Direct Interaction of the Cell Division Cycle 37 Homolog Inhibits Endothelial Nitric Oxide Synthase Activity

M. Brennan Harris, Manuela Bartoli, Sarika G. Sood, Robert L. Matts, Richard C. Venema

Abstract—Endothelial NO synthase (eNOS) via the production of NO in the endothelium plays a key role in cardiovascular biology and is tightly regulated by co- and posttranslational mechanisms, phosphorylation, and protein–protein interactions. The cell division cycle 37 homolog (Cdc37) is a key heat shock protein 90 (Hsp90) co-chaperone for protein kinase clients, and Akt/Hsp90 interaction is dependent on Cdc37. Because both Hsp90 and Akt are key eNOS regulatory proteins, we hypothesized that Cdc37 interacts with eNOS as part of the regulatory complex. In the present study, we demonstrate by coimmunoprecipitation and affinity purification in bovine aortic endothelial cells (BAECs) that Cdc37 is complexed with eNOS, Hsp90, and Akt. In addition, cell fractionation data indicate that Cdc37 is found in caveolae with eNOS. Further analysis by in vitro binding assays reveals a direct interaction between purified Cdc37 and eNOS. Incubation of purified Cdc37 with purified wild-type eNOS decreases eNOS activity in vitro. Overexpression of wild-type Cdc37 in BAECs inhibits eNOS activity and NO release, whereas overexpression of S13A-Cdc37 mutant in BAECs increases eNOS activity and NO release. Taken together, these data suggest that Cdc37 has a direct regulatory interaction with eNOS and may play an important role in mediating the eNOS protein complex formation as well as subsequent eNOS phosphorylation and activation. (Circ Res. 2006;98:335-341.)

Key Words: Cdc37  ■  nitric oxide synthase  ■  endothelium  ■  heat shock protein 90

Endothelial nitric oxide synthase (eNOS), the enzyme responsible for the production of nitric oxide (NO) within the endothelium, plays an important role in cardiovascular biology regulating vasodilation, platelet and leukocyte adhesion to the endothelium, as well as endothelial cell and vascular smooth muscle proliferation. In addition, eNOS uncoupling in which the balance of NO production and superoxide production is shifted in favor of superoxide has been suggested to play a key role in endothelial dysfunction associated with various pathological conditions, including hypertension and diabetes. Precise regulation of eNOS function is, therefore, of vital importance to maintain vascular homeostasis.

eNOS is known to be regulated by co- and posttranslational mechanisms, phosphorylation, and protein–protein interactions. Previously, we and others have demonstrated that eNOS is regulated by and binds directly to heat shock protein 90 (Hsp90) in the middle region or M-domain. Hsp90 increases eNOS activity in vitro and is recruited to the eNOS complex by eNOS-activating agonists including bradykinin, vascular endothelial growth factor (VEGF), histamine, and fluid shear stress. Overexpression of Hsp90 in human umbilical vein endothelial cells increases Akt-mediated phosphorylation of eNOS and acts as scaffold aiding in the recruitment Akt, another key eNOS regulatory protein. Recently, Hsp90 has been shown to play a role in modulating the balance of superoxide and NO production. Inhibition of Hsp90 uncouples eNOS activity in proliferating endothelial cells and dissociation of Hsp90 and impairment of eNOS activity has been observed in a model of persistent pulmonary hypertension.

As mentioned above, the protein kinase Akt is also an important mediator of eNOS activity. eNOS is phosphorylated in endothelial cells at Ser1179 and Ser617 (bovine sequence) by the Akt protein kinase resulting in a 2-fold increase in eNOS catalytic activity and a decrease in the Ca²⁺-calmodulin dependency. Akt binds directly to the middle region (residues 327 to 340) of Hsp90, which appears to maintain Akt activity by preventing Akt dephosphorylation. Follow-up studies reveal that the interaction of Akt with eNOS and subsequent phosphorylation at Ser1179 was dependent on Hsp90.6,7,15 Additional experiments in cell cultures reveal that stimulation of endothelial cells with insulin or VEGF results in increased eNOS activity that is temporally correlated with eNOS/Hsp90/Akt association and eNOS phosphorylation.6,7,15

Interestingly both Hsp90 and Akt interact with another protein, the cell division cycle 37 homolog (Cdc37). Cdc37 was first observed as a 50-kDa phosphoprotein that was coimmunoprecipitated with v-Src and was later identified as...
Cdc37 in *Saccharomyces cerevisiae* by investigations into the role of protein kinases in cell cycle regulation. The significance of the association of Cdc37 with Hsp90 is illustrated by the observation by immunohistochemistry that essentially all cytoplasmic Cdc37 is associated with Hsp90 in cells transformed with the Rous sarcoma virus. The interaction of Hsp90 and Cdc37 has since been investigated in great detail and Cdc37 is believed to be a key Hsp90 co-chaperone for protein kinase clients, acting as a scaffold and facilitating protein kinase binding to Hsp90. Akt has been identified as one of the client protein kinases influenced by the Hsp90/Cdc37 co-chaperone complex, and the interaction ofAkt with Hsp90 is dependent on Cdc37. Based on these studies, we tested the hypothesis that Cdc37 exists within the eNOS regulatory protein complex with Hsp90 and Akt and may interact directly or indirectly with eNOS to alter eNOS activity.

### Materials and Methods

#### Cell Culture

Bovine aortic endothelial cells (BAECs) were passaged from primary cultures and used for experiments during passages 2 to 6. Cultures were maintained in water-jacketed incubators at 37°C and 5% CO₂ and in M-199 medium supplemented with 10% FBS, 5% iron-supplemented calf serum, 20 μg/mL L-glutamine, 1X minimal essential medium, 0.6 μg/mL thymidine, 500 IU/mL penicillin, and 500 μg/mL streptomycin. Serum-containing medium was replaced by serum-free medium 24 hours before experiments involving treatment of BAECs with VEGF.

#### Immunoprecipitation and Immunoblotting

Immunoprecipitation and immunoblotting of BAECs was performed as previously described. Briefly, under basilar conditions or following treatment, BAECs were washed twice with ice-cold PBS containing 1 mmol/L Na₂VO₃. Cells were then lysed in ice-cold buffer containing 20 mmol/L Tris/HCl (pH 7.4), 2.5 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mmol/L NaF, 10 mmol/L Na₃P₂O₇, 1 mmol/L Na₂VO₃, and 1% phenylmethylsulfonyl fluoride. Cell lysates were centrifuged at 10,000 g for 20 minutes to remove insoluble material. For immunoprecipitation, samples were precleared by adding Protein A/G agarose (sc-2003, Santa Cruz Biotechnology) antibody was then added to the supernatant and incubated overnight at 4°C. Protein A/G agarose (sc-2003, Santa Cruz Biotechnology) antibody was then added to the supernatant and incubated overnight at 4°C. Protein A/G agarose (sc-2003, Santa Cruz Biotechnology) antibody was then added to the supernatant and incubated overnight at 4°C.

#### Affinity Purification of eNOS

eNOS was partially purified from BAECs by affinity binding to 2',5'-ADP Sepharose as described previously. Following the lysis of BAECs as described above for immunoprecipitation, 80 μL of a 50:50 2',5'-ADP Sepharose slurry was added to 1 mL of the cell lysate and incubated at 4°C for 2 hours. The beads were then centrifuged and washed 3 times with ice-cold PBS. SDS sample buffer (100 μL) was added to the beads following the last wash and the samples were boiled for 5 minutes to elute the bound proteins from the beads. The samples were centrifuged and the supernatant was subjected to immunoblotting as described above.

### Caveolae Isolation

Caveolae isolation and cell fractionation was conducted by ultracentrifugation of BAEC cell lysates in a discontinuous sucrose gradient as previously described and modified.

#### Construction and Purification of HIS-Cdc37 Fusion Proteins and eNOS

Wild-type (His)₅-p50cdc37 was generated as previously described. Bovine eNOS was expressed in a baculovirus/S9 insect cell system and purified to >95% homogeneity as described previously.

#### In Vitro Binding of His-Cdc37 and eNOS

Because eNOS binds to the Ni-Sepharose beads used to purify Hsp90-tagged proteins, purified His-Cdc37 proteins were eluted from the Ni-Sepharose beads using imidazole and then dialyzed to remove the imidazole from His-Cdc37-containing buffer. The purified His-Cdc37 (57 μg, 2 mL) was injected into a dialysis cassette (Pierce Side-A-Lyzer, product no. 66425) and added to a float. The dialysis was performed in 2 L of buffer (50 mmol/L Tris HCl, pH 7.4+20% glycerol) overnight at 4°C on a stir plate. After the overnight dialysis, the buffer was replaced with 2 L of fresh buffer and the dialysis was continued for an additional 2 hours. The purified His-Cdc37 was then removed from the dialysis cassette with a needle and syringe. Following these steps, equimolar amounts of purified His-Cdc37 and eNOS were incubated overnight at 4°C with or without the alternate protein. Samples were then immunoprecipitated with either anti-Cdc37 or anti-eNOS antibodies as described above. Immunoprecipitated proteins bound to the agarose beads were washed extensively. Lysis buffer was prepared and dialyzed before washing the beads. The lysis buffer used was the same buffer in which the His-Cdc37 was formerly purified. It consisted of 50 mmol/L Na₃PO₄, 300 mmol/mL NaCl, and 10 mmol/L imidazole. Before washing the beads, the above buffer was dialyzed to remove the imidazole. The dialysis process was identical to the previously described procedure. NaCl (0.5 mol/L) was added to this prepared buffer which was then used to wash the immunoprecipitated proteins bound to the agarose beads. The beads were washed 3 times in lysis buffer with 0.5 mol/L NaCl. Bound proteins were eluted by boiling in SDS sample buffer, separated by electrophoresis, and immunoblotted with either anti-Cdc37 or anti-eNOS antibody.

#### Expression of Wild-Type FLAG-Tagged p50cdc37

We have previously generated a wild-type human p50cdc37 in a mammalian expression vector. FLAG-tagged p50cdc37 was then expressed in BAECs using Lipofectamine 2000 (Invitrogen) according to the protocol of the manufacturer. Lipofectamine transfection of BAECs with a construct containing green fluorescent protein (GFP) was also performed to verify and control for the effects of the transfection. Following overnight transfection, BAECs were then used to determine NO release using a CMG reporter cell assay or lysed to determine eNOS activity by 1-[¹⁴C]-arginine to l-[^1⁴C]-citrulline conversion.

#### Expression of Mutant S13A-Cdc37

Using site-directed mutagenesis, we have previously generated the cDNA for a S13A-Cdc37 mutant construct. An adenosine containing the cDNA for S13A-Cdc37 (AdS13A-Cdc37) was generated using the AdEasy system for the production of replication-deficient recombinant adenosine vectors using the method of He et al. An additional adenosine encoding β-galactosidase (Ad-β-gal) was created and used as a negative control 24 hours following incubation of confluent BAECs with these adenoviruses. NO release was determined using a CMG reporter cell assay or eNOS activity was determined in lysates by l-[¹⁴C]-arginine to l-[¹⁴C]-citrulline conversion.
Wild-type bovine eNOS purified as described above were incubated for 5 minutes at 37°C with or without increasing quantities of purified His-Cdc37. Following the incubation, eNOS activity was measured by monitoring the rate of conversion L-[14C]-arginine to L-[14C]-citrulline as previously described. Imidazole used to elute the His-Cdc37 from the Ni-Sepharose beads was dialyzed from the elution buffer as described above. To confirm that the dialyzed buffer had no effect on eNOS activity, a sample containing dialyzed buffer with an equal concentration of BSA instead of His-Cdc37 was used as a control.

In separate experiments, eNOS activity was also determined by monitoring the rate of conversion L-[14C]-arginine to L-[14C]-citrulline as previously described in BAEC cell lysates transfected with wild-type Cdc37 or GFP (negative control) or in BAEC cell lysates transduced with AdS13A-Cdc37 or Ad-/H9252-gal (negative control).

**Statistics**

Descriptive data (means±SD and SE) were calculated for each dependent variable. Overall differences between groups were analyzed using a 2-way ANOVA with Student–Newman–Keuls post hoc analysis for determining differences between the means when comparing more than 2 groups. An independent t test was used when comparing only 2 groups. In all tests, a probability level of \( P<0.05 \) was used as the decision rule for significance testing.

**Results**

**Cdc37 and eNOS Complex Formation**

As illustrated in Figure 1A, immunoblotting reveals that increasing quantities of both Hsp90 (≈90 kDa) and eNOS (≈130 kDa) were immunoprecipitated from increasing amounts of BAEC cell culture lysates using an anti-Cdc37 antibody, suggesting that Cdc37, eNOS, and Hsp90 exist in a complex within BAECs. Using the optimal amount of BAEC cell lysate determined in the first experiment, Figure 1B demonstrates that Cdc37 (≈50 kDa) is also immunoprecipitated using an anti-eNOS antibody, further confirming the suggestion that Cdc37 and eNOS exist in a complex.

In an additional series of experiments, eNOS was partially purified from BAECs by affinity binding to 2',5'-ADP Sepharose as described previously. As shown in Figure 2, immunoblotting of the proteins bound to the 2',5'-ADP Sepharose reveals both Cdc37 and eNOS, further supporting the notion that the two proteins interact.

Further evidence of Cdc37/eNOS association is shown in Figure 3. BAEC cell lysates were fractionated using a sucrose gradient and ultracentrifugation. Analysis of the cellular fractions by immunoblotting shows that a large portion of eNOS is found in the caveolar fractions as determined by immunoblotting with anti–caveolin-1, which is in agreement with previous reports of eNOS localization. Additional analysis of the cellular fractions by immunoblotting with anti-Cdc37 antibody reveals the presence of Cdc37 in the caveolin-1–containing fractions, suggesting that both eNOS and Cdc37 exist in the endothelial cell caveolae.

**Figure 1.** Coimmunoprecipitation of eNOS and Cdc37 in BAECs. Increasing quantities of BAEC cell lysates were immunoprecipitated with anti-Cdc37 antibody (A) or anti-eNOS antibody (B). Precipitated proteins were separated by gel electrophoresis, transferred to nitrocellulose, and immunoblotted with anti-eNOS, anti-Hsp90, and anti-Cdc37 antibodies (A) or anti-Cdc37 and anti-eNOS antibodies (B). Similar results were obtained in 3 separate experiments.

**Figure 2.** Affinity-purified eNOS from BAECs contains Cdc37. eNOS was partially purified from BAEC cell lysates by affinity binding to 2',5'-ADP Sepharose. Proteins bound to the beads were eluted, separated by electrophoresis, transferred to nitrocellulose, and immunoblotted with anti-Cdc37 antibody and anti-eNOS antibody. Similar results were obtained in 3 different experiments.

**Figure 3.** Distribution of eNOS, Cdc37, and caveolin-1 in BAEC cell lysates in sucrose density gradient fractions. BAEC homogenates were fractionated in a discontinuous sucrose density gradient by centrifugation. Eight fractions were collected from the top of each tube, and aliquots of each fraction were analyzed by immunoblotting with anti-eNOS, anti-Cdc37, and anti-caveolin-1 antibodies. Similar results were obtained in 3 separate experiments.
Direct Inhibitory Interaction Between Cdc37 and eNOS

Full-length human Cdc37 (GenBank accession no. R87892) was expressed as a 6 His–tagged fusion protein and purified on Ni-Sepharose by affinity chromatography as previously described.\textsuperscript{23} The purified His-Cdc37 fusion protein was then used in in vitro binding assays with recombinant bovine eNOS, expressed and purified in a baculovirus system. The purified proteins were incubated together or separately overnight at 4°C and then immunoprecipitated with either anti-Cdc37 or anti-eNOS antibodies and protein A/G agarose beads. As shown in Figure 4A, immunoblotting revealed that eNOS was immunoprecipitated with anti-Cdc37 antibody only in samples containing both Cdc37 and eNOS. In Figure 4B, Cdc37 was immunoprecipitated with anti-eNOS antibody only in samples containing both eNOS and Cdc37. These data strongly suggest a direct interaction between Cdc37 and eNOS.

To determine the effects of the direct interaction of Cdc37 on eNOS activity, increasing quantities of purified His-Cdc37 were incubated with purified wild-type eNOS. Following the incubation, eNOS activity was determined by monitoring the rate of conversion L-[\textsuperscript{14}C]-arginine to L-[\textsuperscript{14}C]-citrulline. As shown in Figure 5, His-Cdc37 exerts a dose dependent inhibitory action on eNOS activity in vitro. This effect was not attributable to the buffer or general protein content in the assay as demonstrated by controls containing dialyzed elution buffer and equal concentrations of BSA.

To confirm that the inhibitory effects of Cdc37 on eNOS activity in vitro are representative of the regulatory role of Cdc37 in endothelial cells, we overexpressed both wild-type Cdc37 and a Cdc37 phosphorylation mutant, S13A-Cdc37, in BAECs and determined both eNOS activity and stimulated NO release. As shown in Figure 6A, expression of wild-type Cdc37 significantly (\(P < 0.05\)) reduced eNOS activity in BAEC cell lysates compared with the negative control expressing GFP, whereas in Figure 6B, overexpression of S13A-Cdc37 significantly (\(P < 0.05\)) increased eNOS activity compared with \(\beta\)-gal. Figure 7 provides additional evidence that Cdc37 not only regulates eNOS activity but also alters VEGF-stimulated endothelial NO release. Figure 7A shows that expression of the wild-type Cdc37 significantly (\(P < 0.05\)) attenuates VEGF stimulated NO release compared with cells transfected with GFP. In addition, Figure 7B shows overexpression of S13A-Cdc37 significantly (\(P < 0.05\)) enhances VEGF-stimulated NO release. Taken together, these data strongly suggest that Cdc37 regulates eNOS in endothelial cells.

Discussion

Previous studies have demonstrated that both Hsp90 and Akt exist as part of the eNOS regulatory protein complex.\textsuperscript{4} In

![Figure 4. In vitro binding of Cdc37 and eNOS. His-tagged full-length Cdc37 fusion protein were expressed in Escherichia coli and purified by affinity binding to Ni-Sepharose beads. Purified His-Cdc37 proteins were eluted from the Ni-Sepharose beads using imidazole and then dialyzed to remove the imidazole from His-Cdc37–containing buffer. Equimolar amounts of purified His-Cdc37 and purified wild-type eNOS were incubated overnight at 4°C with or without the alternate protein. Samples were then immunoprecipitated with anti-Cdc37 antibody (A) or anti-eNOS antibody (B). The beads were washed 3 times in lysis buffer with 0.5 mol/L NaCl. Bound proteins were eluted by boiling in SDS sample buffer, separated by electrophoresis, and immunoblotted with anti-Cdc37 and anti-eNOS antibodies. Similar results were obtained in 3 different experiments.

![Figure 5. Inhibition of in vitro eNOS activity by Cdc37. Wild-type bovine eNOS purified from a baculovirus system was incubated for 5 minutes at 37°C with or without increasing quantities of purified His-Cdc37. eNOS activity was then measured by monitoring the rate of conversion L-[\textsuperscript{14}C]-arginine to L-[\textsuperscript{14}C]-citrulline. Imidazole used to elute the His-Cdc37 from the Ni-Sepharose beads was dialyzed from the elution buffer as described above. To confirm that the dialyzed buffer had no effect on eNOS activity, a sample containing dialyzed buffer with an equal concentration of BSA instead of His-Cdc37 was used as a control. *\(P < 0.05\) (n=6).]
addition, it has been shown that Cdc37 acts as a key Hsp90 cochaperone and is a critical component in Hsp90/Akt interaction. For the first time, the results of this study demonstrate that Cdc37 is also part of the eNOS protein complex. More importantly, the results of this study demonstrate that Cdc37 interacts directly with eNOS in vitro and that direct interaction of Cdc37 with eNOS inhibits eNOS activity both in vitro and in endothelial cells. Taken together, these data suggest an important eNOS regulatory function for Cdc37.

As indicated above, it has previously been shown that Akt is part of ternary complex with eNOS and Hsp90. Key early studies revealed that eNOS is phosphorylated at Ser1179 (bovine sequence) and activated by the Akt protein kinase in response to stimulation of endothelial cells by various agonists. Additional landmark studies also showed that eNOS interacts directly with and is activated by Hsp90. Recently, these 2 regulatory pathways were integrated revealing that Hsp90 and Akt synergistically activate eNOS, in part, because Akt dephosphorylation and deactivation is blocked by Hsp90. More importantly, several investigations have reported that Akt interaction with eNOS and subsequent eNOS phosphorylation and activation is dependent on Hsp90.

Further analysis of the functional significance of the interactions among Hsp90, Akt, and eNOS have also been conducted. The addition of purified Hsp90 to purified active Akt and eNOS increased eNOS phosphorylation in vitro and can be blocked by the addition of the Hsp90 inhibitor, geldanamycin. Hsp90, however, did not increase Akt-mediated phosphorylation of a short eNOS peptide containing the Akt phosphorylation site, suggesting that enhanced phosphorylation of full-length eNOS in the presence of Hsp90 involves spatial and conformational components. Overexpression of Hsp90 in either COS cells cotransfected with eNOS or in BAECs resulted in increase Ser1179 phosphorylation, which was associated with an increase in basal and VEGF stimulated NO release. Finally, in an impressive series of experiments, Fontana et al confirmed our observation that eNOS binds within the middle domain of Hsp90 (residues 442 to 600), which is adjacent to the reported Akt binding site (residues 327 to 340). In addition, they report that Hsp90 interacts with eNOS between residues 300 and 400. Taken together, these data confirm that Hsp90 is required for the efficient and productive Akt-dependent phosphorylation of Ser1179 and activation of eNOS, which is likely attributable to Hsp90 acting as a protein scaffold holding Akt in eNOS in close proximity.

Although the effective interaction of Akt with eNOS is clearly dependent on complex formation with Hsp90, Basso et al provide evidence that the stable association of Hsp90 and Akt requires the presence of another protein, Cdc37. Immunoblotting of both Akt and Cdc37 immunoprecipitates revealed the presence of Akt, Cdc37, and Hsp90 in the human breast cancer cell line MCF-7. Further analysis revealed that the Akt in the Cdc37/Hsp90 complex was both phosphorylated and active. More importantly, cotransfection of MCF-7 cells with FLAG-tagged Cdc37 and HA-tagged Akt revealed that the association of Akt and Hsp90 required the presence of Cdc37 and that the HA-Akt/Hsp90 complex was only found when Cdc37 was overexpressed. These data led the authors to conclude that Cdc37 acts to stabilize the Akt/Hsp90 interaction and/or is required for the binding of Akt to Hsp90. Furthermore, these authors suggested that Cdc37 may act as a scaffold similar to the Hsp90 scaffold facilitating Akt/eNOS interaction.

Experiments conducted in the present study in endothelial cells show that anti-Cdc37 antibody immunoprecipitates eNOS and Hsp90 (Figure 1A). These data support the report of a Cdc37/Hsp90/Akt complex by Basso et al. Whereas the functional significance of Hsp90 and Akt complex formation with eNOS has been explored in great detail, as described above, the role of Cdc37 in the Hsp90/Akt/eNOS complex is unknown. Based on the observation that Cdc37 stabilizes the interaction of Hsp90 and active Akt and on the report that Akt association with Hsp90 prevents dephosphorylation and deactivation of Akt, it is possible to hypothesize that Cdc37 indirectly enhances eNOS phosphorylation and subsequent activity. However, additional experiments performed in this study using purified Cdc37 and eNOS in vitro suggest that interaction of Cdc37 and eNOS is not simply attributable to the indirect relationships with Hsp90 and Akt but may instead be attributable to a direct interaction between the 2 proteins (Figure 4). Furthermore, our results show that Cdc37 inhibits eNOS activity in vitro (Figure 5), and these data are further supported by experiments in endothelial cells transfected with wild-type Cdc37 that demonstrate a reduction in eNOS activity (Figure 6A) as well as VEGF-stimulated NO release (Figure 7A). Taken together, these data support the assertion of MacLeans and Picard that Cdc37 is not merely an Hsp90 cochaperone but may have additional independent effects and contradicts the hypothesis that Cdc37 enhances eNOS activation.

Additional data collected in this study suggest that Cdc37 also impacts the regulatory interactions of Hsp90 and Akt with eNOS. Previously, we have established that the proper interaction of Cdc37 with Hsp90 is dependent on phosphorylation of Cdc37 at Ser13. Mutation of Ser13 to alanine disrupts recruitment of Cdc37 to Hsp90-kinase complexes and may compromise the ATPase function and conformational changes necessary for normal Hsp90 function. Interestingly, in the present study, overexpression of the S13A-Cdc37 mutant in BAECs resulted in an enhancement of eNOS activity (Figure 6B) and an increase in VEGF-stimulated NO release (Figure 7B). These data suggest that the regulation of eNOS activity by Cdc37 also involves alterations in Hsp90 function. However, the mechanism by which Cdc37 affects eNOS activity and its interactions with these other regulatory proteins remains unclear.

To postulate a potential model for the observed phenomenon, it is useful to examine our results in the context of previous studies examining the interactions of Cdc37/Hsp90 cochaperone complex with their client proteins. In an elegant series of experiments combined with observations by ourselves and others, Roe et al described the interaction of Hsp90 and Cdc37 in detail. Briefly, the C-terminal domain of Cdc37 (specifically residues 138 to 378) binds to the N-terminal domain of Hsp90, which is also the site of ATP binding. Binding of Cdc37 to the N-terminal domain of
Hsp90 appears to arrest the ATPase cycle of Hsp90, locking Hsp90 in an open conformation, which facilitates client protein binding, particularly to the middle or M-domain of Hsp90. Crystallographic analysis indicates that interaction of Cdc37 with Hsp90 results in a position in which the N-terminal domain of Cdc37 is directed toward M-domain of Hsp90 allowing for simultaneous interaction of Cdc37 with a client protein.18 As mentioned above, the M-domain of Hsp90 is the precise region to which both eNOS5,7 and Akt bind28 and suggests that Cdc37 could, therefore, directly interact simultaneously with Hsp90, Akt, and eNOS. In addition, the observation that Cdc37 binds to the N-terminal domain of Hsp90 arresting the ATPase cycle is particularly interesting in light of studies showing that the Hsp90 inhibitor geldanamycin, which interacts with Hsp90 in a similar manner, either blocks Akt-dependent eNOS phosphorylation and activation4,15 or leads to eNOS uncoupling following phosphorylation and activation.9 With this in mind, we hypothesize that Cdc37 bound to the eNOS in the absence of other regulatory proteins serves a protective function inhibiting uncoupled or unregulated eNOS activity and that overexpression of Cdc37 interferes with the proper interactions of eNOS with Hsp90 and Akt and subsequent activation. In addition, the S13A-Cdc37 may enhance eNOS activity either directly or indirectly by maintaining Hsp90 in an open conformation. Therefore, Cdc37 may act both independently or as an important Hsp90 cochaperone promoting or inhibiting eNOS activity, depending on the presence, absence, or concentrations of other members of the eNOS regulatory complex.

In conclusion, the results of the present study are the first to integrate information from 2 separate areas of investigation, the interaction of Hsp90 with the cochaperone Cdc37 and the interaction Hsp90 and eNOS. More importantly, although we initially hypothesized that Cdc37 would positively modulate eNOS activity, the results of this study led us to conclude that Cdc37 independently inhibits eNOS activity joining caveolin-1 and G-protein–coupled receptors as eNOS inhibitory proteins. Knowledge of the regulatory role of Cdc37 in the Hsp90 chaperone system may aid our understanding of the eNOS complex, and the identification of a direct regulatory interaction of Cdc37 with eNOS also opens many additional possibilities for future investigations. One important area of investigation will be defining the true temporal interactions between these proteins in endothelial cells following agonist stimulation, which will enable us to piece together each step in the formation of the eNOS regulatory complex. Having a more complete picture of the overall complex will help us determine which interactions are critical for efficient, coupled eNOS activity and whether disruption of this complex plays a role in any number of diseases characterized by endothelial dysfunction.

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