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Chaperone-dependent Regulation of Endothelial Nitric-oxide Synthase Intracellular Trafficking by the Co-chaperone/Ubiquitin Ligase CHIP*

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Endothelial nitric-oxide synthase (eNOS), the enzyme responsible for production of endothelial NO, is under tight and complex regulation. Proper cellular localization of eNOS is critical for optimal coupling of extracellular stimulation with NO production. In addition, the molecular chaperone Hsp90 interacts with eNOS and positively regulates eNOS activity. Hsp90 is modulated by physical interaction with its co-chaperones. CHIP (carboxyl terminus of Hsp70-interacting protein) is such a co-chaperone that remodels the Hsp90 heterocomplex and causes protein degradation of some Hsp90 substrates through the ubiquitin-protein isopeptide ligase activity of CHIP. Here we show that CHIP incorporated into the eNOS·Hsp90 complex and specifically decreased soluble eNOS levels in transiently transfected COS cells. Surprisingly, in contrast to the effects of the Hsp90 inhibitor geldanamycin, which induces eNOS ubiquitylation and its subsequent protein degradation, CHIP did not target eNOS for ubiquitylation and proteasome-dependent degradation. Instead, CHIP partitioned soluble eNOS into an insoluble and inactive cellular compartment, presumably through its co-chaperone activity. This effect seems to be due to displacement of eNOS from the Golgi apparatus, which is otherwise required for trafficking of eNOS to the plasmalemma and subsequent activation. Consistent with observations from overexpression studies, eNOS localization to the membrane and activity were increased in mouse lung endothelial cells lacking CHIP. Taken together, these results demonstrate a novel co-chaperone-dependent mechanism through which eNOS trafficking is regulated and suggest a potentially generalized role for CHIP in protein trafficking through the Golgi compartment.

The nitric-oxide synthases (NOSs)[1] are a family of mammalian enzymes that catalyze the oxidation of L-arginine to produce NO and L-citrulline. Three NOS isoforms exist in mammalian cells, neuronal (nNOS; NOS1), inducible (iNOS; NOS2), and endothelial (eNOS; NOS3), named after the cell types in which they were originally discovered. All NOS isoforms have similar primary structures, including an oxygenase domain at the N terminus, a reductase domain at the C terminus, and a hinge calmodulin domain in between. eNOS is unique among the NOS isoforms in that it is dually acylated by myristate and palmitate. Cysteine palmitoylation is necessary for targeting of eNOS to the specific plasmalemmal microdomain, caveolae (1), and both fatty acylations are required for specific targeting of eNOS to the Golgi (2). Correct subcellular trafficking and localization to the plasmalemma is necessary for eNOS function. eNOS produces NO (and/or other reactive nitrogen species) in vascular endothelial cells and cardiomyocytes in response to a variety of agonists and mechanical stimuli (*i.e.* shear) (3, 4). Mislocalization of the enzyme to either domain impairs agonist-stimulated eNOS activation and optimal NO release from cells, implying that the proper subcellular localization of eNOS is critical for optimal coupling of extracellular stimulation with nitric oxide production (5, 6).

The 90-kDa heat shock proteins (Hsp90) are one of the most abundant proteins in cells, accounting for 1–2% of cytosolic protein. Hsp90 is a ubiquitous molecular chaperone with essential roles in stress tolerance and protein folding. Most of its known substrates are signaling proteins, including steroid receptors, some transcription factors (aryl hydrocarbon receptor, Sim, MyoD1, etc.), and a variety of tyrosine kinases (ErbB-2, Src, insulin receptor, focal adhesion kinase, etc.) and serine/threonine kinases (Raf, Cdk4, MEK (mitogen-activated protein kinase/extracellular signal-regulated kinase kinase), etc.) (reviewed in Ref. 7). eNOS has recently been recognized as another Hsp90 client (3). Hsp90 is physically associated with eNOS in resting endothelial cells. Activation of endothelial cells by vascular endothelial growth factor, histamine, fluid shear stress, and estrogen enhances the interaction between eNOS and Hsp90 and increases eNOS activity. The mechanism of this activation is presently unclear. Hsp90 may act as an allosteric activator of eNOS (3) and/or as a scaffold of eNOS and the serine/threonine kinase Akt (8), the recruitment of which to the eNOS·Hsp90 complex leads to eNOS phosphorylation and activation (9, 10). There are also studies suggesting that Hsp90

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[1] The abbreviations used are: NOSs, nitric-oxide synthases; nNOS, neuronal nitric-oxide synthase; iNOS, inducible nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; TPR, tetratricopeptide repeat; GR, glucocorticoid receptor; CFTR, cystic fibrosis transmembrane conductance receptor; GA, geldanamycin; E3, ubiquitin-protein isopeptide ligase; Ni-NTA, nickel-nitrilotriacetic acid; E1, ubiquitin-activating enzyme; MOPS, 4-morpholinepropanesulfonic acid; E2, ubiquitin carrier protein.

may couple eNOS oxidation (11) or facilitate the replacement of caveolin by calmodulin as a binding partner for eNOS (12). nNOS is an Hsp90 client as well. Hsp90 associates with nNOS and chaperones its maturation (13, 14). Whether similar effects are involved in eNOS regulation remains to be determined, although both proteins have similar structure.

In vivo, Hsp90 is not functional unless associated in heterocomplexes with a range of accessory proteins. The tetratricopeptide repeat (TPR)-containing proteins are one major group of Hsp90 partner proteins. Various TPR proteins compete for binding to a TPR acceptor site at the C terminus of Hsp90 and thus regulate the chaperone function of Hsp90. CHIP (carboxyl terminus of Hsp70-interacting protein) is a recently identified Hsp90 cofactor (15). CHIP interacts with Hsp90 through its N-terminal TPR domain and stimulates protein degradation of the following Hsp90 substrates: the glucocorticoid receptor (GR), the cystic fibrosis transmembrane conductance receptor (CFTR), and ErbB-2 (15–18). Moreover, CHIP-induced protein degradation is not simply a consequence of interference with chaperone function, as is thought to be the case for the Hsp90 inhibitor geldanamycin (GA). CHIP contains a U-box domain at its C terminus and directly targets diverse chaperone substrates to the ubiquitin-proteasome system through its ubiquitin-protein isopeptide ligase (E3) activity (18–20). In this way, CHIP shifts the balance from protein folding to protein degradation during protein quality control, although, at the present time, the range of substrates for CHIP ubiquitination activity is not known.

Although CHIP is a ubiquitous protein, its high level expression in heart and endothelial cells (21) suggests that CHIP may interact with proteins that play important roles in cardiovascular function. In this study, we sought to determine whether the effects of CHIP on Hsp90 substrates such as GR and ErbB-2 could be generalized to the Hsp90 substrate eNOS in consideration of eNOS modulation by Hsp90 and its significant role in the cardiovascular system. Instead, we found that CHIP elicited partitioning of eNOS to the insoluble compartment and impaired trafficking through the Golgi apparatus, indicating a novel chaperone-dependent mechanism for regulation of eNOS activity.

EXPERIMENTAL PROCEDURES

Cell Culture—COS-7 cells were obtained from American Type Culture Collection and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. COS-7 cells were transiently transfected using FuGENE (Roche Applied Science) as described previously (19). Bovine aortic endothelial cells were harvested and cultured as described previously (22). Recombinant adenoviruses were constructed with the Ad-Easy system, and cultures were routinely infected at a multiplicity of infection of 5. Western blotting and indirect immunofluorescence were performed as described previously (15).

Mouse lung endothelial cells were isolated from CHIP$^{-/-}$ or wild-type mice.[2] Lungs from mice were finely minced and incubated in 0.1% collagenase for 1 h at 37 °C. Cells were further homogenized by passage through a 14-guage needle and sieved to remove undigested material. Isolated cells were washed and then incubated with rat anti-mouse ICAM-2 (intercellular adhesion molecule-2) antibody coupled to magnetic beads (Dynabeads M-450, Dynal, Inc.). After magnetic selection, cells were plated in tissue culture flasks and cultured as described previously (22). The endothelial phenotype of isolated cells was confirmed by staining for PECAM (platelet/endothelial cell adhesion molecule).

Antibodies and Chemicals—The following antibodies were used for immunoblotting: mouse anti-eNOS (clone H32; BIOMOL Research Labs Inc.), mouse anti-iNOS and rabbit anti-nNOS (Transduction Laboratories), mouse anti-ubiquitin (Babco), mouse anti-Hsp90 and mouse anti-Hsc/Hsp70 (Stressgen Biotech Corp.), mouse anti-β-actin (Santa Cruz Biotechnology), and rabbit anti-CHIP (21). Mouse anti-Hsp90 (3G3), mouse anti-eNOS, and mouse anti-Myc antibody-agarose conjugates (Santa Cruz Biotechnology) were used for immunoprecipitation. GA was purchased from Calbiochem and used at 2 μM. The proteasome inhibitors MG132, lactacystin, and proteasome inhibitor I were purchased from Calbiochem and used as indicated. nickel-nitrilotriacetic acid (Ni-NTA)-agarose was from QIAGEN Inc.

Western Blotting and Immunoprecipitation—Transfected COS cells were harvested 36–48 h after transfection and lysed with modified radioimmune precipitation assay buffer (100 mM Tris (pH 7.4), 1% Nonidet P-40, 10 mM NaF, and 1 mM Na_3VO_4) (23) supplemented with protease inhibitors and 50 mM *N*-ethylmaleimide to inhibit ubiquitin-cleaving isopeptidase. Cell lysates were clarified by centrifugation at 16,000 $\times$ *g* for 10 min at 4 °C, and protein concentration was determined with a protein assay kit (Bio-Rad). In some experiments, proteins in the pellets were recovered by resuspension of pellets in the lysis buffer and sonication. Immunoprecipitation was performed as described previously (19). For some immunoprecipitations, 20 mM sodium molybdate was added in the lysis buffer to stabilize the interaction between Hsp90 and its substrate eNOS. Immunoprecipitated proteins or cell lysates were mixed with SDS sample buffer and separated by SDS-PAGE.

RNA Isolation and Northern Blot Analysis—Total RNA (2 μg) was prepared as described previously (21). eNOS and 18 S cDNAs were labeled with ^{32}P by random priming and used to hybridize filters. Filters were autoradiographed.

In Vitro Ubiquitylation Reactions—Bacterially expressed eNOS (0.25 μg) was incubated in reactions containing 0.1 μM purified rabbit ubiquitin-activating enzyme (E1) (Calbiochem), 8 μM UBCH5a, 4 μM CHIP, 2.5 mg/ml ubiquitin (Sigma), and 5 mM ATP in 20 mM MOPS (pH 7.2), 100 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride for 2 h at 30 °C. In some reactions, 2 μM Hsp70 and 4 μM Hdj1 or Hdj2 were included. Reactions were stopped with SDS loading buffer, subjected to SDS-PAGE, and then immunoblotted with appropriate antibodies. For ubiquitylation of *in vitro* translated eNOS, eNOS was transcribed and translated with the TnT coupled reticulocyte lysate system (Promega). Following translation, ^{35}S-labeled eNOS was immunoprecipitated with mouse anti-eNOS antibody, and the immunocomplex was immobilized on anti-mouse IgG-agarose conjugate. The washed immobilized complex was used as a substrate in ubiquitin conjugation reactions. Reactions were stopped with SDS loading buffer, separated by SDS-PAGE, and autoradiographed.

Ni-NTA-Agarose Conjugate Pull-down Assay—His-tagged proteins were precipitated following a previously described method (24) as modified. Briefly, 45 h after transfection, COS cells were treated with 2.5 μM MG132 for 2.5 h and lysed in 1 ml of buffer A (6 M guanidinium chloride, 0.1 M Na_2HPO_4/NaH_2PO_4 (pH 8.0), and 10 mM imidazole)/100-mm dish. Lysates were passed through a 26-gauge needle to reduce viscosity and then mixed on a rotator with 15 μl (settled volume) of Ni-NTA-agarose/250 μg of lysates for 2 h at room temperature. The beads were washed three times with 1 ml of buffer A, twice with 1 ml of buffer A diluted in 25 mM Tris-HCl (pH 6.8) and 20 mM imidazole (1:4), and twice with 1 ml of 25 mM Tris-HCl (pH 6.8) and 20 mM imidazole. His-tagged proteins were eluted by boiling beads in 1$\times$ SDS sample buffer supplemented with 100 mM EDTA and then analyzed by immunoblotting.

Cellular Fractionation—Transfected or primary culture cells were lysed in Tris/Triton extraction buffer (100 mM Tris-HCl (pH 7.4), 2% Triton X-100, 1 mM Na_3VO_4, 0.5 mM phenylmethylsulfonyl fluoride, protease inhibitors, and 10 mM EGTA (pH 8.0)) (25, 26), and the cytoskeleton was pelleted by centrifugation at 15,000 $\times$ *g* for 5 min. The supernatant from this low speed centrifugation was centrifuged at 100,000 $\times$ *g* for 5 h at 4 °C in a Beckman SW 60 Ti rotor. The supernatant from this centrifugation was considered the cytosol, and the insoluble pellet was considered the membrane fraction. The pellet was resuspended in 100 μl of Tris/Triton extraction buffer and sonicated until fully dissolved. Fractions were resuspended in SDS sample buffer, and immunoblot analysis was performed.

NOS Activity Assay—NOS activity from transiently transfected COS cell lysates or mouse lung endothelial cells was measured by the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline with the NOS activity kit from Calbiochem-Novabiochem. Briefly, COS cells were transfected as described above and harvested 48 h after transfection. The cells were homogenized in homogenization buffer (25 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 1 mM EGTA) supplemented with protease inhibitors and pelleted at 16,000 $\times$ *g* for 2 min at 4 °C. Both supernatant and pellets were used for eNOS activity determinations (25–50 μg of protein/reaction).

Statistical Analysis—Data were analyzed using a one-way analysis of variance, followed by an unpaired Student's *t* test.

[2] Q. Dai and C. Patterson, submitted for publication.

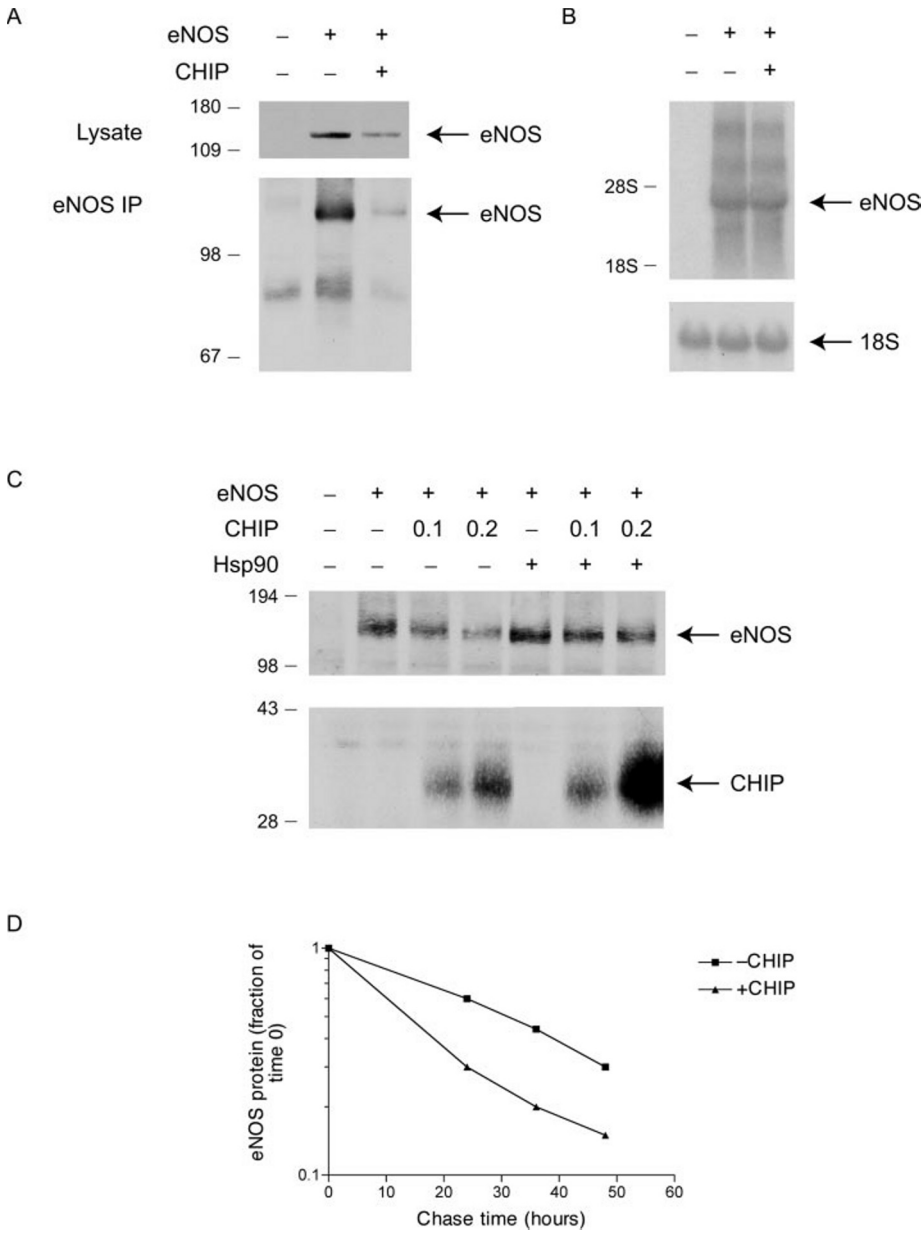

FIG. 1. **Detergent-soluble eNOS protein levels are decreased by CHIP.** *A*, COS-7 cells were transiently transfected with eNOS with or without CHIP expression plasmids as indicated. Detergent-soluble fractions of lysate and eNOS immunoprecipitations (*IP*) were subjected to Western blotting with anti-eNOS antibody. *B*, COS-7 cells were transfected as described for *A*, and Northern blots were probed with a [^{32}P]dCTP-labeled eNOS probe. *C*, COS-7 cells were transiently transfected with eNOS and different amounts of CHIP plasmids with or without Hsp90 as indicated. Detergent-soluble fractions of lysates were immunoblotted for eNOS (*upper panel*) and CHIP (*lower panel*). *D*, COS-7 cells transfected with eNOS with or without CHIP were metabolically labeled with 50 μCi/ml [^{35}S]methionine for 20 min and chased at different time points as indicated. eNOS was immunoprecipitated from the detergent-soluble fraction of lysate and identified by SDS-PAGE, followed by autoradiography.

RESULTS

Detergent-soluble eNOS Protein Levels Are Decreased by CHIP in Transfected COS Cells—To test the hypothesis that CHIP regulates eNOS through an Hsp90-dependent mechanism, we first examined whether CHIP has effects on eNOS expression in COS cells. COS cells are a good system for this purpose because previous studies have shown that exogenously expressed eNOS in COS cells behaves similarly to endogenous eNOS with respect to intracellular trafficking and activation (3). COS cells (which do not express NOS) were cotransfected with eNOS and CHIP, and the expression of eNOS protein was measured by Western blotting. Detergent-soluble eNOS levels in both cell lysates and immunoprecipitates were markedly reduced in cells overexpressing CHIP (Fig. 1*A*), whereas eNOS mRNA expression was not altered (Fig. 1*B*). The effects of CHIP were dose-dependent; with increasing amounts of CHIP, eNOS levels decreased further (Fig. 1*C*).

Kinetics of Detergent-soluble eNOS Stability Is Altered by CHIP—Having shown previously that CHIP targets chaperone substrates for ubiquitylation and rapid degradation *in vivo* (15, 16, 18), we examined whether the stability of eNOS is altered in the presence of increased levels of CHIP using pulse-chase experiments. We cotransfected eNOS with or without CHIP, pulse-labeled the cells with [^{35}S]methionine, and harvested at different time points. The detergent-soluble fraction of the lysate was immunoprecipitated with an antibody recognizing eNOS. At time 0, similar levels of eNOS were present in control cells and in cells that overexpressed CHIP. An increased rate of eNOS disappearance was observed in cells that overexpressed CHIP (Fig. 1*D*), suggesting either that CHIP induced an accelerated rate of eNOS degradation or that CHIP caused eNOS redistribution into a detergent-insoluble pool.

Effect of CHIP on eNOS Expression Is Hsp90-dependent—To determine whether CHIP elicits its effect through Hsp90-dependent mechanisms, we overexpressed Hsp90 in transfected cells. These studies were designed to test whether an increase in the cellular ratio of Hsp90 to CHIP would be able to rescue eNOS expression, as should be the case if this event is mediated through interactions between CHIP and eNOS·Hsp90 complexes. Cotransfection of Hsp90 with CHIP blocked the ability of CHIP to decrease eNOS levels in a dose-dependent manner (Fig. 1*B*). In addition, we compared the effects of CHIP

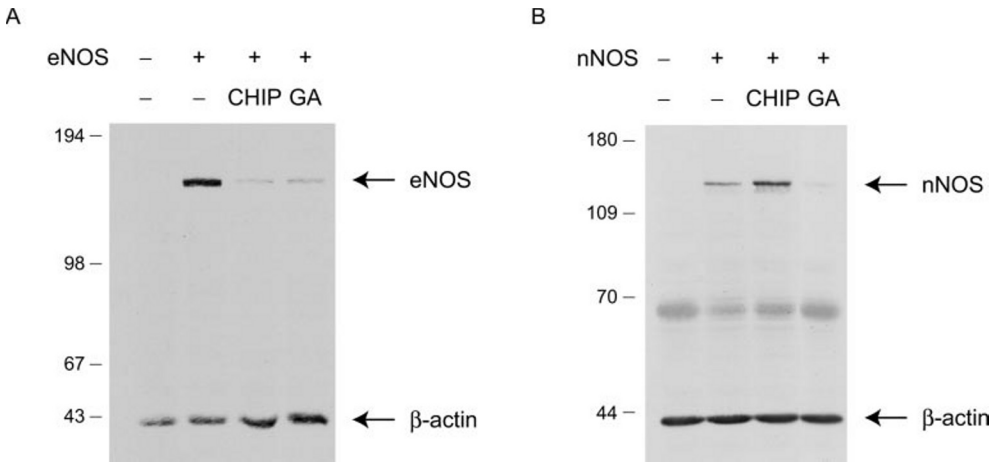

FIG. 2. **Differential effects of CHIP and GA on NOS isoforms.** COS-7 cells were transfected with eNOS (*A*) or nNOS (*B*) with or without CHIP or pretreatment with 2 μM GA for 16 h. Detergent-soluble lysates were immunoblotted for the respective NOS proteins and for endogenously expressed β-actin.

with those of GA, a specific inhibitor of Hsp90, on eNOS levels. Both CHIP and GA caused similar decreases in eNOS protein levels (Fig. 2*A*), whereas CHIP increased nNOS levels and GA decreased nNOS levels (Fig. 2*B*). Having shown previously that CHIP interacts with the TPR acceptor site of Hsp90 through the CHIP N-terminal TPR domain (15), we examined the requirement of this domain for CHIP action. Deletion of the TPR domain (ΔTPR) markedly reduced the effects of CHIP on eNOS (Fig. 3, *left panel*), suggesting that the effects of CHIP are TPR domain- and most likely Hsp90-dependent. Several lines of evidence suggested to us that decreased eNOS expression by CHIP is likely mediated through its inhibition of Hsp90 function: 1) increasing Hsp90 overcomes the effect of CHIP, and 2) CHIP action is dependent on its ability to interact with Hsp90 (*i.e.* it is TPR domain-dependent).

To test the specificity of this effect, we examined the activity of CHIP on other NOS isoforms. The effects of CHIP on soluble eNOS and iNOS levels were similar (Fig. 3). In contrast, we noticed a consistent increase in nNOS protein levels in cells overexpressing CHIP (Fig. 3, *right panel*), the consequence and mechanisms of which are unclear at present. We found as well that endogenous β-actin expression was unaffected by CHIP (Fig. 3). Together, these data demonstrate a surprising specificity of the effects of CHIP on NOS isoforms. We therefore explored the mechanisms underlying the effects of CHIP on steady-state eNOS levels in more detail.

CHIP Interacts with eNOS and Hsp90—Previous studies have shown that Hsp90 interacts with eNOS and activates eNOS activity (3). If CHIP directly modulates eNOS expression through the chaperone activity of Hsp90, then we should be able to detect stable interactions between CHIP and eNOS. We examined interactions between these proteins by co-immunoprecipitation in COS cells cotransfected with Myc-tagged CHIP (which enabled us to measure only ectopically expressed CHIP) and eNOS and harvested cells with a buffer containing molybdate (which mimics nucleotide binding and stabilizes the association of Hsp90 and its client proteins (27)). Although eNOS levels were decreased in CHIP-expressing cells, CHIP was still present in a stable complex with eNOS and Hsp90 (Fig. 4, *A* and *B*). These data indicate the likely formation of a ternary complex containing these three proteins. Binding domains of eNOS and Hsp90 have recently been mapped to the oxygenase domain of eNOS and the M domain of Hsp90 (which is far away from the C-terminal CHIP-binding site) (8). To further characterize the spatial arrangement among these three proteins, we conducted the same experiment with a molybdate-free buffer. Association of Hsp90 and its substrate eNOS could not be detected with the molybdate-free buffer. Although the interaction between eNOS and CHIP was abrogated, Hsp90 could still bind CHIP (Fig. 4*C*). These results indicate that the association of CHIP and eNOS is probably not direct, but is instead mediated by Hsp90 (Fig. 4*D*).

eNOS Is Not a Target for CHIP-mediated Ubiquitylation—CHIP is a U-box-dependent E3 ligase (18–20) that directly participates in the ubiquitylation of chaperone substrates such as GR, CFTR, and ErbB-2 and their transfer to the proteasome for degradation (15–17). Ubiquitylation of GR and CFTR can be recapitulated by an *in vitro* reconstitution reaction with purified components of the ubiquitin system. In addition to the ΔTPR mutant, we noted that a deletion mutant lacking the U-box domain (ΔU), a domain first identified in the yeast protein Ufd2 and known to facilitate protein polyubiquitylation (28), also inhibited the effects of CHIP on eNOS expression (Fig. 3, *left panel*). To determine whether CHIP modulates eNOS expression through mechanisms similar to those observed with GR and CFTR, we tested the ability of CHIP to elicit ubiquitylation of eNOS in an *in vitro* ubiquitin conjugation system. Reactions were carried out with recombinant proteins, including rabbit E1, UBCH5a (a collaborating ubiquitin-conjugating enzyme for CHIP) (19), and CHIP; and the capacity of these components to mediate ubiquitylation of bacterially expressed eNOS in the presence of purified ubiquitin and ATP was examined. We found that CHIP did not increase ubiquitylated high molecular mass forms of eNOS in the presence of E1, ubiquitin carrier protein (E2), and ubiquitin (Fig. 5*A*, *first* and *second lanes*).

The requirement of chaperones for ubiquitylation of some chaperone substrates has been reported (29, 30). To test whether this is the reason why we failed to detect ubiquitylation of eNOS, recombinant Hsp70 was included in the reactions with or without its co-chaperone Hdj1 or Hdj2, the mammalian Hsp40 homologs. In contrast to the observation of CHIP-mediated Hsp70 ubiquitylation, no obvious increase in eNOS ubiquitylation was observed in the same reaction (Fig. 5*A*, *third* through *fifth lanes*), although these conditions efficiently increased the ubiquitylation of other CHIP substrates (data not shown). Because the above reactions did not include Hsp90, we used eNOS generated in reticulocyte lysate (which has sufficient quantities of Hsp90 heterocomplex components (31)) as a source of substrate to test the ability of CHIP to ubiquitylate eNOS. With an approach similar to that used for characterizing CHIP-mediated ubiquitylation of GR (15), we incubated eNOS with CHIP and ubiquitin after its *in vitro* transcription and

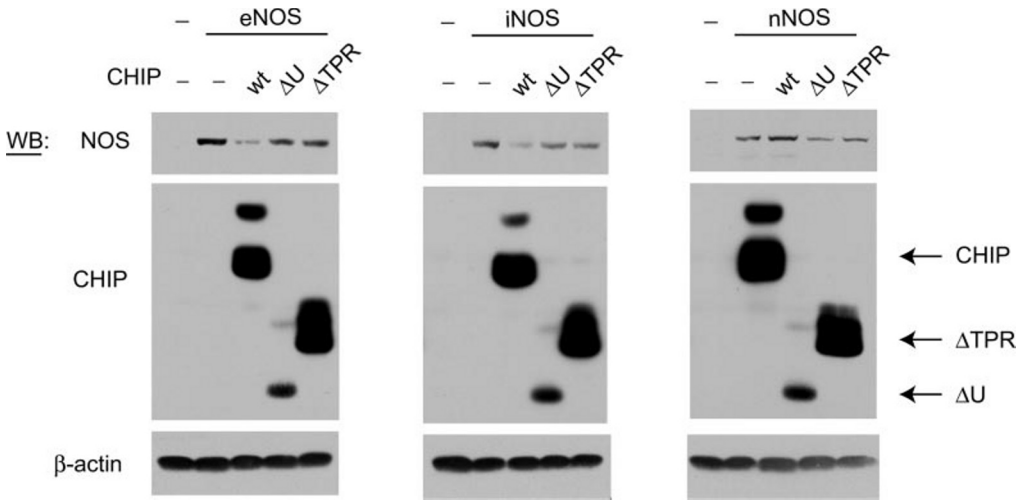

FIG. 3. **Effects of CHIP require both the TPR and U-box domains of CHIP, and CHIP shows different effects on different NOS isoforms.** COS-7 cells were transfected with eNOS (*left panels*), iNOS (*middle panels*), or nNOS (*right panels*) expression plasmids with or without the wild type or deletion mutants lacking the TPR (residues 1–145; ΔTPR) or U-box (residues 196–303; ΔU) domain of CHIP. Western blots (*WB*) were probed with antibodies to the respective NOSs (*upper panels*), to CHIP (*middle panels*), or to endogenously expressed β-actin (*lower panels*).

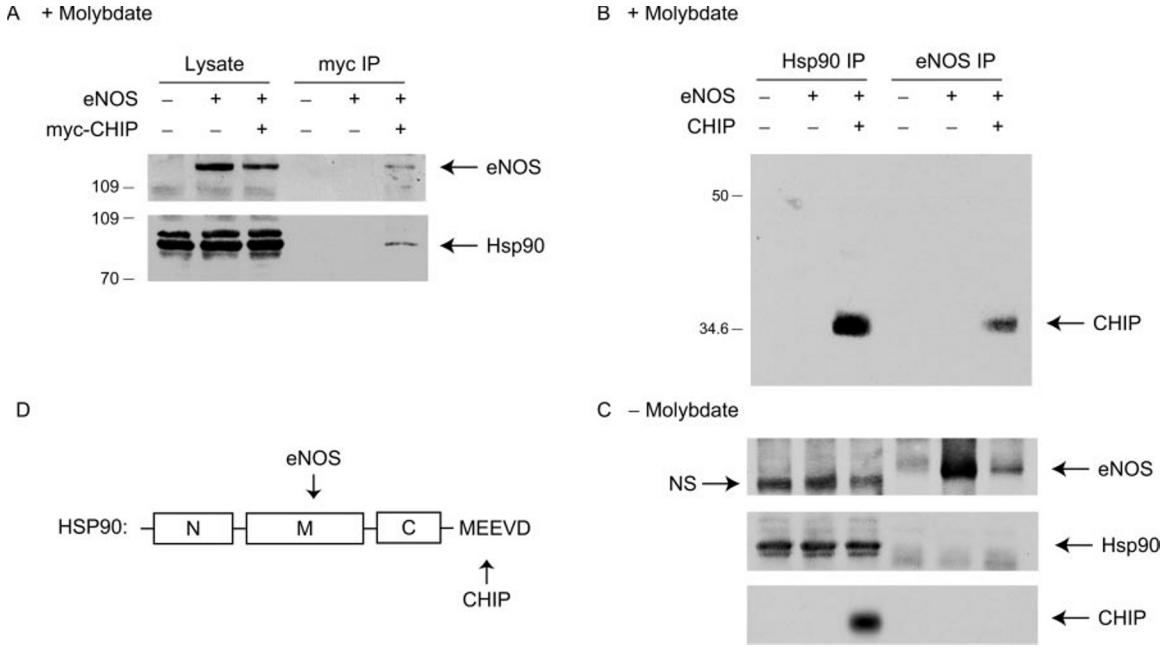

FIG. 4. **Formation of a ternary CHIP·eNOS·Hsp90 complex.** COS-7 cells were transfected with eNOS with or without Myc-tagged CHIP (*A*) or non-tagged CHIP (*B*). Detergent-soluble lysates were immunoprecipitated (*IP*) with anti-Myc, anti-Hsp90, or anti-eNOS antibody and blotted for eNOS, Hsp90, and CHIP, respectively. In *C*, COS-7 cells were transfected and immunoprecipitated as described for *B*, except that lysates were harvested in a molybdate-free buffer. A putative model of the ternary complex is shown in *D*. Hsp90 scaffolds the interaction between eNOS and CHIP. *NS*, nonspecific. *M*, middle.

translation of eNOS in reticulocyte lysate (which contains hemin to inhibit the proteasome). Low levels of eNOS ubiquitylation were observed after translation; but again, we did not observe any augmentation with CHIP (Fig. 5*B*).

We utilized an established assay for the detection of ubiquitylated proteins (32) to further examine whether CHIP ubiquitylates eNOS *in vivo*. COS cells were transfected with eNOS plus His_6- or hemagglutinin-tagged ubiquitin (serving as a control) with or without CHIP. Cell extracts were prepared under denaturing conditions (6 M guanidinium chloride) and purified on Ni-NTA beads. Ubiquitylated proteins represent intermediates of degradation and are thus both rare and labile. Denaturing conditions were used to prevent degradation or de-ubiquitylation of protein-ubiquitin conjugates as well as any noncovalent protein-protein interactions. Because the interaction of His_6 sequences with the NTA affinity resin is not impeded by the presence of guanidinium chloride, tagged ubiquitin and its modified derivatives could be directly purified from the lysates by NTA chromatography. Treating cells with GA increased eNOS ubiquitylation in the presence of a proteasome inhibitor (Fig. 5*C*, *sixth lane*). However, contrary to our expectation, we found no increase (in fact, a decrease) in ubiquitin-modified forms of eNOS by CHIP under the same conditions (*fifth lane*), although reprobing the same blot with ubiquitin revealed an increase in overall ubiquitylated forms of proteins in cells overexpressing CHIP (which is consistent with our previous observations (19)) (Fig. 5*C*). We observed similar results in COS cells overexpressing Myc-tagged ubiquitin, harvested with nondenaturing buffer, and detected by immunoprecipitation (data not shown).

Proteasome-dependent Protein Degradation Is Not Required for Regulation of eNOS by CHIP—CHIP promotes the protea-

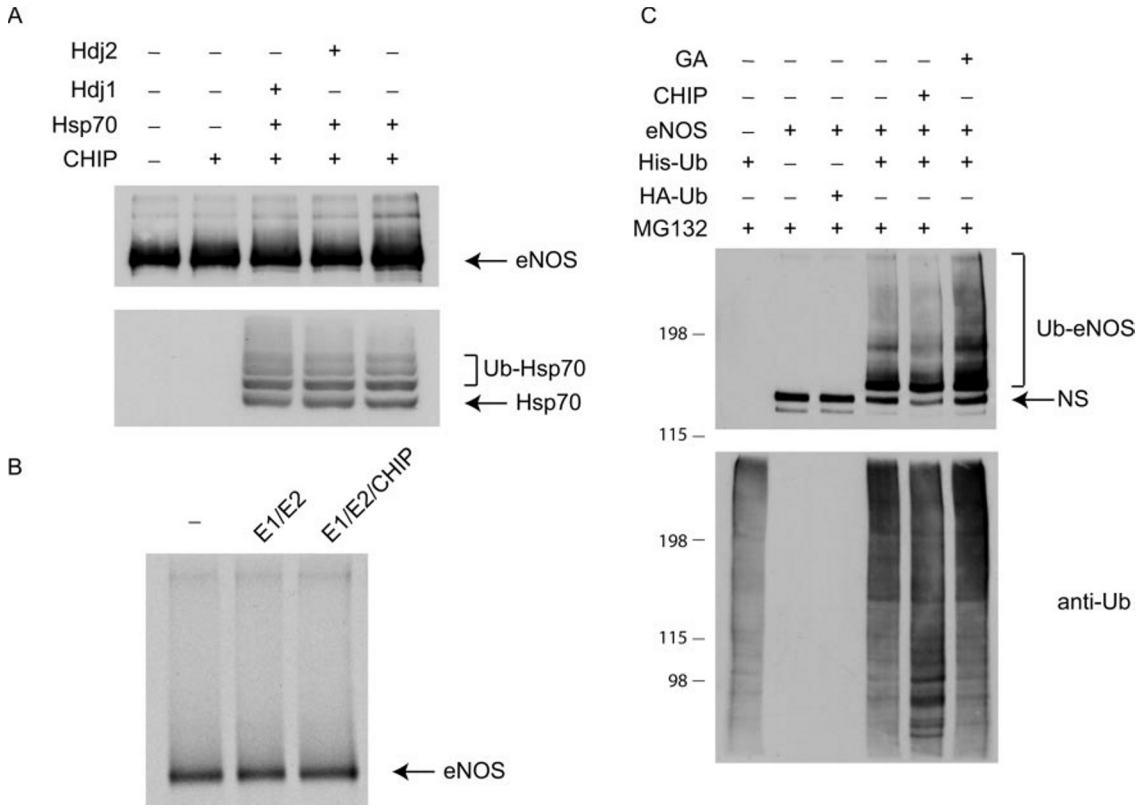

FIG. 5. **CHIP does not increase eNOS ubiquitylation.** *A*, *in vitro* ubiquitylation reactions were performed with purified proteins. Purified ubiquitin, E1, the E2 UBCH5a, and eNOS were present in each reaction. In some reactions, Hsp70, Hdj1, or Hdj2 was included as indicated. Following SDS-PAGE, blots were probed with anti-eNOS and anti-Hsp70 antibodies. *B*, *in vitro* ubiquitin conjugations were performed with *in vitro* transcribed and translated ^{35}S-labeled eNOS. ^{35}S-Labeled eNOS was synthesized in reticulocyte lysate, followed by immunoprecipitation with anti-eNOS antibody; and ubiquitin conjugation was performed with ^{35}S-labeled eNOS, purified E1, or the E2 UBCH5a with or without CHIP. Reactions were resolved by SDS-PAGE, followed by autoradiography. *C*, COS-7 cells were transiently transfected with eNOS, His-tagged ubiquitin (*Ub*), or hemagglutinin-tagged ubiquitin (as a control) with or without CHIP or pretreatment with 2 μM GA for 16 h as indicated. Two days after transfection, cells were incubated with 2.5 μM MG132 for 2.5 h and harvested with a denaturing buffer. His-tagged ubiquitylated forms of eNOS were precipitated with Ni-NTA-agarose, resolved by SDS-PAGE, and detected with anti-eNOS antibody (*upper panel*). The same blot was stripped and reprobed with anti-ubiquitin antibody (*lower panel*). *NS*, nonspecific.

some-dependent degradation of chaperone substrates such as GR and CFTR (15, 16, 18). To determine whether this mechanism could be generalized to the Hsp90 substrate eNOS, we examined the requirement of the proteasome pathway for CHIP actions on eNOS. Because it is well accepted that proteasome inhibitors block proteasome-mediated proteolysis and that they lead to an accumulation of proteins metabolized by this pathway, we utilized selective proteasome inhibitors to test this hypothesis. Proteasome inhibitors should be able to restore eNOS expression decreased by CHIP if the proteasome pathway is involved. Treating transfected COS cells with the proteasome inhibitors lactacystin, MG132, and proteasome inhibitor I for 2.5 h did not result in restoration of eNOS expression in cells cotransfected with CHIP, even though a slowly migrating smear of high molecular mass ubiquitylated proteins was more abundant in treated cells (indicating that the inhibitors were functional) (Fig. 6*A*). Because eNOS has a relatively long half-life of 15–20 h (33), it is possible that this treatment (2.5 h) was not long enough. We therefore treated cells for a longer time; but again, a 12-h treatment did not rescue the effect of CHIP on eNOS expression (Fig. 6*B*). In addition, inhibitors of the other two major cellular protein degradation systems, the lysosome and calcium-dependent calpain proteases, were tested, and neither of these blocked the effects of CHIP on eNOS (data not shown). Our results indicate that the degradation of eNOS does not account for the effects of CHIP observed in our preceding studies.

Taken together, multiple lines of evidence demonstrate that, although disruption of Hsp90 function by GA induces eNOS ubiquitylation, the Hsp90-interacting CHIP is not the E3 ligase responsible for this event, indicating that other chaperone-associated E3 ligases exist and are responsible for eNOS ubiquitylation. In addition, the effects of CHIP on eNOS do not involve ubiquitin-dependent degradation, suggesting that CHIP has previously unsuspected effects on protein trafficking and maturation.

CHIP Alters Compartmental Partitioning of eNOS—The aforementioned results do not support our initial hypothesis that CHIP inhibits eNOS maturation and promotes eNOS degradation via the ubiquitin-proteasome system. CHIP is a co-chaperone of Hsp70 that inhibits most of the chaperoning activities of Hsp70 (including refolding activity) (21). In addition, CHIP interacts with Hsp90 and prevents the assembly of an Hsp90 chaperone complex required for protein folding and maturation (15). Based on this knowledge, we proposed alternatively that CHIP inhibits chaperone-mediated protein refolding of eNOS *in vivo* and instead diverts it to an insoluble and inactive state, perhaps through deviations in trafficking that are known to be required for eNOS activity (1, 2). To test this hypothesis, transfected COS cells were fractionated into membrane (high speed pellet), soluble, and insoluble (low speed pellet) fractions, and eNOS protein levels in each fraction were measured by Western blotting (Fig. 7*A*). In cells transfected with eNOS, eNOS was found primarily in the high speed pellet (*fifth lane*) relative to the low speed pellet (*second lane*) and soluble fraction (*eighth lane*), consistent with the known tight

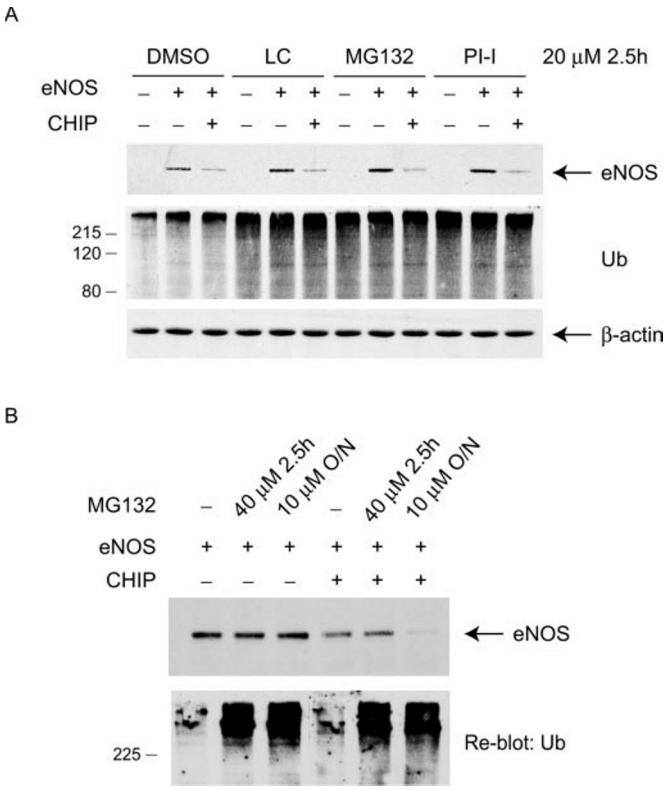

FIG. 6. **Proteasome inhibitors do not block the effects of CHIP on eNOS expression.** *A*, COS-7 cells were transfected with eNOS with or without CHIP and incubated with a proteasome inhibitor (20 μM lactacystin (*LC*), MG132, or proteasome inhibitor I (*PI-I*)) or vehicle (dimethyl sulfoxide (*DMSO*)) for 2.5 h. *B*, alternatively, transfected cells were incubated with MG132 under different conditions as indicated. Detergent-soluble fractions of lysates were subjected to Western blotting for eNOS, ubiquitin (*Ub*), and β-actin. *O/N*, overnight.

membrane association of this protein. Cotransfection of eNOS with CHIP resulted in the redistribution of eNOS from membrane and soluble pools to the insoluble pool (note the increase in eNOS levels in the *third lane* and the decrease in the *sixth* and *ninth lanes* in CHIP-transfected cells). The effects of CHIP were specific, as the cellular distribution of endogenous Hsp70 and Hsp90 was not changed by CHIP (Fig. 7*A*). In addition, the cellular localization of neither iNOS nor nNOS was modified by CHIP; CHIP decreased iNOS and increased nNOS protein levels in all three fractions, consistent with its overall effects on these NOS isoforms (Fig. 7*B*). Despite the ability of CHIP to direct eNOS into a detergent-insoluble fraction, eNOS activity in this fraction was not increased; in fact, a decrease in the apparent specific activity was observed (Fig. 7*C*), thus indicating that CHIP caused accumulation of inactive eNOS in the detergent-insoluble fraction. Interestingly, we also noted that the cellular localizations of Hsp70 and Hsp90 were different. Hsp90 was limited to membrane and soluble pools, whereas Hsp70 was present in all three fractions. (The differential cellular localization of Hsp90 and Hsp70 may correlate with their different cellular functions.) The deficiency of Hsp90 in the detergent-insoluble fraction where eNOS accumulated suggests that the interaction between Hsp90 and eNOS was disrupted by CHIP.

CHIP Disrupts Golgi Trafficking of eNOS—To gain more insight into the effects of CHIP on eNOS, we examined the consequences of altering CHIP expression on endogenous eNOS localization in bovine aortic endothelial cells. As expected, a major fraction of eNOS (*green* filter, Fig. 8, *upper row*) co-localized with GM130 (*red* filter), a resident protein of the Golgi apparatus, in the perinuclear region (merge is *yellow*).

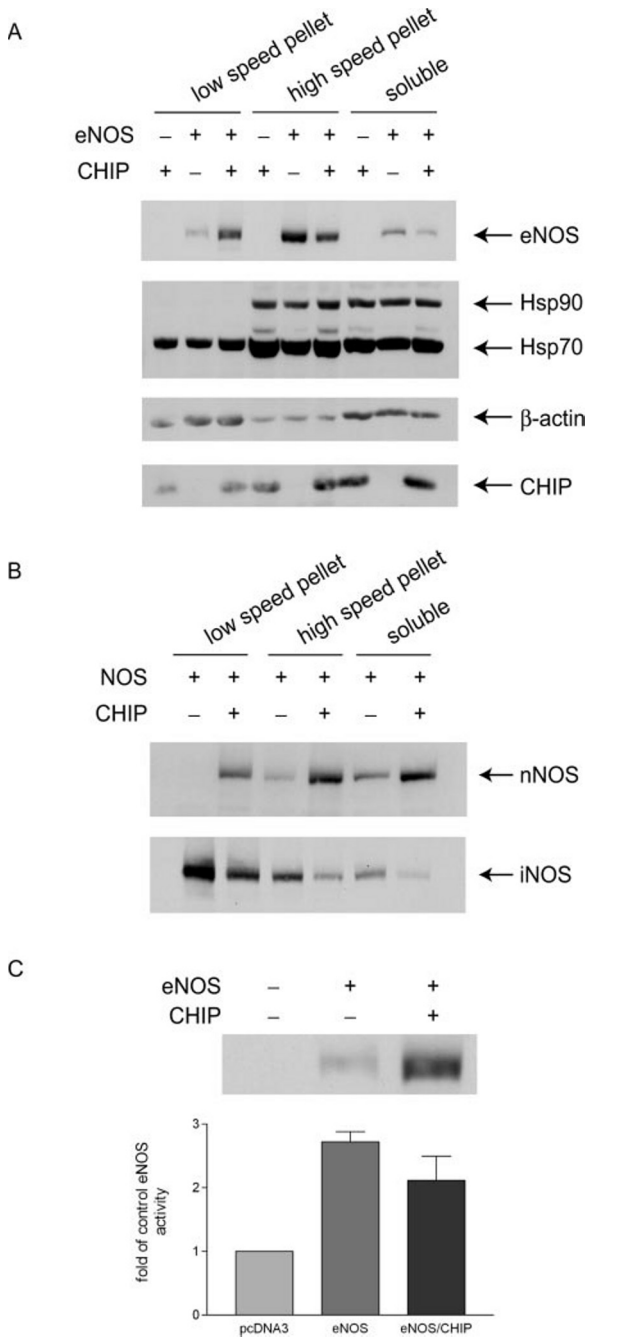

FIG. 7. **Accumulation of inactive detergent-resistant eNOS by CHIP.** COS cells were transfected with eNOS (*A*) or with nNOS or iNOS (*B*) with or without CHIP. Lysates were fractionated as described under "Experimental Procedures" and resolved by SDS-PAGE. Expression of NOS in each fraction was measured with the corresponding anti-NOS antibodies (*A*, *upper panel*; and *B*). The same membrane blotted for eNOS was reprobed with anti-Hsp90, anti-Hsp70, anti-β-actin, and anti-CHIP antibodies (*A*). In *C*, COS cells were transiently transfected as indicated. eNOS protein levels and activity in the detergent-insoluble fractions of lysates were determined by Western blotting and NOS activity assays, respectively. Data of the eNOS activity assay are means ± S.E. (n = 4).

Infection of cells with an adenovirus expressing red fluorescent protein (*middle row*, showing red fluorescent protein-expressing cells) did not influence the distribution of either eNOS (*green* filter, *left panel*) or GM130 (*green* filter, *right panel*). However, in cells infected with an adenovirus expressing CHIP (*lower row*), eNOS was redistributed from the perinuclear location to discrete foci within the cytoplasm (*left panel*; eNOS is *green*, and CHIP is *red*). Remarkably, the peripheral mem-

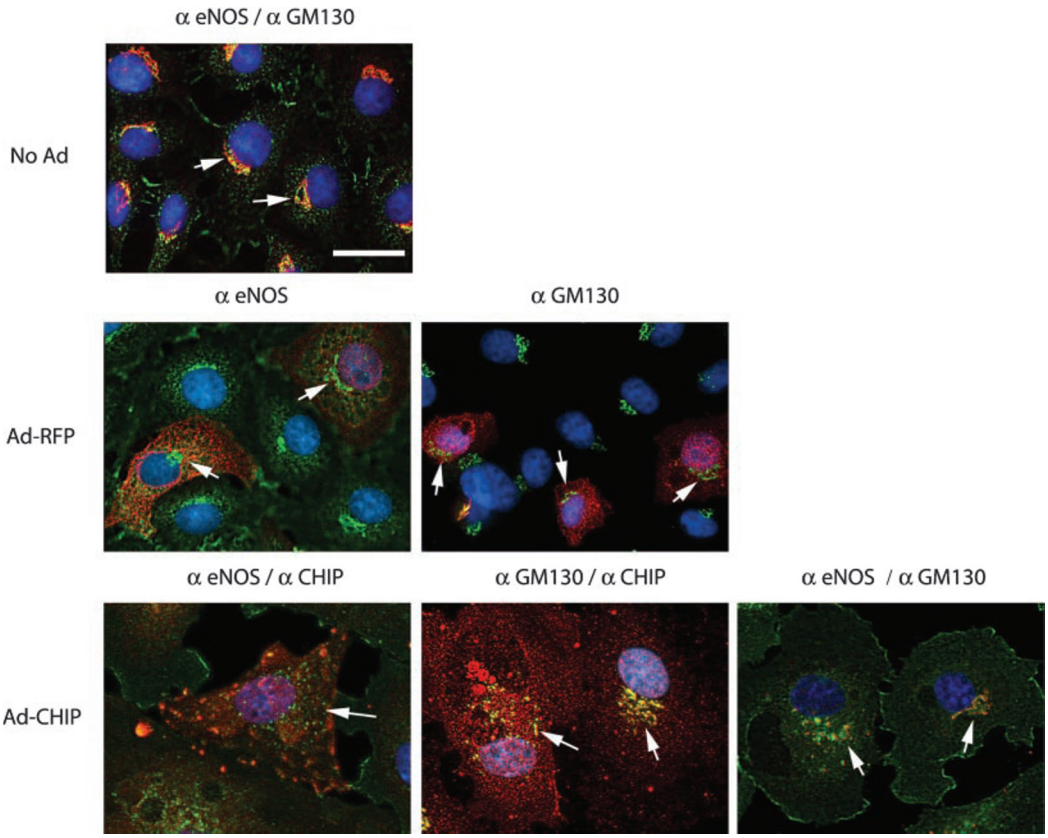

FIG. 8. **Displacement of eNOS from the Golgi by CHIP in endothelial cells.** In the *upper row*, bovine aortic endothelial cells were fixed and immunolabeled with an antibody that recognizes eNOS (fluorescein isothiocyanate-labeled secondary antibody) or GM130 (rhodamine-labeled secondary antibody), and the merged image is shown. The merge of the two colors to *yellow* and *arrows* depict co-localization of eNOS and GM130. In the *middle row*, bovine aortic endothelial cells were infected with an adenovirus-expressing red fluorescent protein (*Ad-RFP*) and labeled for eNOS (*left panel*) or GM130 (*right panel*) decorated with fluorescein isothiocyanate-labeled secondary antibody. *Arrows* depict intact perinuclear eNOS and GM130 in the cells infected with an adenovirus-expressing red fluorescent protein (*red*). In the *lower row*, bovine aortic endothelial cells were infected with an adenovirus expressing CHIP, and eNOS/CHIP, GM130/CHIP, and eNOS/GM130 co-localization was examined. The presence of CHIP caused the redistribution of eNOS (*left panel*) and GM130 (*middle panel*). The *right panel* demonstrates the merged image showing co-labeling for eNOS (fluorescein isothiocyanate) and GM130 (rhodamine). Data are representative of at least seven individual experiments.

brane Golgi protein GM130 was similarly redistributed (*middle panel*; GM130 is *green*, and CHIP is *red*), and eNOS and GM130 merged in the cytoplasm (*right panel*; eNOS is *green*, and CHIP is *red*, with *yellow* reflecting the merged images). These results indicate that CHIP impairs the Golgi targeting of eNOS, which accounts for its redistribution in an insoluble fraction and its lower specific activity. In addition, these observations suggest that the chaperone system may play a generalized and unanticipated role in maintenance of Golgi integrity and the trafficking of peripheral membrane proteins.

Deficiency of CHIP in Mouse Lung Endothelial Cells Increases Membrane-bound eNOS and Enhances eNOS Activity—To firmly establish the importance of the observations of CHIP-dependent eNOS regulation, we isolated lung endothelial cells from adult CHIP$^{-/-}$ mice (created by homologous recombination of embryonic stem cells)[2] and similar cells from wild-type littermates to determine eNOS localization and activity. In CHIP$^{-/-}$ cells, increased levels of eNOS were found in the soluble and membrane-associated (high speed pellet) fractions, and slightly lower levels were present in the insoluble (low speed pellet) fraction (Fig. 9*A*). Consistent with this observation of increased translocation of eNOS to the membrane compartment in the absence of CHIP, we found that both basal and vascular endothelial growth factor-induced eNOS activities were increased by ~2-fold in CHIP$^{-/-}$ endothelial cells (Fig. 9*B*). A 2-fold increase in eNOS activity in CHIP$^{-/-}$ endothelial cells is likely to exert a profound physiological effect on function because the dose-response relationship to NO in intact blood vessels is very steep, with small changes in NO levels exerting great changes on vasomotion (34). These observations provide further support for a model in which CHIP regulates eNOS partitioning under physiological conditions and indicate that eNOS is more efficiently translocated to the membrane compartment, where it is maximally active, in the absence of CHIP. Additionally, because the proper subcellular localization of eNOS is critical for activation by mechanical forces and growth factors (6, 35) and subsequent eNOS phosphorylation (36), the levels or activity of CHIP may exert an unappreciated control mechanism for the partitioning/trafficking of eNOS to the Golgi and plasma membrane.

DISCUSSION

Previous studies have shown that CHIP is a U-box-dependent E3 ligase that interacts with both Hsp70 and Hsp90 and promotes ubiquitylation of some chaperone substrates in a chaperone-dependent fashion (15, 16, 18, 19). We found that inhibition of Hsp90 function by GA, a specific Hsp90 inhibitor, increases ubiquitylation of another Hsp90 client protein, eNOS. These results indicate the presence of Hsp90-mediated pathways that regulate eNOS stability; and therefore, it is reasonable to speculate that the Hsp90-binding ubiquitin ligase CHIP might be involved in the regulation of eNOS degradation. To our surprise, although we observed a decrease in eNOS protein levels by CHIP, we demonstrated through several lines of evidence, contrary to our expectation, that this phenomenon is not due to increasing ubiquitin-mediated deg-

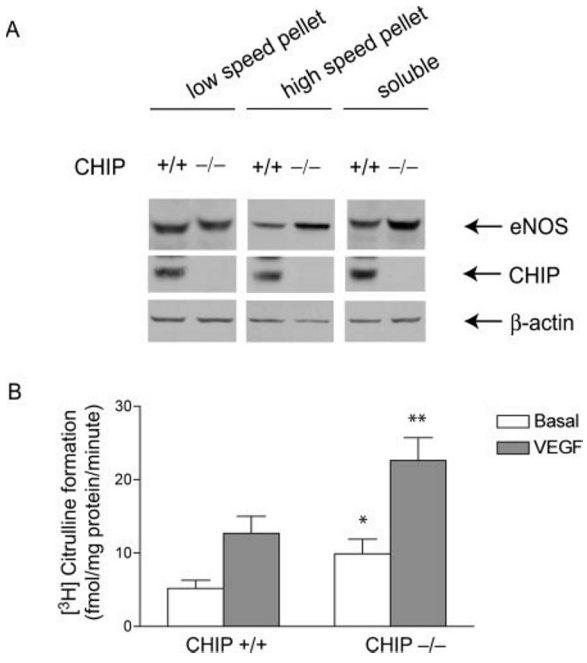

FIG. 9. **eNOS localization and activity in mouse lung endothelial cells.** *A*, cell lysates from $CHIP^{+/+}$ and $CHIP^{-/-}$ murine lung endothelial cells were separated into the indicated fractions and probed for eNOS, CHIP, and β-actin by Western blotting. *B*, NOS activity was determined in total cell lysates from $CHIP^{+/+}$ and $CHIP^{-/-}$ murine lung endothelial cells before and after stimulation with vascular endothelial growth factor (*VEGF*; 20 ng/ml). Data are expressed as means ± S.E. ($n = 6$). *, $p < 0.05$ compared with basal levels from $CHIP^{+/+}$ cells; **, $p < 0.05$ compared with vascular endothelial growth factor-inducible levels from $CHIP^{+/+}$ cells.

radation of eNOS. CHIP does not elicit eNOS ubiquitylation, but rather causes eNOS to redistribute into an inactive detergent-insoluble pool by preventing eNOS targeting or by displacing eNOS from the Golgi.

It is now well established that molecular chaperones play essential roles in protein quality control not only by aiding protein folding and refolding, but also by directly contributing to protein degradation when proteins are no longer needed or permanently damaged (37). Disruption of Hsp90 function by GA induces ubiquitylation and proteasome-dependent degradation of several Hsp90 client proteins, including the steroid hormone GR, the plasma membrane chloride channel CFTR (38), the serine/threonine kinase Raf-1 (39), the receptor tyrosine kinase ErbB-2 (40), and the basic helix-loop-helix/PAS family transcription factor hypoxia-inducible factor-1α (41), indicating the involvement of molecular chaperones in their ubiquitin-mediated degradation. These studies also imply potential roles for CHIP, the chaperone-dependent ubiquitin ligase, in these events. Interaction of CHIP with molecular chaperones appears to enable CHIP to gain access to a broad spectrum of chaperone-bound substrates for subsequent ubiquitylation and targeting to the proteasome for degradation. This raises the question of whether CHIP ubiquitylates substrates captured by chaperones in a general manner, or if this is otherwise a selective process. CHIP promotes polyubiquitin chain formation on some of these Hsp90 clients, such as GR (15), CFTR (16), Raf-1 (18), and ErbB-2 (17), which leads to their subsequent degradation by the proteasome. In contrast, eNOS is not a target for the ubiquitin ligase activity of CHIP. Taken together with our data, these results suggest that CHIP does not generally ubiquitylate all substrates presented by the molecular chaperones. Given the limited substrate set presently known, it is difficult to speculate on common structural features that characterize CHIP substrates. Nevertheless, the present data showing the selectivity of CHIP for misfolded, abnormal, and/or mutant proteins (16, 17, 20) indicate that the conformation and folding state of these proteins may serve as recognition signals for CHIP. Given this likelihood, it is of interest to note that the allosteric activation (3) and scaffold effect (8) of Hsp90 on eNOS suggest that it is the eNOS dimer that interacts with Hsp90. This conformation of Hsp90-bound eNOS is relatively stable and thus might not be recognized by the E3 ligase CHIP for ubiquitylation. Such a model would explain the ubiquitylation-independent effects of CHIP observed in our studies. In addition, the observation that the Hsp90 inhibitor GA induced the ubiquitylation of eNOS (Fig. 5*C*), whereas CHIP did not, argues strongly in favor of the existence of another chaperone-associated ubiquitin ligase in addition to CHIP.

Our observations that CHIP modulated eNOS partitioning within cells are, to our knowledge, a novel co-chaperone-dependent mechanism through which eNOS may be regulated. That Hsp90 was not present in the detergent-insoluble fraction is informative regarding the mechanism through which CHIP acts. It is possible that CHIP, via its ability to interact with and to modify the function of the molecular chaperone Hsp90, causes a conformational change in Hsp90 that dissociates plasma membrane-associated eNOS from Hsp90 (3) and that Hsp90-free eNOS becomes unstable, inactive, and perhaps prone to aggregation. Alternatively, CHIP may interfere with chaperone-mediated solubility of eNOS during biosynthesis from its cytoplasmic state (*i.e.* during co-translational myristoylation and post-translational palmitoylation) to its final destination on the Golgi and plasma membrane (1, 2, 36). It is even possible that CHIP may interact with eNOS after eNOS has been inserted into the Golgi because eNOS is targeted to the cytoplasmic face of the Golgi and is therefore accessible to CHIP at all times during its biosynthesis and activation (6). This latter observation is particularly notable, as increased levels of CHIP appear to elicit a global disassembly of the Golgi complex, based on the re-localization of GM130, a well characterized peripheral membrane Golgi marker. This effect does not seem to be due to a general inhibition of protein processing pathways because similar increased levels of CHIP do not generally alter endoplasmic reticulum structure or processing of the T-cell receptor, which requires the integrity of the endoplasmic reticulum.[3] In addition, the folding of some proteins is actually enhanced when CHIP levels are elevated (42). To date, there are few indications of a role for molecular chaperones in Golgi function or integrity. It is possible that CHIP participates in the regulation of Golgi function, either directly or (more likely) through interactions with chaperones, although this model will need to be tested in further studies.

In any event, the contrast between the effects of CHIP on eNOS and nNOS is striking. Although eNOS and nNOS isoforms share very similar primary structures, functional domains, and biochemical features, CHIP exhibits totally opposite effects on them. The significance and mechanisms by which CHIP increases nNOS protein expression are unknown at present. The primary structure of nNOS is very similar to that of eNOS except for a distinct N-terminal 220-amino acid leader sequence (43). The nNOS leader sequence contains a PDZ domain-binding motif that interacts with several proteins that may target the enzyme to signal transduction hot spots within cells. It also contains a binding site for a highly conserved and widely expressed protein, PIN (protein inhibitor of NO synthases), which, when bound to nNOS, appears to destabilize its dimeric structure (44). nNOS is an Hsp90 substrate as well. Disruption of Hsp90 by GA increases nNOS turnover (45). The

[3] D. Cyr, unpublished data.

effect of CHIP on nNOS is different from that of GA, implying an Hsp90-independent effect. Therefore, it is reasonable to postulate that with respect to its effects on steady-state nNOS levels, the direct target of CHIP might be some protein other than nNOS itself, and the most likely candidates lie in unique nNOS-interacting proteins that affect nNOS protein stability.

Structurally, both the TPR and U-box domains of CHIP are required for its effects on eNOS distribution (Fig. 3). That the TPR domain is required is not surprising because this domain is required for the interaction of CHIP with Hsp90 (15). The requirement of the U-box in this process is less clear, as CHIP does not induce ubiquitylation of eNOS. It is possible that the ubiquitin ligase activity is required for ubiquitylation of a protein or proteins other than eNOS and that this contributes in part to the redistribution of eNOS. Alternatively, it is possible that the U-box has functions (in addition to its ubiquitin ligase activity) that participate in the regulation of eNOS by CHIP. Further studies will be required to distinguish between these possibilities.

Recently, two additional proteins that modulate eNOS distribution have been identified. NOSTRIN (eNOS traffic inducer) and NOSIP (eNOS-interacting protein) were both identified by their interactions with the eNOS oxygenase domain in yeast two-hybrid screens (46, 47). Both of these proteins appear to affect eNOS localization by redistribution from the plasma membrane into insoluble cytoplasmic compartments in overexpression studies similar to those performed here. The activities of NOSTRIN and NOSIP stand in contrast to that of CHIP, which diverts eNOS from its normal trafficking pathway at the Golgi or pre-Golgi level. Whether the final destination of eNOS is to a similar compartment after diversion by each of these proteins is not clear, although this is a distinct possibility. In any event, these separate studies indicate the existence of multiple mechanisms for diversion of eNOS from its "normal" trafficking pathway and provide further evidence for the precise regulation of intracellular localization and activity of eNOS.

Having previously identified CHIP as an E3 ligase for the chaperone substrates GR and CFTR (15, 16, 19), our studies demonstrate that CHIP modulates the chaperone substrate eNOS in a manner independent of its ubiquitin ligase activity. In these studies, we have identified a novel co-chaperone-dependent mechanism of eNOS regulation. CHIP inactivates eNOS by uncoupling its interaction with Hsp90 and by partitioning eNOS from the normal Golgi transit pool. Reduction in the expression or activity of eNOS is a hallmark of cardiovascular diseases such as hypertension, diabetes, heart failure, and arteriosclerosis. Thus, negative regulation of eNOS by CHIP suggests that it might serve as a new target for intervention in the treatment of cardiovascular disease. In addition, we have shown for the first time that eNOS can be regulated through the ubiquitin-proteasome pathway under certain circumstances (*i.e.* chaperone dysfunction induced by GA). Our studies indicate the potential existence of novel chaperone-dependent ubiquitin ligases for eNOS and provide new directions for future research.

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