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Ubiquitylation of Neuronal Nitric-oxide Synthase by CHIP, a Chaperone-dependent E3 Ligase*

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It is established that neuronal nitric-oxide synthase (nNOS) is ubiquitylated and proteasomally degraded. The proteasomal degradation of nNOS is enhanced by suicide inactivation of nNOS or by the inhibition of hsp90, which is a chaperone found in a native complex with nNOS. In the current study, we have examined whether CHIP, a chaperone-dependent E3 ubiquitin-protein isopeptide ligase that is known to ubiquitylate other hsp90-chaperoned proteins, could act as an ubiquitin ligase for nNOS. We found with the use of HEK293T or COS-7 cells and transient transfection methods that CHIP overexpression causes a decrease in immunodetectable levels of nNOS. The extent of the loss of nNOS is dependent on the amount of CHIP cDNA used for transfection. Lactacystin (10 μM), a selective proteasome inhibitor, attenuates the loss of nNOS in part by causing the nNOS to be found in a detergent-insoluble form. Immunoprecipitation of the nNOS and subsequent Western blotting with an anti-ubiquitin IgG shows an increase in nNOS-ubiquitin conjugates because of CHIP. Moreover, incubation of nNOS with a purified system containing an E1 ubiquitin-activating enzyme, an E2 ubiquitin carrier protein conjugating enzyme (UbcH5a), CHIP, glutathione *S*-transferase-tagged ubiquitin, and an ATP-generating system leads to the ubiquitylation of nNOS. The addition of purified hsp70 and hsp40 to this *in vitro* system greatly enhances the amount of nNOS-ubiquitin conjugates, suggesting that CHIP is an E3 ligase for nNOS whose action is facilitated by (and possibly requires) its interaction with nNOS-bound hsp70.

Nitric-oxide synthases (NOSs)[1] are cytochrome P450-like hemoprotein enzymes that catalyze the conversion of L-arginine to citrulline and nitric oxide by a process that requires NADPH and molecular oxygen. NOS is bidomain in structure with an oxygenase domain, which contains the cysteine residue that is coordinated to the prosthetic heme as well as the tetrahydrobiopterin binding site, and a reductase domain, which contains the binding sites for FMN, FAD, and NADPH. The NOS is a highly regulated enzyme requiring homodimerization and association with Ca^{2+}-calmodulin for activity. Another mechanism for regulation involves the selective ubiquitin-dependent proteasomal degradation of dysfunctional NOS (1). This is evident, for example, when metabolism-based or suicide inactivators cause the covalent alteration and inactivation of neuronal NOS (nNOS) and trigger enhanced proteasomal degradation of the enzyme (2). The nature of the factor(s) that selectively recognize dysfunctional nNOS is unknown; however, inhibition of hsp90 leads to enhanced proteasomal degradation of nNOS, implicating the hsp90-based chaperone machinery as a potential regulator of nNOS protein levels (3).

Hundreds, perhaps thousands, of cellular proteins are chaperoned by the hsp90/hsp70-based chaperone machinery (4), and it has been proposed that these chaperones play a key role in protein triage decisions that maintain quality control of cellular proteins. CHIP is a U-box-containing E3 ubiquitin ligase that binds through its tetratricopeptide repeat (TPR) domain to independent TPR acceptor sites on hsp90 and hsp70 (5, 6). CHIP facilitates the ubiquitylation of chaperone substrates, including the glucocorticoid receptor (7), the cystic fibrosis transmembrane conductance regulator protein (8), and Raf-1 kinase (9). Because a substantial fraction of all cellular proteins is degraded by the ubiquitin-proteasome pathway, the interaction of CHIP with hsp90 and/or hsp70 is an attractive mechanism for the control of protein triage decisions. The exact mechanism by which CHIP elicits this action is still unclear, although at least three general mechanisms have been investigated: the interaction of CHIP with hsp70 to ubiquitinate chaperoned proteins (5, 8–10), a displacement of p23 from hsp90 by CHIP that remodels the hsp90-heterocomplex to favor degradation (7), and a combination of the hsp70 and hsp90 mechanisms (6, 10).

In the current study we found that CHIP acts to ubiquitylate nNOS and that hsp70 and hsp40 can facilitate this conjugation reaction. The transient transfection of CHIP into HEK293T cells or COS-7 cells causes the loss of nNOS that is partially attenuated by inhibition of the proteasome with lactacystin. Immunopurification of nNOS from these cells reveals an increased level of nNOS-ubiquitin conjugates because of CHIP. Moreover with the use of an *in vitro* system containing purified proteins, we show that CHIP in concert with hsp70 and hsp40 effectively ubiquitylates nNOS. This is of special interest in light of a recent discovery that CHIP causes not the ubiquitylation but rather the redistribution of soluble endothelial NO synthase (eNOS) to an insoluble compartment and impairs

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[1] The abbreviations used are: NOS, nitric-oxide synthase; nNOS, neuronal NOS; CHIP, carboxyl terminus of hsp70-interacting protein; E3, ubiquitin-protein isopeptide ligase; TPR, tetratricopeptide repeat; eNOS, endothelial NOS; GST, glutathione *S*-transferase; Ub, ubiquitin; E1, ubiquitin-activating enzyme; HEK, human embryonic kidney; E2, ubiquitin carrier protein.

eNOS trafficking through the Golgi apparatus (11). This difference of the two enzymes in trafficking *versus* degradation may relate to the differences in the structure of the N termini, which include a PDZ domain in nNOS but not eNOS and myristylation and palmitylation sites on eNOS but not nNOS.

EXPERIMENTAL PROCEDURES

Materials

Untreated rabbit reticulocyte lysate was from Green Hectares (Oregon, WI). (6R)-5,6,7,8-Tetrahydro-L-biopterin (BH_4) was purchased from Dr. Schirck's laboratory (Jona, Switzerland). Protein A-Sepharose, ubiquitin, ATP, creatine phosphokinase, L-arginine, radicicol, rabbit polyclonal anti-nNOS, mouse monoclonal anti-GST, and mouse anti-β tubulin were purchased from Sigma. The affinity-purified rabbit IgG used for Western blotting of nNOS was from Transduction Laboratories (Lexington, KY), which used C-terminal peptide (residues 1095–1289) as immunogen. The affinity-purified IgG used for Western blotting and immunoprecipitation of Ub was from Dako Corp. (Carpinteria, CA). The rabbit antiserum used to immunoprecipitate nNOS was raised against rat neuronal NOS and was the generous gift of Dr. Lance Pohl (National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD). Rabbit anti-CHIP antibody was from Affinity BioReagents (Golden, CO). The mouse monoclonal anti-c-Myc IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Lactacystin and MG132 were purchased from Biomol (Plymouth Meeting, PA). The cDNA for rat neuronal NOS was kindly provided by Dr. Solomon Snyder (Johns Hopkins Medical School, Baltimore, MD). The cDNA for His-HA-tagged ubiquitin was from Dr. Yi Sun (University of Michigan). GST-tagged ubiquitin, ubiquitin aldehyde, ubiquitin-activating enzyme (E1), and GST-tagged UbcH5a were from Boston Biochem (Cambridge, MA). Creatine phosphate was from Fluka. Nickel-nitrilotriacetic acid-agarose was from Qiagen, Inc. (Valencia, CA).

Methods

Cell Culture and Transient Transfection—Human embryonic kidney (HEK) 293T cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum. COS-7 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Transient transfections of 293T or COS-7 cells were carried out with the use of a standard calcium phosphate method as described previously (12) in 6-well plates. The rat nNOS cDNA was subcloned from PVL1393 (3) into the EcoRI and NotI sites of pcDNA3.1+. Human CHIP cDNA and myc-CHIP cDNA was in pcDNA3 (11, 13). Indicated amounts of CHIP cDNA and nNOS cDNA were transfected into 70–80% confluent cells such that the total amount of cDNA was kept constant with vector plasmid. Cells were transfected for 28 h, and where indicated were treated with lactacystin (10 μM) for an additional 20 h. As indicated in experiments where CHIP was immunoprecipitated, myc-CHIP cDNA was used.

SDS-Polyacrylamide Gel Electrophoresis, Western Blotting, and Immunoprecipitation—Cells from each well of a 6-well plate were harvested 48 h after transfection, washed with ice-cold phosphate-buffered saline, and homogenized with a TenBroeck ground glass homogenizer in 0.1 ml of HS buffer (10 mM Hepes, pH 7.4, 0.32 M sucrose, 2 mM EDTA, 6 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, 15 mM sodium vanadate, 1% Nonidet P-40, and 5 mM *N*-ethylmaleimide). Where indicated, the transfected cells were treated with 10 μM lactacystin or water vehicle for 20 h before harvesting. In some studies, the transfected cells were treated with 20 μM radicicol or an equivalent amount of Me_2SO vehicle for 24 h before harvesting. Homogenates were centrifuged for 15 min at 16,000 $\times$ *g*, and the supernatant was taken as the "soluble" fraction of cells. The remaining pellet was considered the "insoluble" fraction and was washed once with phosphate-buffered saline and then sonicated after the addition of 0.1 ml of HS buffer. An aliquot (60 μl) of the resulting soluble or insoluble sample was added to 40 μl of sample buffer containing 5% SDS, 20% glycerol, 6 mg/ml dithiothreitol, and 0.02% bromphenol blue in 125 mM Tris-HCl, pH 6.8. After boiling, 40 μl of the soluble and insoluble protein samples were resolved on 6% SDS-polyacrylamide gels and transferred to nitrocellulose membranes for 2 h at 100 volts. Immunoreactive bands were visualized with the use of enhanced chemiluminescence reagent (Super Signal, Pierce) and X-Omat film (Eastman Kodak Co.). In some experiments, the total cell lysates were prepared by direct sonication in HS buffer and subsequent boiling in sample buffer. In studies where nNOS or ubiquitin was immunopurified, the cells were grown in 10-cm plates and transfected with 6 μg of nNOS cDNA, 6 μg of His-HA-Ub cDNA, and 0.6 μg of CHIP cDNA. The nNOS was immunoprecipitated from the soluble fraction with a rabbit polyclonal antibody as described previously (14).

Expression and Purification of nNOS, CHIP, hsp70, and hsp40—Rat nNOS was expressed in Sf9 insect cells using a recombinant baculovirus and purified by 2′5′-ADP-Sepharose and gel-filtration chromatography as described previously (3). Heme was added as an albumin conjugate during the expression as described (3). His-CHIP was bacterially expressed and purified by nickel-nitrilotriacetic acid affinity chromatography as described previously (5). hsp70 was purified from rabbit reticulocyte lysate by sequential chromatography on DE52, hydroxylapatite, and ATP-agarose as described previously (15). YDJ-1, the yeast ortholog of hsp40, was expressed in bacteria and purified by sequential chromatography on DE52 and hydroxylapatite as described previously (16).

In Vitro Ubiquitylation of nNOS—To conjugate Ub to nNOS, purified nNOS (0.24 μg) was incubated with a purified system containing an E1 ubiquitin-activating enzyme (0.1 μM), an E2 GST-tagged UbcH5a (4.0 μM), His-tagged CHIP (4.0 μM), GST-tagged ubiquitin (8.3 μM), 1 mM dithiothreitol, and an ATP-generating system consisting of 4 mM ATP, 20 mM creatine phosphate, 10 mM $MgCl_2$, and 20 units/ml creatine phosphokinase, expressed as final concentrations, for 2 h at 30 °C in a total volume of 20 μl of 50 mM Tris-Cl, pH 7.5. After incubation, 20 μl of sample buffer was added, and an aliquot (10 μl) was loaded for Western blotting. In some studies, nNOS was pretreated with hsp70 and hsp40 and then ubiquitylated as above. In these experiments, 5.0 μM hsp70 and 0.5 μM hsp40 were incubated for 5 min at 30 °C with 1.5 μM nNOS and 2.5 μl of an ATP-generating system (50 mM ATP, 250 mM creatine phosphate, 20 mM magnesium acetate, and 100 units/ml creatine phosphokinase) in a total volume of 25 μl of 50 mM Hepes, pH 7.5. The reaction mixture was placed on ice and diluted 5-fold with 10 mM Hepes buffer, pH 7.4, containing 100 mM KCl, and 5 mM dithiothreitol. An aliquot (5 μl) of this reaction mixture was substituted for nNOS in the ubiquitylation reaction mixture containing purified ubiquitin ligases described above and was probed for ubiquitin conjugation.

RESULTS

CHIP Enhances the Proteasomal Degradation of nNOS in HEK293T Cells—With the use of transient transfection methods, we tested to see if CHIP, a known ubiquitin E3 ligase for some other hsp90-chaperoned proteins, could act to enhance the ubiquitylation and proteasomal degradation of nNOS. We have previously used the HEK293 cell system to characterize the ubiquitylation and proteasomal degradation of nNOS (14). In this system, both functional inactivation of the nNOS with suicide inactivators and inhibition of hsp90 enhance the ubiquitin-proteasomal degradation of nNOS (2, 3). HEK293T cells were transiently transfected with CHIP and nNOS for 48 h, and cellular lysates were prepared by sonication in detergent-containing buffer. The amount of nNOS, CHIP, and tubulin in these lysates was determined by Western blotting. In the experiment shown in Fig. 1, transfection with increasing amounts of CHIP cDNA decreases the amount of immunodetectable nNOS (*lanes 1–4*). The total amount of cDNA used was made constant by use of the vector plasmid. Treatment of the cells with 10 μM lactacystin, a highly selective inhibitor of the proteasome, for the last 20 h of transfection increased the amount of nNOS observed (*lanes 5–8*). The bands corresponding to nNOS in all samples were quantified by laser densitometry, and the results are plotted in Fig. 1*B*. From this analysis, it is clear that the loss of nNOS caused by CHIP is greatly diminished by lactacystin (*cf. open circles* with *closed circles*), an indication that CHIP acts to enhance nNOS degradation by the proteasome.

In light of a recent finding that CHIP directs eNOS to an insoluble fraction that is not easily extracted by detergent unless sonicated (11), we chose to investigate the effect of CHIP on nNOS distribution to a detergent insoluble fraction prepared from cells in a manner similar to that described for eNOS. HEK293T cells were lysed manually by use of a TenBroeck glass homogenizer in detergent-containing buffer, and the 16,000 $\times$ *g* supernatant (soluble) and pellet (insoluble)

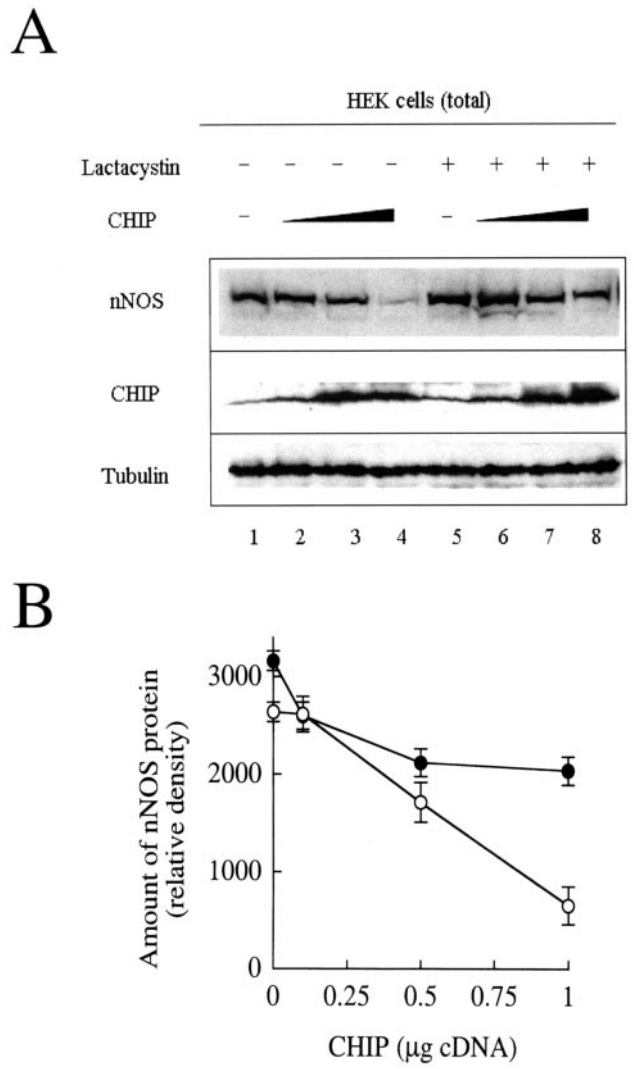

FIG. 1. **Lactacystin protects against the CHIP-mediated decrease of nNOS protein in HEK293T cells.** The effect of CHIP transfection on nNOS, which was detected in the sonicated cell lysates, was determined in co-transfection studies of HEK293T cells as described under "Experimental Procedures." CHIP and tubulin levels were also determined. *A*, cells were transfected with nNOS (0.1 μg) and different amounts of CHIP cDNA (0, 0.1, 0.5, and 1.0 μg) and subsequently treated with (+) or without (−) 10 μM lactacystin. An experiment typical of three independent experiments is shown. *B*, the band corresponding to nNOS in *A* was analyzed by laser densitometry. The effect of CHIP transfection on nNOS levels without lactacystin (*open circles*) or with lactacystin (*closed circles*) is plotted. The values are the mean ± S.E. (n = 3).

fractions were prepared. The insoluble fraction was sonicated and boiled in sample buffer so that this fraction could be analyzed by Western blotting. As shown in Fig. 2*A*, CHIP causes a decrease in the nNOS present in the soluble fraction. Lactacystin had little effect on the soluble nNOS (see samples under *Soluble*) unlike that observed for the sonicated cell lysates. CHIP appears to slightly increase the amount of nNOS present in the insoluble fraction, whereas a large increase in insoluble nNOS is found after treatment with lactacystin (*Insoluble*). Lactacystin has been shown to cause the accumulation of proteins that are destined for proteasomal removal in an ordered but aggregated state that is resistant to solubilization by detergent (17, 18).

In addition to HEK293T cells, we investigated the effect of CHIP in COS-7 cells (Fig. 2*B*). CHIP causes a loss of nNOS in the soluble fraction of COS-7 cells that is partially attenuated by lactacystin. Lactacystin also causes the accumulation of nNOS in the insoluble fraction of COS-7 cells. Thus, the decrease in nNOS protein caused by CHIP is not unique to the HEK293T cells.

Effect of Radicicol and CHIP on nNOS—CHIP directly interacts with hsp90 and hsp70, and it is thought that the functional role of CHIP in mediating protein levels are caused by these interactions (5, 6). To confirm that nNOS is regulated by chaperones in our cell model, we tested the effect of radicicol, a specific hsp90 inhibitor, and compared it to the effect of CHIP. As shown in Fig. 3*A*, treatment of HEK293T cells with radicicol alone causes a decrease in the amount of nNOS in the soluble fraction (*cf. lane 1* and *lane 4*) and greater amounts of nNOS in the insoluble fraction (*cf. lane 7* and *lane 10*). The loss of nNOS in the soluble fraction is consistent with our previous observation after inhibition of hsp90 with geldanamycin (2). The appearance of nNOS in the insoluble fraction has not been reported previously. To discern the effect of CHIP with radicicol, the bands corresponding to nNOS in the soluble and insoluble fractions were quantified by laser densitometry, and the results are plotted in Fig. 3, *B* and *C*, respectively. In the soluble fraction, CHIP causes the loss of nNOS in both radicicol treated (*solid circles*) and untreated cells (*open circles*) such that at the highest amount of CHIP similar amounts of nNOS are present. In the insoluble faction, there is an accumulation of nNOS in both radicicol-treated (*solid circles*) and untreated cells (*open circles*). Thus, CHIP is able to decrease nNOS in the soluble fraction and increase it in the insoluble fraction even in the presence of radicicol. It remains to be shown whether the hsp90 is not fully inhibited by radicicol and CHIP acts on the remaining hsp90 or CHIP causes a further decrease by a process independent of binding to hsp90. Studies to be described below suggest that the CHIP interaction with hsp70 is important.

CHIP Enhances the Ubiquitylation of nNOS in HEK293T Cells—We wanted to obtain direct evidence of the effects of CHIP on the ubiquitylation of nNOS. The nNOS present in HEK293T cells was immunopurified and blotted for nNOS and ubiquitin. The nNOS is specifically precipitated from soluble fraction of HEK293T cells by the immune (*IP*) IgG antibody but not by the non-immune (*NI*) IgG (Fig. 4 *middle panel*, *cf. lanes 1–4* with *lanes 5–8*). The nNOS is observed in cells as mono- and polyubiquitin conjugates (14), and the levels of these conjugates are increased after treatment with lactacystin (, *cf. lane 5* with *lane 6*). CHIP acts to increase the level of nNOS-Ub conjugates with a more pronounced increase in the presence of lactacystin (*lanes 7* and *8*). The effect may be underestimated because of the lower amounts of nNOS immunopurified from CHIP-transfected cells as well as the finding that most of the lactacystin-sensitive pool of nNOS ends up in the insoluble fraction, which is not amenable to the immunopurification procedure. Interestingly, CHIP is selectively immunoprecipitated by the anti-nNOS antibody (*cf. lanes 7 and 8* with *lanes 3 and 4*), indicating that CHIP exists in a complex with nNOS. This is expected because nNOS is found in native complexes with large amounts of hsp70 and small amounts of hsp90 (3), and CHIP binds to hsp70 and hsp90 through TPR acceptor sites. The association of nNOS with CHIP and other chaperones is explored in further detail later.

To further validate that nNOS-Ub conjugates have formed, we immunoprecipitated with anti-ubiquitin and blotted for nNOS (Fig. 4*B*). This is important because the Western blot with anti-ubiquitin above does not differentiate between nNOS-ubiquitin conjugates and ubiquitin conjugates of proteins associated with nNOS. Several higher molecular mass nNOS bands were observed after immunoprecipitation with anti-ubiquitin but not in the non-immune controls. The most

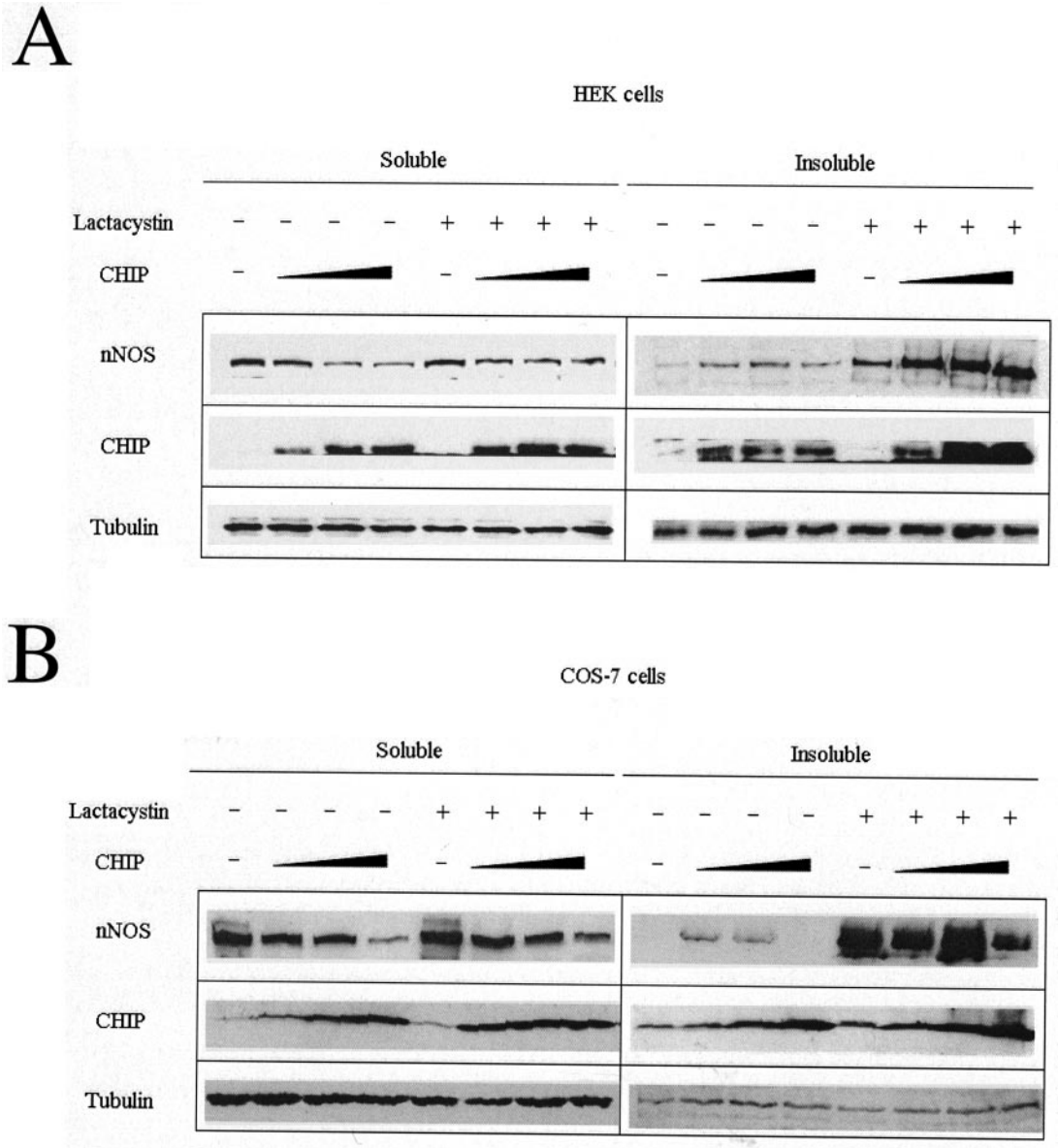

FIG. 2. **Effect of CHIP and lactacystin on the accumulation of detergent-resistant nNOS in HEK293T and COS-7 cells.** Cells were treated as described in Fig. 1 except that the samples were homogenized with TenBroeck ground glass homogenizer, and the soluble and insoluble fractions were prepared and probed for nNOS, CHIP, and β-tubulin by Western blotting as described under "Experimental Procedures." The signal from samples of the insoluble fraction was much weaker than that of the soluble fraction, and thus the film was exposed for a greater duration for visualization of the insoluble samples. The cells were transfected with nNOS (1.0 μg) and different amounts of CHIP cDNA (0, 0.1, 0.5, and 1.0 μg) and subsequently treated with (+) or without (−)10 μM lactacystin. *A*, effect of CHIP and lactacystin on nNOS transfected in HEK293T cells. An experiment typical of four independent experiments is shown. *B*, effect of CHIP and lactacystin on nNOS transfected in COS-7 cells. An experiment typical of three independent experiments is shown.

intense signals were observed in samples transfected with CHIP and treated with lactacystin, consistent with the formation of nNOS-ubiquitin conjugates.

CHIP Enhances the Ubiquitylation of nNOS in an in Vitro System Containing Purified Ub Ligases—The ubiquitin ligase activity of CHIP has been observed in an *in vitro* system containing other known components of a ligase system, including purified E1 and E2 (10, 11, 13). To more definitively assess whether CHIP is a ubiquitin ligase for nNOS, we utilized a similar *in vitro* system containing purified nNOS, E1, E2, and CHIP. We used GST-tagged ubiquitin so that the conjugates could be more easily detected by Western blotting with anti-nNOS IgG. As shown in Fig. 5*A* in the *left panel*, nNOS treated with E1, E2, and GST-Ub gives some faint higher molecular mass nNOS immunoreactive bands consistent with conjugation with GST-Ub (*lane 2*). This may reflect the ability of E2 enzymes to carry out limited ligase reactions with target proteins even in the absence of E3 ligases such as CHIP (19). The addition of CHIP to this system greatly increases the level of ubiquitin conjugates (*lane 3*). The addition of purified hsp70 and hsp40 to the CHIP-containing reaction further enhances the level of nNOS-ubiquitin conjugates (*lane 4*). Probing of the same blot with anti-GST also revealed a banding pattern similar to that for nNOS, although other protein-GST-Ub conjugates are probably present. As shown in Fig. 5*B*, our purified preparation of nNOS contains substantial amounts of insect hsp70 derived from baculovirus expression in Sf9 cells consistent with our previous observation (3). This hsp70 co-immunoabsorbs with nNOS, and thus a portion of nNOS is in complex with this chaperone (data not shown). Thus, it may be that hsp70 is required for the binding and action of CHIP and that the lower levels of conjugate formed in the absence of added hsp70 is caused by the low levels of hsp70 in our nNOS preparations.

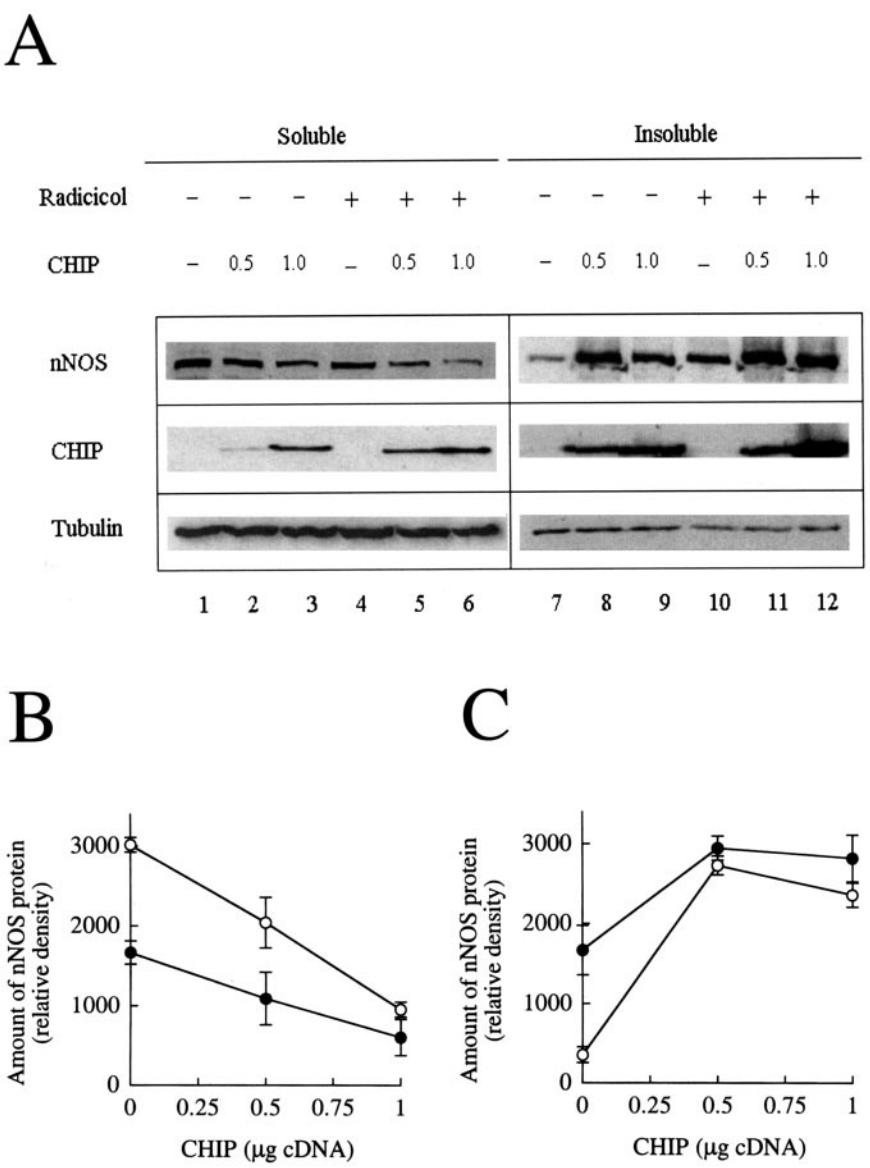

FIG. 3. **Radicicol and CHIP enhance the accumulation of detergent-resistant nNOS protein.** HEK293T cells were transfected with nNOS (0.5 μg) and CHIP and then treated with (+) or without (−) 20 μM radicicol for 24 h. *A*, the amount of CHIP cDNA used for transfection is indicated in μg of cDNA. The soluble and insoluble fractions were prepared and Western blotted for nNOS, CHIP, and tubulin. *B*, the band corresponding to nNOS in the soluble fraction from *A* was analyzed by laser densitometry. The effect of CHIP transfection on nNOS levels without radicicol (*open circles*) or with radicicol (*closed circles*) is plotted. The values are the mean ± S.E. (n = 3). *C*, the band corresponding to nNOS in the insoluble fraction from *A* was analyzed by laser densitometry. The effect of CHIP transfection on nNOS levels without radicicol (*open circles*) or with radicicol (*closed circles*) is plotted. The values are the mean ± S.E. (n = 3).

nNOS Exists in Heterocomplexes with CHIP and hsp70—We wanted to address the biochemical interaction between nNOS and CHIP as well as other chaperones. As shown in Fig. 6*A*, immunoprecipitation of nNOS (*IP*) from lactacystin-treated cells is accompanied by co-adsorption of hsp70 and CHIP. These proteins are not observed in the non-immune samples (*NI*). Consistent with our earlier report (3), there is a faint signal for hsp90. As shown in Fig. 6*B*, anti-myc specifically immunoprecipitates myc-CHIP (*cf. IP* with *NI*). The anti-myc immunoprecipitates from lactacystin-treated cells contain nNOS and hsp70 consistent with the finding that CHIP binds to hsp70 through a TPR acceptor site (5).

DISCUSSION

We have previously shown that hsp90 and hsp70 are associated with nNOS and that inhibition of hsp90 by geldanamycin or suicide inactivation of nNOS both lead to enhanced proteasomal degradation of the enzyme (2, 3, 14). One potential mechanism that explains these observations of nNOS degradation is the selective recognition of a dysfunctional form of nNOS by chaperones that in turn recruit ubiquitin ligases. Because CHIP is a U-box-containing E3 ubiqutin ligase that binds through its TPR domain to independent TPR acceptor sites on hsp90 and hsp70 and mediates ubiquitylation of other hsp90-chaperoned proteins (5, 6), we wondered if CHIP could act as an ubiquitin ligase for nNOS. In the current study, we have shown that overexpression of CHIP causes the enhanced proteasomal degradation of nNOS. Treatment with lactacystin, a selective inhibitor of the proteasome, attenuates the loss of nNOS caused by CHIP and leads to the accumulation of nNOS-Ub conjugates. Under these conditions, the nNOS in cell cytosols is found associated with CHIP. Moreover, in studies with the use of an *in vitro* ubiquitylating system containing purified nNOS and ubiquitin ligase enzymes, CHIP increases the amount of nNOS-Ub conjugates. We also show here that hsp70, in the presence of hsp40, greatly facilitates nNOS-ubiquitin conjugation *in vitro*. Our preparation of nNOS has substantial amounts of insect hsp70, which could not be removed, and thus the absolute requirement for hsp70 could not be tested. Nonetheless, the addition of exogenous hsp70 greatly increases the number of nNOS-ubiquitin conjugates. Thus, it is clear that CHIP acts in concert with hsp70 and hsp40 to enhance the ubiquitylation of nNOS, consistent with the established role of CHIP as a chaperone-dependent E3-ubiquitin ligase.

Ubiquitylation and proteasomal degradation is a critical component of protein quality control in cells. How proteins are targeted for ubiquitylation and degradation is fundamental to

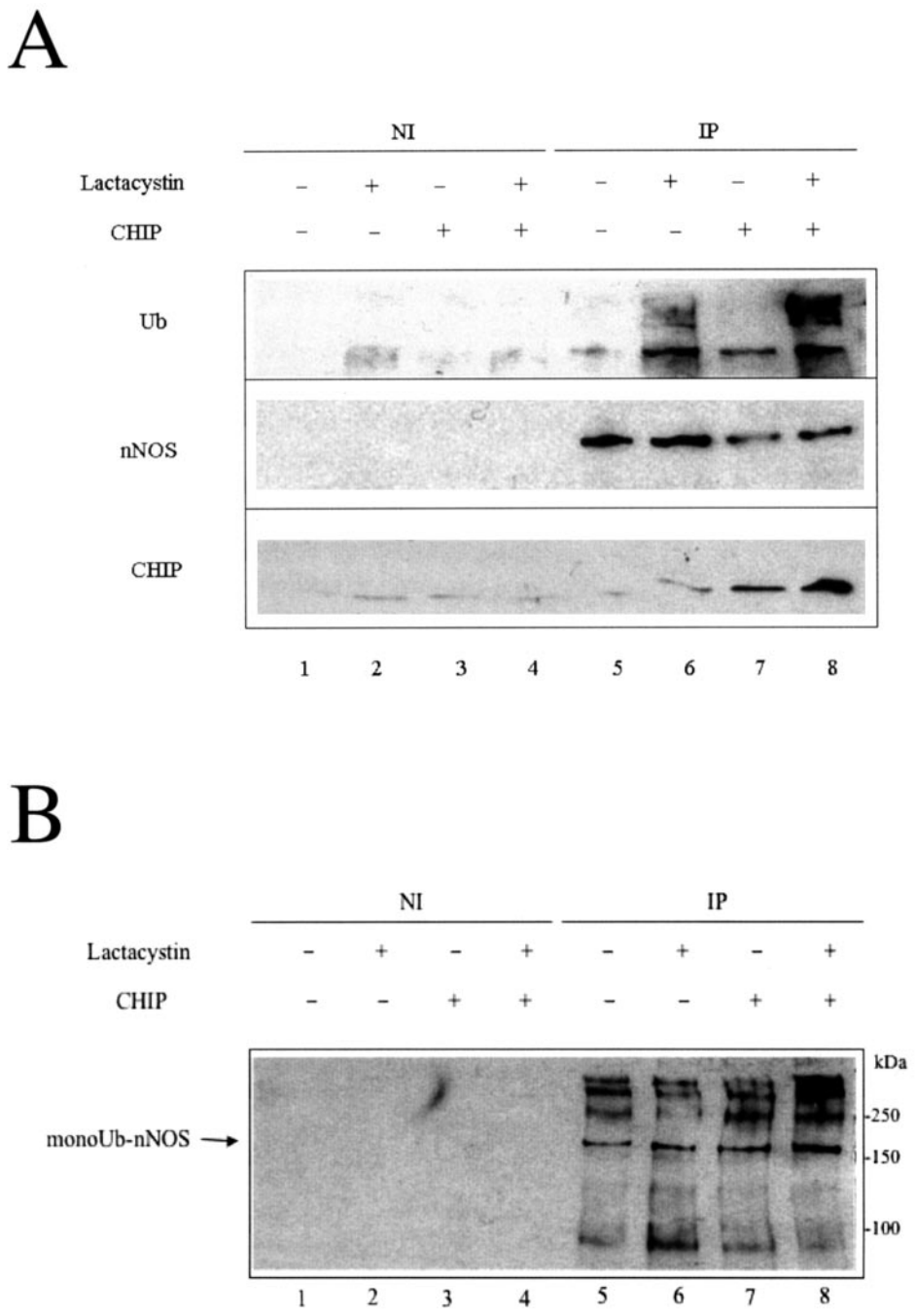

FIG. 4. **CHIP increases ubiquitylation of nNOS proteins in HEK293T cells.** HEK293T cells (10-cm dishes) were transfected for 28 h with His-HA-ubiquitin and nNOS in the presence or absence of CHIP. The cells were then treated with 10 μM lactacystin or water vehicle for another 20 h before lysis. *A*, the soluble fraction was prepared from the cells and used for immunoprecipitation with non-immune (*NI*) or an anti-nNOS (*IP*) antibody followed by Western blot analysis with anti-ubiquitin (*top panel*), anti-nNOS (*middle panel*), and anti-CHIP (*bottom panel*) antibodies. The data are representative of three identical experiments. *B*, the soluble fraction was used for immunoprecipitation with non-immune or an anti-ubiquitin (*IP*) antibody, followed by Western blot analysis with anti-nNOS antibody. The data are representative of three identical experiments.

understanding the control of the cellular protein triage machinery. It is widely thought that the E3 ubiquitin ligases select target proteins for ubiquitylation and thus commit the target protein to proteasomal degradation. CHIP is not only an E3 ligase but is also a co-chaperone for hsp90 and hsp70, and thus CHIP has been proposed to play a role in protein triage decisions through chaperones (6). CHIP has been described to act via hsp70, hsp90, or a combination of the two chaperones (6–8, 10). The exact mechanism of how this occurs is not known.

CHIP is known to inhibit the hsp90-dependent binding of steroid to the glucocorticoid receptor and decrease p23 bound to the glucocorticoid receptor·hsp90 heterocomplex (7). It was proposed that this remodeling of the hsp90 heterocomplex explains the enhanced ubiquitylation of the glucocorticoid receptor caused by CHIP. Another proposed mechanism is the inhibition of hsp90 in a manner similar to ansamycin drugs, which bind to the ATP binding site of hsp90 (6). The inhibition of hsp90 by ansamycin drugs and the enhanced proteasomal degradation of the target protein are some of the most convenient and commonly used indicators of an hsp90 client protein. Thus, the inhibition of hsp90 would explain the decreased levels of target protein caused by CHIP. However, in studies with purified proteins, the ubiqui-

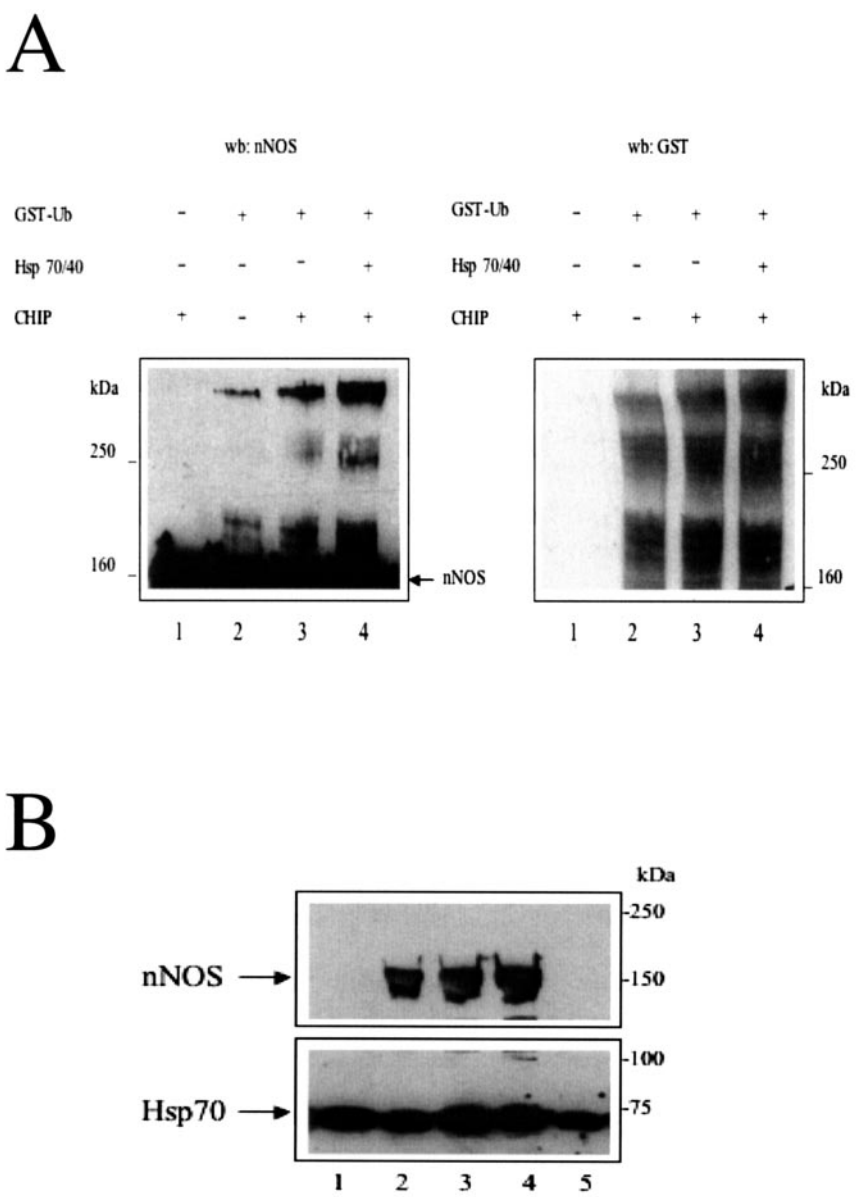

FIG. 5. **CHIP increases ubiquitylation of nNOS in an *in vitro* system containing purified proteins.** *A*, purified nNOS was treated in reaction mixture containing purified E1 ubiquitin-activating enzyme, E2 conjugating enzyme, and GST-Ub. As indicated, some of the samples contained purified CHIP, and some of the nNOS samples were pretreated with hsp70 and hsp40. The samples were Western blotted by probing with anti-nNOS IgG (*left panel*) or anti-GST IgG (*right panel*), and higher molecular mass Ub-conjugates of nNOS were visualized. The data are representative of six similar experiments. *B*, the purified nNOS preparation contains insect hsp70. Samples of purified nNOS were resolved by SDS-PAGE and immunoblotted with an antibody that reacts with insect hsp70. *Lane 1*, 0.2 μg of purified rabbit hsp70; *lane 2*, 1.0 μg of nNOS; *lane 3*, 2.0 μg of nNOS; *lane 4*, 3.0 μg of nNOS; *lane 5*, 0.1 μg of purified rabbit hsp70. The immunoblots were performed four times.

tylation of heat-denatured luciferase by CHIP is enhanced when purified hsp90 is added, suggesting a direct role of hsp90 in the ubiquitylation process (10).

We raise here the possibility that hsp70 directly mediates protein triage decisions by recognition of nNOS and recruiting an ubiquitin ligase machinery that involves CHIP. Bercovich *et al.* (20) first described the requirement of hsp70 for ubiquitin-dependent degradation of actin, α-crystallin, glyceraldehyde-3-phosphate dehydrogenase, α-lactalbumin, and histone H2A. These authors concluded that hsp70 was needed for ubiquitin conjugation to occur but that hsp70 was not an E3-ubiquitin ligase. Consistent with this view, the cystic fibrosis transmembrane conductance regulator protein is ubiquitylated by CHIP and hsp70 but not hsp90 (8). Indeed heat-denatured luciferase is ubiquitylated by CHIP in the presence of hsp70 in an *in vitro* system consisting of purified proteins (10). Recently it was shown that Parkin, an E3 ubiquitin ligase, also associates with hsp70 and enhances ubiquitylation (21).

At any time, only a small percentage (<1%) of nNOS is found in the ubiquitylated state, and the ubiquitylated products are thought to represent protein in a non-native conformation. It makes sense that hsp70 makes the triage decision to proceed in the direction of client protein degradation *versus* stabilization. In the assembly of hsp90 heterocomplexes, the first step is an ATP-dependent interaction with hsp70 and hsp40 to form a "primed" client protein·hsp70 complex that then binds hsp90 in a second ATP-dependent step to form the client protein·hsp90 heterocomplex (4). When the client protein is bound by hsp90, it is stabilized, and inhibition of hsp90 binding by geldanamycin or radicicol uniformly results in client protein destabilization, manifest as degradation via the ubiquitin-proteasomal pathway (22). In the early stages of protein damage and unfolding, hydrophobic regions to which hsp70 can bind are exposed. In this case, instead of forming a "primed" complex that can interact with hsp90 and be stabilized, binding of CHIP to the TPR acceptor site on the client protein-bound hsp70 can lead to ubiquitylation and ultimately degradation as shown here with nNOS.

Because of the dynamic nature of the interaction of hsp90 and hsp70 with nNOS (3, 23), we are unable to test in detail the relative roles of these chaperones in ubiquitylation and degradation of nNOS, specifically by the CHIP-chaperone-nNOS heterocomplexes. However, we intend to pursue this line of investigation with the glucocorticoid receptor, where much more stable heterocomplexes with hsp90 and hsp70 can be formed in stepwise fashion using a purified five-protein assembly system.

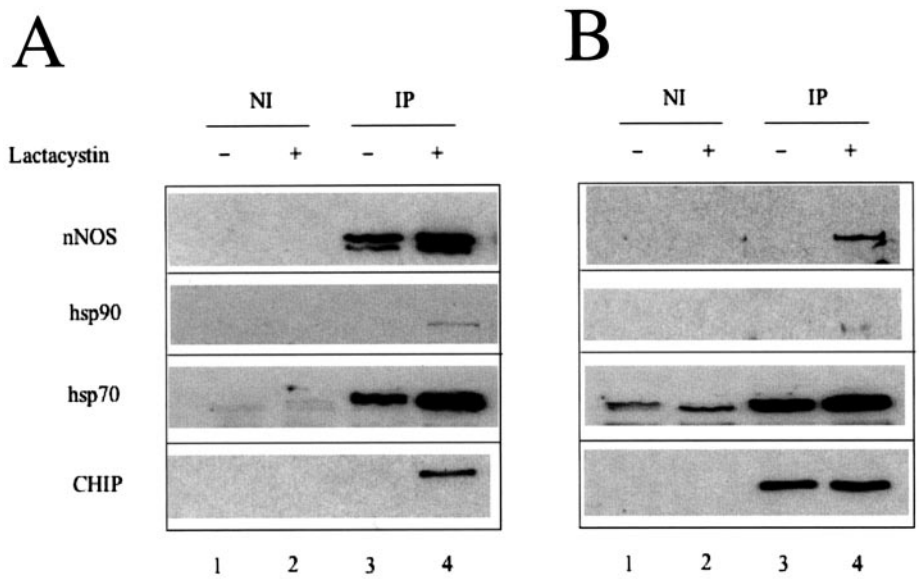

FIG. 6. **CHIP is found in heterocomplexes with nNOS and hsp70.** HEK293T cells (10-cm dishes) were transfected for 28 h with myc-CHIP and nNOS. The cells were then treated with 10 μM lactacystin or water vehicle for another 20 h before lysis. *A,* the soluble fraction was prepared from the cells and used for immunoprecipitation with non-immune (*NI*) or an anti-nNOS (*IP*) antibody followed by Western blot analysis with anti-nNOS, anti-hsp90, anti-hsp70, and anti-myc antibodies. The anti-myc was used to blot for myc-CHIP. The data are representative of three identical experiments. *B*, the soluble fraction was used for immunoprecipitation with non-immune or an anti-myc (*IP*) antibody followed by Western blot analysis with anti-nNOS, anti-hsp90, anti-hsp70, and anti-CHIP antibodies as indicated. The data are representative of two identical experiments.

This can be coupled with the purified ubiquitylation system used here to determine whether hsp70, hsp90, or both can direct CHIP-dependent ubiquitylation of a client protein.

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