washed with buffers containing low salt (L) or high salt (H) as previously described (6,18). Samples analyzed by Western blotting (Hsp90) and autoradiography ([35S]Cdc37).

Figure 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, and Cdc37/S13E (lanes 8-14) were synthesized in RRL and subsequently mixed with lysate
containing (lanes 2-7 & 9-14) or lacking (lanes 1 & 8) His-tagged HRI. Samples were then chased into 5 volumes of normal RRL containing (+molybdate: lanes 5-7 & 12-14) or lacking (no additions: lanes 1-4 & 8-11) 20 mM sodium molybdate. His-tagged HRI was subsequently immunoadsorbed after 0 (lanes 2, 5, 9 & 12), 5 (lanes 3, 6, 10, & 13) and 10 (lanes 1, 4, 7, 8, 11 & 14) min of chase incubation. HRI heterocomplexes were washed and analyzed by SDS-PAGE and Western blotting (Hsp90) or autoradiography (wtCdc37 & Cdc37/S13E).
**Fig. 1**

(A) WT, S13A, S13E

- Hsp90
- HRI
- Cdc37

INPUT

WT, S13A, S13E

(B) WT, S13A, S13E

- Hsp90
- Cdc37

INPUT

WT, S13A, S13E

(C) NT, WT, S13A, S13E

- Hsp90
- Cdc37

salt L, H
Figure 2
Fig. 3
Fig. 4
Phosphorylation of serine-13 is required for the proper function of the Hsp90 Co-chaperone, Cdc375
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J. Biol. Chem. published online August 20, 2003

Access the most updated version of this article at doi: 10.1074/jbc.C300330200

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