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Interaction between the 90-kDa Heat Shock Protein and Soluble Guanylyl Cyclase: Physiological Significance and Mapping of the Domains Mediating Binding[S]

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ABSTRACT

The 90-kDa heat shock protein (hsp90) regulates the stability and function of many client proteins, including members of the NO-cGMP signaling pathway. Soluble guanylyl cyclase (sGC), an NO receptor, was recently reported to be an hsp90-interacting partner. In the present study, we show that hsp90 binds to both subunits of the most common sGC form ($\alpha_1\beta_1$) when these are expressed individually but only interacts with β_1 in the heterodimeric form of the enzyme. Characterization of the region of hsp90 required to bind each subunit in immunoprecipitation experiments revealed that residues 310 to 456 of hsp90 interact with the sGC subunits. The region of β_1 responsible for binding to hsp90β was mapped using in vitro binding assays and immunoprecipitation experiments and was found to lie in the regulatory domain. The physiological importance of the hsp90/sGC interaction was investigated by treating rat smooth muscle cells with the hsp90 inhibitors radicicol and geldanamycin (GA) and determining both sGC activity and protein levels. Long-term (24 or 48 h) inhibition of hsp90 resulted in a strong decrease of both α_1 and β_1 protein levels and sGC activity. Moreover, incubation of smooth muscle cells with the proteasome inhibitor *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal (MG132) blocked the GA-induced down-regulation of sGC. We conclude that the N-terminal region of the β_1 subunit mediates binding of the heterodimeric form of sGC to hsp90 and that this interaction involves the M domain of hsp90. Hsp90 binding to sGC regulates the pool of active enzymes by affecting the protein levels of the two subunits.

The 90-kDa heat shock protein (hsp90) is one of the most abundant cytosolic proteins in eukaryotes, amounting to 1 to 2% of the total soluble protein, even under resting conditions. Two isoforms of hsp90 exist: hsp90α is inducible by heat shock and other stressful stimuli, whereas hsp90β is expressed constitutively (Sreedhar et al., 2004). The primary function of hsp90 is to participate in the folding of newly synthesized proteins and the stabilization and refolding of denatured proteins after stress (Buchner, 1999; Pearl and Prodromou, 2001; Picard, 2002); hsp90 is aided in this task by a number of cochaperones, the identity of which depends on the protein being folded (Pratt, 1998; Buchner, 1999; Pratt and Toft, 2003).

Unlike most members of the heat shock family of proteins, hsp90 has been implicated not only in "housekeeping" functions but also in the dynamic regulation of cell signaling (Pratt, 1998). One of the most studied hsp90-bound signaling molecules is the glucocorticoid receptor that needs to interact

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ABBREVIATIONS: hsp90, 90-kDa heat shock protein; eNOS, endothelial nitric-oxide synthase; GA, geldanamycin; GST, glutathione *S*-transferase; IBMX, 3-isobutyl-1-methylxanthine; NOS, NO synthase; RAD, radicicol; RASMC, rat aortic smooth muscle cell; SNP, sodium nitroprusside; sGC, soluble guanylyl cyclase; MG132, *N*-benzoyloxycarbonyl (*Z*)-Leu-Leu-leucinal; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HA, hemagglutinin; PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; TBST, Tris-buffered saline/Tween 20; IB, immunoblotted; IP, immunoprecipitated; 17-AAG, 17-allylamino 17-demethoxygeldanamycin.

with hsp90 to exhibit ligand-binding activity (Bresnick et al., 1989). The hsp90 client protein list has expanded substantially over the past few years to include transcription factors, protein kinases, and proteins involved in the control of cell cycle (Pratt and Toft, 2003). Most of hsp90 exists as homodimers (α/α or β/β), with each hsp90 form being divided in three domains with discrete functions (Sreedhar et al., 2004). The N-terminal domain of hsp90 contains an ATP-binding pocket that is also the binding site for hsp90 inhibitors (geldanamycin, 17-AAG, and radicicol) (Sreedhar et al., 2004). Most client proteins, including endothelial nitric-oxide synthase (eNOS), the protein kinase Akt, and the glucocorticoid receptor, bind to the middle domain (Pratt and Toft, 1997; Fontana et al., 2002). On the other hand, the C-terminal domain binds proteins with tetratricopeptide repeats (e.g., immunophilin) and facilitates homodimer formation (Sreedhar et al., 2004).

Soluble guanylyl cyclase (sGC) is the best studied receptor for the labile signaling molecule NO (Moncada et al., 1991). NO produced from the constitutive NO synthases diffuses through cell membranes or through the cytosol and activates sGC, increasing its cGMP-forming ability up to 400-fold (Hobbs, 1997). The most common form of sGC is $\alpha_1\beta_1$, which is expressed in all tissues studied so far (Budworth et al., 1999). sGC subunits are divided into three domains: an N-terminal domain, which is also termed regulatory; a central domain; and a C-terminal domain (Hobbs, 1997; Koesling, 1999). The N-terminal domain of sGC contains the heme-binding region rendering the enzyme NO-sensitive (Wedel et al., 1994, 1995; Foerster et al., 1996). The central domain contains the information needed for subunit dimerization (Zhou et al., 2004). Finally, residues that are important for substrate recognition and catalytic activity are distributed on the C-terminal domains of the α_1 and β_1 subunits (Sunahara et al., 1998).

sGC interacts with hsp90 in both endothelial and smooth muscle cells (Venema et al., 2003). Moreover, using in vitro binding assays, we determined that sGC binds directly to hsp90 with high affinity, because the hsp90/β_1 heterocomplex is resistant to high salt concentrations (Venema et al., 2003). In the present study, we set out to determine the regions of the two proteins that participate in the interaction and to evaluate the effect of hsp90 inhibition on sGC function. We observed that the regulatory domain of β_1 in the α_1/β_1 sGC heterodimer mediates binding to hsp90 and that this interaction facilitates NO-signaling because it preserves high levels of sGC expression.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were obtained from Invitrogen BRL (Paisley, UK). Cell-culture plastic ware was from Greiner Bio-One GmbH (Frickenhausen, Germany); BL21-codon plus strain of *Escherichia coli* was from Stratagene (La Jolla, CA); monoclonal anti-V5 antibody, platinum *Pfx* DNA polymerase, and pcDNA3.1 Directional TOPO Expression kit were from Invitrogen; dNTPs were purchased from MBI Fermentas (St. Leon-Rot, Germany); restriction enzymes were obtained from New England Biolabs (Frankfurt, Germany); the nucleospin plasmid kits for the isolation of cDNA were obtained from Macherey-Nagel (Düren, Germany); cGMP enzyme immunoassay kits were from R&D Systems (Minneapolis, MN); SuperSignal West Pico chemiluminescent substrate was from Pierce Chemical (Rockford, IL); *DC* Protein assay kit, Tween 20, and other immunoblotting reagents were from Bio-Rad (Munich, Germany); jetPEI transfection reagent was from Polyplus transfection (Illkirch, France); and protein G-agarose beads and nitrocellulose membrane Hybond ECL were from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Radicicol was purchased from Tocris Cookson Inc. (Avonmouth, UK). The anti-HA was obtained from Roche Diagnostics (Mannheim, Germany); the anti-hsp90 was from StressGen Biotechnologies (Victoria, BC, Canada); the anti-β_1 was from Cayman Chemical (Ann Arbor, MI); and MG132 was from Calbiochem-Novabiochem (Schwalbach, Germany). All other reagents, including agarose beads coupled to glutathione or Ni^{2+}, antibodies to myc, FLAG and α_1, penicillin, streptomycin, 3-isobutyl-1-methylxanthine (IBMX), sodium nitroprusside (SNP), bovine serum albumin, phenylmethylsulfonyl fluoride (PMSF), aprotinin, EGTA, EDTA, and pepstatin were from Sigma Chemical (St. Louis, MO).

Construction of Expression Plasmids for sGC Subunit Mutants and Transfections. The cDNAs for rat α_1 and β_1 and bovine hsp90β, as well as deletion mutants thereof, were N-terminally tagged with either the myc epitope (sGC subunits) or the HA and FLAG epitope (hsp90) using polymerase chain reaction and cloned into the pcDNA3.1/V5-His TOPO vector using standard methodology (Zhou et al., 2004). The β_1 N-terminally truncated mutants were tagged with V5/His_6. All cDNA constructs used in this study were sequenced before use. African green monkey kidney COSm6 cells were cultured in DMEM supplemented with 10% FCS. Cells plated in six-well plates at a density of 2×10^5 cells per well were grown overnight. They were transfected with appropriate plasmids using the jetPEI transfection reagent according to the manufacturers' in-

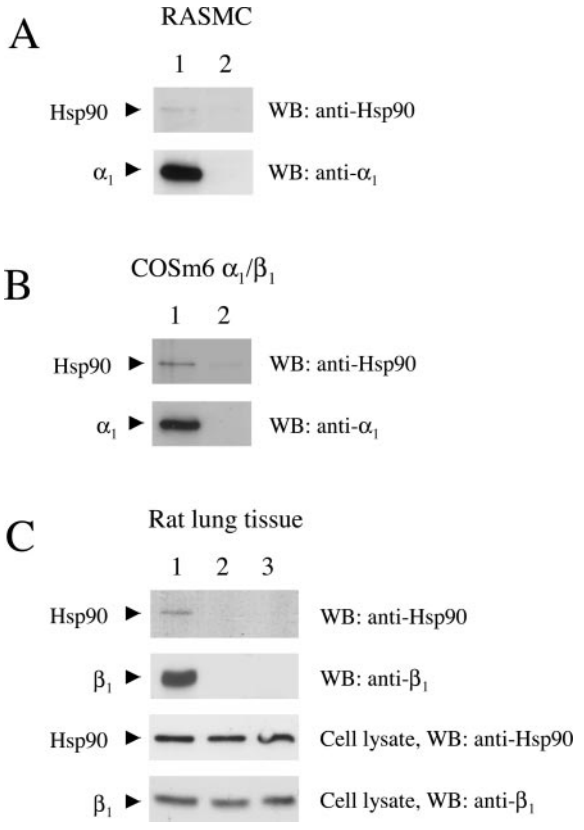

Fig. 1. Evidence for sGC binding to hsp90 in cells and tissue. Protein extracts (300–400 μg) from RASMC (lane 1 in A), COSm6 cells transfected with α_1 and β_1 (COSm6 α_1/β_1; lane 1 in B), or rat lung (lane 1 in C) were immunoprecipitated using anti-β_1 (1 μg) and were blotted for either hsp90 or the α_1 sGC subunit. Protein G beads (lane 2 in A–C) or rabbit IgG (lane 3 in C) were used as a control for the immunoprecipitation. Blots shown are representative of experiments repeated at least twice with identical results.

structions, applying a total of 3 μg of DNA and 6 μl of jetPEI per well. For cotransfection experiments, equal amounts of DNA were used for each plasmid.

Immunoprecipitation and Western Blotting. Cells were harvested 30 to 48 h after transfection and lysed in a buffer containing 1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 0.1 mM EGTA, 1 mM Na_3VO_4, 0.5% deoxycholic acid, 0.1% SDS, 10 mg/ml aprotinin, 10 mg/ml pepstatin, and 20 mM PMSF. Cellular debris was pelleted at 12,000*g* for 10 min, the supernatants were collected, and their protein concentrations were determined. Cell lysates containing 100 μg of protein were incubated overnight at 4°C with the antibody followed by protein G-coupled agarose beads; alternatively, myc-conjugated agarose beads or Ni^{2+}-conjugated agarose beads were used for myc-tagged and His-tagged proteins. Immunoprecipitated proteins or cell lysates were subjected to SDS-PAGE on 10% polyacrylamide gels and were transferred to nitrocellulose membranes. The membranes were blocked with 5% dry milk in TBST (10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween 20) for 1 h at room temperature, rinsed, and incubated overnight at 4°C with primary antibody in TBST. Thereafter, the blots were incubated with secondary antibody for 2 h at room temperature. Immunoreactive proteins were detected using the SuperSignal chemiluminescence kit.

In Vitro Binding Assays. Plasmid constructs encoding a chimeric protein consisting of glutathione *S*-transferase (GST) fused to the N terminus of sGCβ_1 were created by subcloning the full-length rat cDNA into the GST-fusion protein cloning vector pGEX-Kg. Deletion constructs for GST-β_1 were produced by polymerase chain reaction and were cloned into the same vector. GST-fusion proteins were expressed in *E. coli* and purified using standard methodology (Venema et al., 2003). For the binding assays, COSm6 cells lysates were prepared using the following buffer: 50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 0.1 mM EGTA, 1 mM Na_3VO_4, 0.1% SDS, 1% deoxycholic acid, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml leupeptin, and 20 mM PMSF for 20 min at 4°C under constant rotation. The samples were centrifuged for 30 min (12,000*g*) at 4°C. Lysates (1 mg of protein) were then incubated overnight at 4°C with beads containing 300 pmol GST-β_1 constructs in a total volume of 500 μl. After binding, the beads were washed (five to eight times) with wash buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 5 mM $MgCl_2$. To determine whether eNOS and sGC binding to hsp90 is mutually exclusive, GST-β_1 (5 μg) was mixed with purified hsp90 (0.25 μg) in the presence or absence of eNOS (1 μg). Hsp90 was preincubated with eNOS for 2 h at 4°C, then combined with the GST-β1, and incubated for an additional 2 h at 4°C in binding buffer (50 mM Tris-Cl, pH 7.4, and 20% glycerol). The

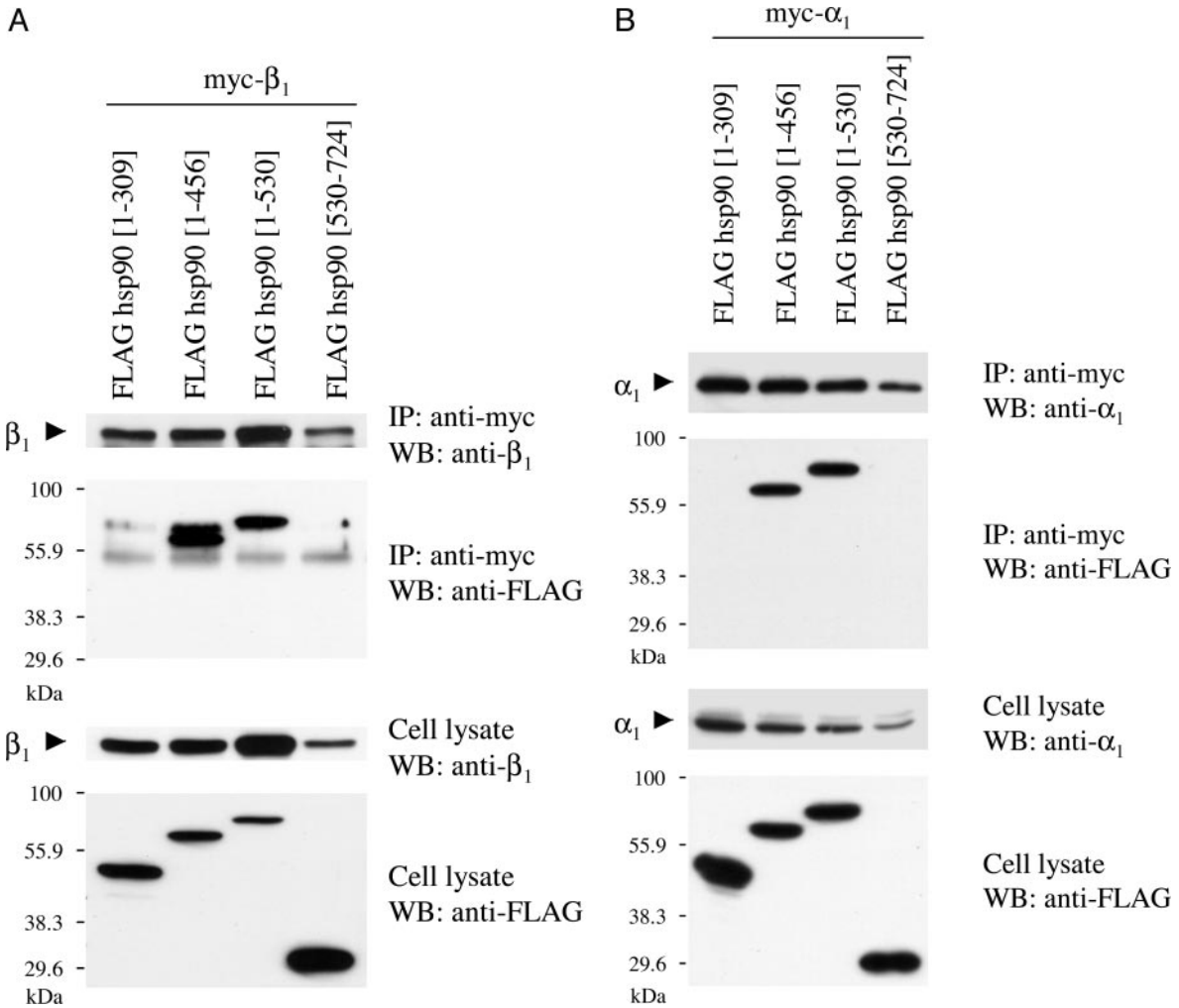

Fig. 2. Individually expressed sGC subunits bind to the M domain of hsp90. A, COS cells were cotransfected with the cDNAs encoding myc-tagged full-length β_1 and various deletion mutants of hsp90. IP was performed using an myc antibody, and the precipitates were analyzed by SDS-PAGE and Western blotting (WB) with an antibody to either β_1 or FLAG. Equal expression of β_1 and the hsp90 deletion mutants was monitored in the lysates. B, experimental setup is as in A, only cells were transfected with cDNAs encoding myc-tagged full-length α_1 and the deletion mutants of hsp90. Blots are representatives of experiments repeated at least twice with identical results. Numbers identify the relative positions in the amino acid sequence. Retained sequences are bracketed.

beads were washed five times with washing buffer (containing 400 mM NaCl). Beads were eluted by boiling in SDS sample buffer and subjected to SDS-polyacrylamide gel electrophoresis. Interacting proteins were identified by Western blotting with an hsp90 antibody.

sGC Binding to hsp90 in Smooth Muscle Cells. Cells lysates (20 μg) were immunoblotted (IB) for hsp90 or sGC. Another 100 μg of cell lysate was immunoprecipitated (IP) with an hsp90 antibody and blotted for either hsp90 or sGC. After transferring on the same membrane, membranes were cut and exposed to sGC and hsp90 antibodies separately, and the two films were developed. After scanning, optical density was calculated using image analysis software. The fraction of hsp90 recovered by IP/IB was determined as the ratio of hsp90 IP/IB to hsp90 whole-cell lysate divided by 0.2 (20/100 μg). The fraction of sGC bound to hsp90 was determined as the ratio of hsp90 IP/sGC IB to sGC whole-cell lysate divided by the ratio of hsp90 IP/IB to hsp90 whole-cell lysate.

cGMP Enzyme Immunoassay. Rat aortic smooth muscle (RASMC) cells (passages 3 to 7) were grown in 24 multiwell clusters with DMEM containing 10% FCS, 100U/ml penicillin, 100 mg/ml streptomycin, and 2 mM glutamine. Cells were treated for 24 h with 20 μM radicicol (RAD) or 1 μg/ml geldanamycin (GA) or both of the corresponding vehicles (ethanol for RAD and DMSO for GA); RAD-treated cells were also treated with DMSO, and GA-treated cells were also exposed to ethanol. In the experiments performed to assess the involvement of the proteasome in GA-induced down-regulation of sGC, RASMC were pretreated for 30 min with 10 μM MG132 before being exposed to the hsp90 inhibitor for 24 h. After the treatments, cells were washed with Hanks' balanced salt solution and incubated in Hanks' balanced salt solution in the presence of 1 mM concentration of the phosphodiesterase inhibitor IBMX for 15 min with or without 10 μM sodium nitroprusside. Media were aspirated, and 200 μl of 0.1 N HCl was added to extract cGMP. After 30 min, HCl extracts were collected, and cGMP was quantified by enzyme immunoassay using the cGMP enzyme immunoassay low pH kit according to the manufacturer's instructions.

Data Analysis and Statistics. Results are presented as means ± S.E.M. of the number of observations. Statistical comparisons between groups were made using the one-way analysis of variance followed by a post hoc test. Statistical differences were considered significant when $p < 0.05$.

Results

Mapping of the hsp90 Domain that Binds to sGC Subunits. To confirm the previously described interaction between hsp90 and sGC, we used native or transfected cultured cells and tissue. Immunoprecipitation of sGC β_1 from rat aortic smooth muscle cells, transfected COSm6 cells, and rat lung indicated that endogenous hsp90 exists in heterocomplexes with sGC under all of the conditions studied (Fig. 1). To characterize the region of the hsp90 involved in the interaction with sGC, we used the heterologous expression system and cotransfected cells with FLAG-tagged truncation mutants of hsp90 and myc-tagged full-length β_1. Anti-myc immunoprecipitates were immunoblotted with an anti-FLAG antibody. Despite similar levels of expression for the various forms of hsp90, only the longer variants 1 to 456 and 1 to 530, but not the shortest variant 1 to 309 or the C-terminal fragment 530 to 724, were present in anti-myc immunoprecipitates (Fig. 2A). Identical results were obtained using the reverse approach (i.e., by immunoprecipitating hsp90 from cell lysates using a FLAG antibody and analyzing them with an anti-β_1) (Supplemental Fig. S1). To test whether the α_1 subunit has the potential to directly bind to hsp90, we used the same approach that was used for β_1 (i.e., cotransfected cells with FLAG-tagged hsp90 and myc-α_1 in the absence of the β_1 subunit). In these experiments, we observed the presence of the hsp90 M domain-containing fragments in α_1 immunoprecipitates (Fig. 2B), indicating that in addition to the small sGC subunit, the large subunit of the most frequently occurring sGC isoform interacts, too, with hsp90 when expressed alone. Because sGC subunits interact with the same domain of hsp90 that eNOS interacts with, we sought to determine whether β_1 and eNOS binding to hsp90 is mutually exclusive. Data from these experiments suggest that GST-β_1 retains the ability to interact with hsp90/eNOS complexes (Fig. 3).

Mapping of the β_1 Region Involved in hsp90/sGC Heterocomplex Formation. To identify the region of β1 that binds to hsp90, we used two different approaches: in vitro binding assays, and immunoprecipitation studies. For the first approach, we generated GST-fusion proteins of β_1 and expressed them in *E. coli*. After purification, the fusion proteins were incubated with cell lysates from COS cells, precipitated, and subjected to SDS-PAGE and Western blotting. Using an antibody that recognizes hsp90, we observed the binding of this heat shock protein to GST-β_1, GST- β_1 (1–408), and GST-β_1 (67–377) but not to GST-β_1 (1–100), suggesting that β_1 uses its regulatory domain and possibly its dimerization region to bind to hsp90 (Fig. 4A). To determine whether the dimerization region is involved in binding to hsp90, cells were cotransfected with full-length HA-tagged hsp90 and full-length β_1 or mutants of β_1 lacking parts of the dimerization region tagged with V5/His_6. Using Ni^{2+}-conjugated agarose beads, we precipitated the β_1 subunit and analyzed the precipitates with an HA antibody. We observed that even the shortest N-terminal truncation mutant containing the entire dimerization region did not coprecipitate with hsp90 (Fig. 4B), indicating that the dimerization region of β_1 does not participate in hsp90/sCG heterocomplex formation. To more precisely define the area in the β_1 regulatory domain that binds to hsp90, we progressively deleted the first 61, 78, 140, or 160 residues of β_1 (Fig. 5A). Analysis of these N-terminally truncated mutants in cotransfection immunoprecipitation assays revealed that deletion of residues 63 to 78 reduced binding compared with 62 to 619 β_1 and that additional deletion of amino acids 79 to 140 did not further reduce binding to hsp90. A shorter N-terminally truncated mutant (161–619) displayed marked reduction of hsp90 binding and could only be clearly seen in overexposed film. Fi-

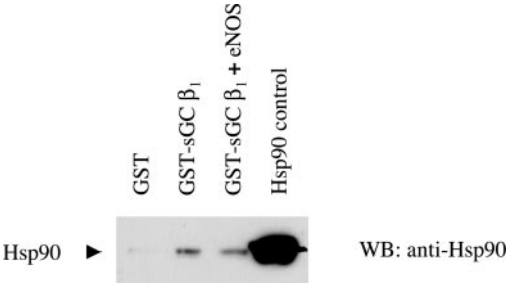

Fig. 3. Binding of eNOS and sGC to hsp90 is not mutually exclusive. A GST-fusion protein of the full-length β_1 was incubated with purified hsp90 or hsp90 and eNOS. After incubation at 4°C, samples were precipitated, washed, and eluted from the beads by boiling in SDS sample buffer. Protein samples were then subjected to SDS-PAGE, and membranes blotted with an hsp90 antibody. The last lane contains 10 ng of hsp90. Blots shown are representative of experiments repeated at least twice with identical results.

nally, the 204 to 619 mutant of β_1 did not exhibit any hsp90 binding ability. Internal deletion of residues 111 to 140, 141 to 159, 191 to 210, or 141 to 210 in the context of full-length β_1 did not affect binding to hsp90 (Fig. 5, B and C), whereas deleting residues 63 to 210 reduced but did not abolish the binding of β_1 to hsp90 (data not shown). These results taken together suggest that multiple residues dispersed throughout the regulatory domain participate in the interaction of β_1 and hsp90.

Binding of Heterodimeric sGC to hsp90. To estimate the amount of sGC bound to hsp90 in native cells, we performed immunoprecipitations and immunoblotting experiments in lysates from rat aortic smooth muscle cells. From the calculations described under *Materials and Methods,* approximately 20% of sGC can be found in complex with hsp90.

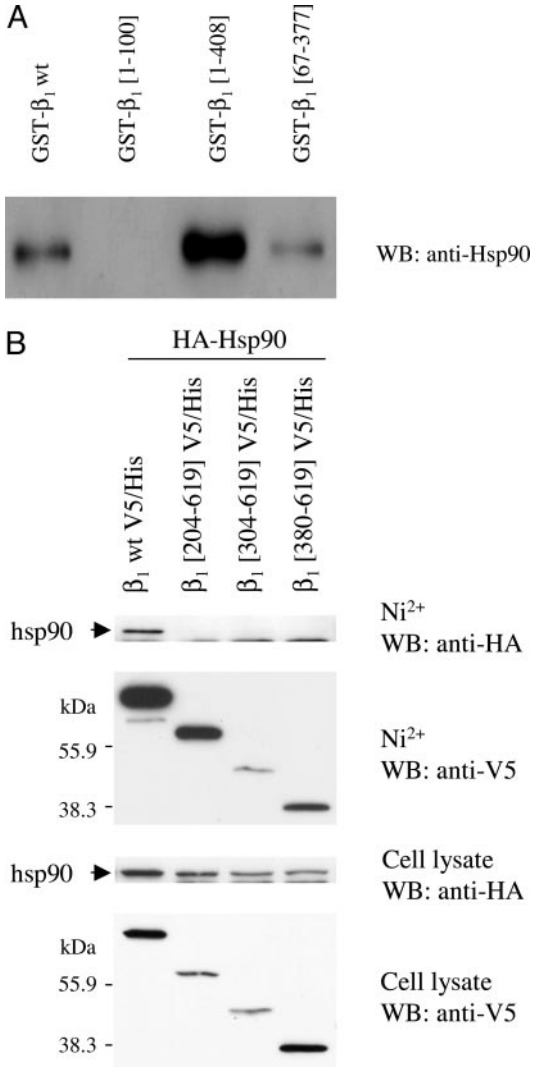

Fig. 4. The regulatory domain of β_1 mediates the interaction with hsp90. A, GST fusion proteins of the β_1 subunit were expressed in *E. coli* and purified; 300 pmol of each protein was incubated with COSm6 lysate (1 mg) overnight at 4°C. Samples were precipitated and eluted from the beads by boiling in SDS sample buffer. Protein samples were then subjected to SDS-PAGE, and membranes were blotted with an hsp90 antibody. B, cells were cotransfected with the cDNAs encoding HA-tagged full-length hsp90 and full-length or N-terminally deleted forms of β_1 tagged with V5/His_6. Ni^{2+}-agarose–bound His_6-tagged β_1 or total cell lysates were subjected to SDS-PAGE and blotted with anti-HA or anti-V5. Retained β_1 sequences are bracketed. Blots shown are representative of experiments repeated three times with identical results.

To evaluate whether the presence of the α_1 subunit affects β_1 binding to hsp90, we cotransfected full-length myc-tagged α_1 with the β_1 204 to 619 that lacks the ability to bind hsp90

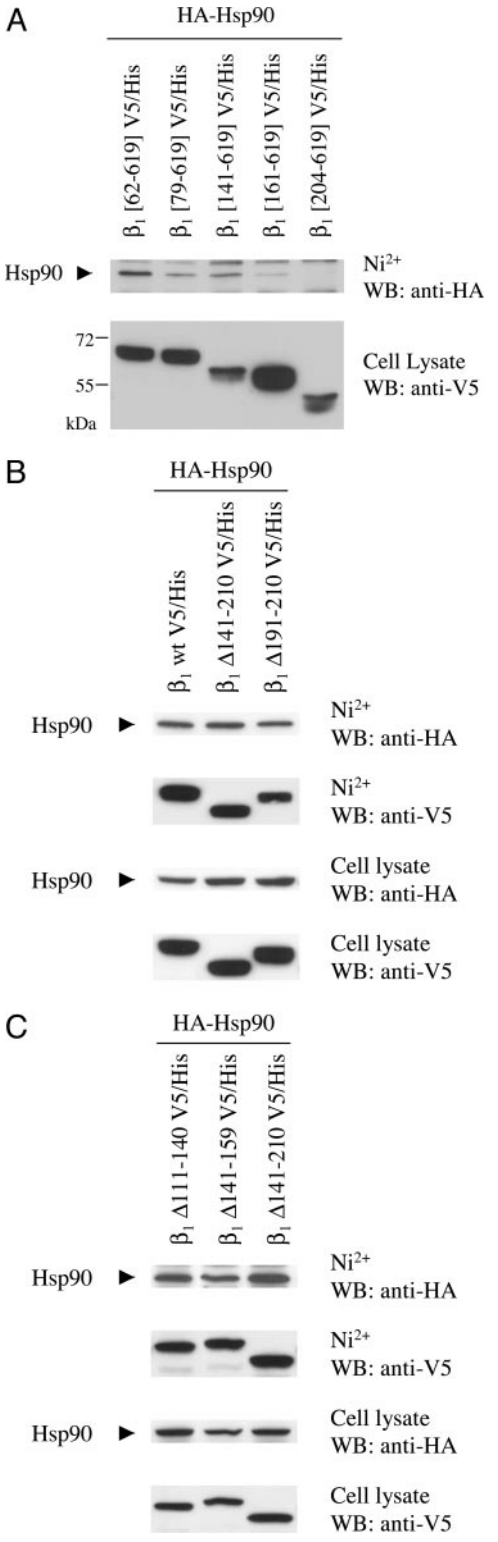

Fig. 5. A discontinuous region within the regulatory domain of β_1 is responsible for sGC/hsp90 binding. COS cells were cotransfected with cDNAs encoding the indicated mutant of β1 and full-length HA-tagged hsp90. Ni^{2+}-agarose–bound His_6-tagged β_1 or total cell lysates were subjected to SDS-PAGE and blotted with anti-HA or anti-V5. Numbers identify the relative positions in the amino acid sequence. Retained sequences are bracketed, and deleted sequences are indicated by Δ.

(Fig. 6A). As shown previously, this β_1 mutant binds to α_1; however, under these conditions, the sGC heterodimer did not exhibit hsp90 binding, suggesting that the native sGC heterodimer interacts with hsp90 only through the β_1 subunit. In addition, when full-length β_1 was coexpressed with α_1, reduced amounts of hsp90 bound to sGC were noted (Fig. 6B).

Effects of hsp90 Inhibition on sGC Levels and Activity. To examine the functional significance of the hsp90/sGC interaction, we treated RASMC cells that endogenously express sGC with the hsp90 inhibitors GA (1 μg/ml) and RAD (20 μM) and assessed sGC subunit protein levels and cGMP accumulation. When cells were treated for up to 1 h with either GA or RAD, protein levels were not altered. On the other hand, long-term treatment (24 and 48 h) of the cells with the hsp90 inhibitors resulted in a profound decrease of both α_1 and β_1 protein levels (Fig. 7A). The decrease in sGC subunit levels brought about by the hsp90 inhibitors was paralleled by a reduction in NO-stimulated cGMP accumulation in the smooth muscle cells (Fig. 7B). It is interesting that pretreatment of smooth muscle cells with the proteasome inhibitor MG132 prevented the GA-induced reduction in α_1 and β_1 (Fig. 8A), suggesting that when not associated with hsp90, the sGC subunits became degraded through the proteasome pathway. Pretreatment of smooth muscle cells with the proteasome inhibitor before GA exposure also restored their responsiveness to nitric oxide (Fig. 8B).

Discussion

The NO-cGMP pathway plays an important role in cardiovascular homeostasis by regulating smooth muscle tone, reducing platelet aggregation, and modulating angiogenesis and vascular remodeling (Moncada et al., 1991; Ziche et al., 1994; Papapetropoulos et al., 1997; Rudic et al., 1998). Components of this pathway include 1) the NO synthases (NOS) that produce NO; 2) sGC, which acts as the NO receptor; and 3) effector molecules such as cGMP-dependent protein kinases, channels, and phosphodiesterases (Forstermann et al., 1994; Andreopoulos and Papapetropoulos, 2000; Lucas et al., 2000). Members of the NOS family and sGC have already been found to bind hsp90. Hsp90 is important for heme binding, folding, and NO synthesis by neuronal NOS and also suppresses the generation of superoxide anions by this enzyme (Bender et al., 1999; Billecke et al., 2002). Hsp90 also binds eNOS and promotes its activation by reducing binding of the inhibitory protein caveolin-1 (Garcia-Cardena et al., 1998; Gratton et al., 2000). In addition, hsp90 recruits the serine/threonine kinase Akt to the hsp90/eNOS complex, facilitating eNOS phosphorylation by Akt, an event associated with increased NO production (Fontana et al., 2002).

Venema et al. (2003) recently showed that sGC coimmunoprecipitates with hsp90 in vascular cells. Moreover, this interaction is regulated by endothelial cell activators such as bradykinin and vascular endothelial growth factor, both of which promote recruitment of sGC to the hsp90/eNOS complex in a geldanamycin-sensitive manner. In the present study, we confirmed the hsp90/sGC interaction in native tissue (lung) and cultured cells and used a heterologous expression system to structurally characterize hsp90 binding to sGC. We have found that hsp90/sGC heterocomplex formation is mediated by the M region of hsp90, the same region that mediates binding of hsp90 to eNOS. Although it is unknown whether binding of eNOS changes the affinity of hsp90 for sGC, we have observed both previously (Venema et al., 2003) and in the current set of experiments that binding of the two proteins (eNOS and sGC) to hsp90 is not mutually exclusive. The existence of a hsp90/eNOS/sGC complex in endothelial cells would increase the efficacy of NO as the proximity of eNOS to sGC would prevent inactivation of NO by superoxide anions. In a previous study, data from in vitro binding assays suggested that only the β_1 subunit has the ability to interact with purified hsp90 (Venema et al., 2003), whereas in the present study, both sGC subunits were shown to bind hsp90 when expressed individually. This discrepancy could be accounted for if binding of α_1 to hsp90 requires an accessory protein. On the other hand, the differences observed could be attributed to the fact that in the in vitro binding assays, the α_1 used was as a GST fusion and not in its native form.

To better characterize the region of the β_1 subunit that

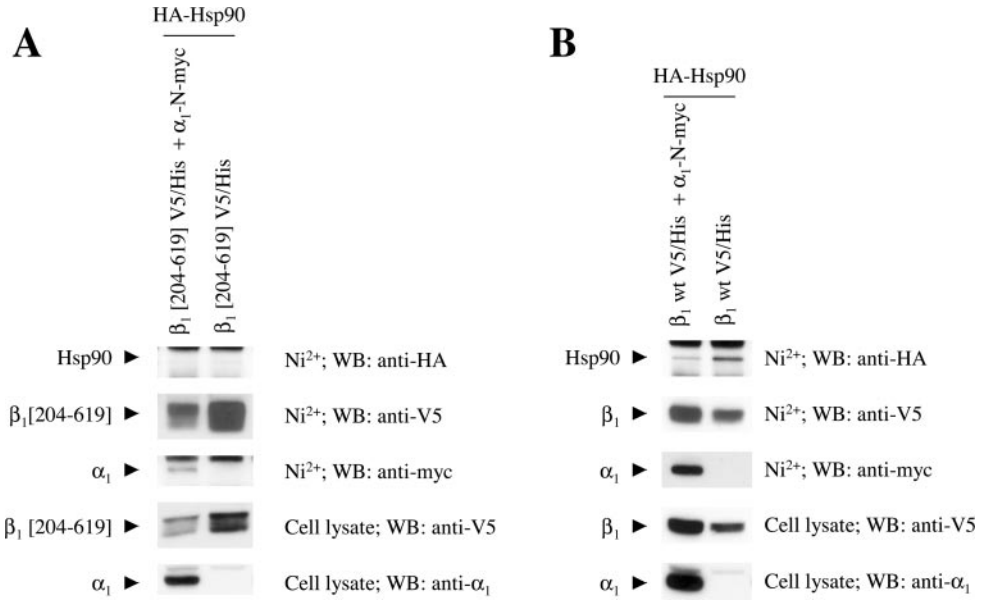

Fig. 6. The sGC heterodimer binds hsp90 through the β_1 subunit. A, COS cells were cotransfected with the cDNAs encoding HA-tagged hsp90, the 204–619 truncation mutant of β_1 (tagged with V5/His) in the presence or absence of full-length α_1. The β_1 was precipitated using a Ni^{2+} column, and precipitates were blotted with anti-HA, V5, or myc. B, same as in A, only full-length β_1 was used instead of the truncation mutant. Blots shown are representative of experiments repeated twice with identical results.

participates in the binding with hsp90, we used deletion mutants of this subunit and incubated them with cytosolic proteins from COS, as a source of hsp90. In line with our previous observations (Venema et al., 2003), full-length β_1 expressed as a GST-fusion protein bound hsp90. Moreover, we observed that the catalytic domain of β_1 is not required for the interaction of the two proteins, because a C-terminally truncated version of β_1 lacking this domain also bound hsp90. It is interesting that the 1-to-408 truncation form of β_1 seemed to have higher hsp90 binding affinity; if this does not represent an in vitro binding artifact, it could perhaps be caused by the fact that in the β_1 mutant lacking the catalytic domain, the hsp90-binding region is more accessible to the chaperone.

The central part of sGC is involved in the formation of heterodimers, which is a prerequisite for the exhibition of catalytic activity (Buechler et al., 1991). We identified a sequence segment spanning positions 204 to 408 that mediates the binding of β_1 to α_1 (Zhou et al., 2004); within this region, two distinct segments contribute to α_1 binding: an N-terminal site (residues 204–244) and a C-terminal site (residues 379–408). To determine whether either of these areas of β_1 participates in heterocomplex formation with hsp90, we used truncated variants of β_1 carrying deletions of the N-terminal site and/or C-terminal site. All three N-terminally truncated β_1 mutants used (204–619, 304–619, and 380–619) showed no hsp90-binding ability. In line with this observation, deletion of residues 204 to 303 or 346 to 408 in the context of full-length β_1 did not alter hsp90 binding (data not shown), further suggesting that the dimerization region is not important for the hsp90/β_1 interaction. Although the dimerization region was not necessary for the hsp90/β_1 binding, we can not rule out that association of sGC subunits with hsp90 aids in the formation of mature heterodimers. To more precisely map the amino acid sequence within the regulatory domain that mediates the hsp90/β_1 interaction, we used additional β_1 mutants that lacked parts of the regulatory domain (β_1 N-terminal truncation mutants or mutants carrying internal deletions in the context of a full-length subunit). Taken together, observations from these experiments suggest that a discontinuous region which spreads throughout the first 200 amino acids of the regulatory domain participates in β_1 binding to hsp90 and that preservation of some of

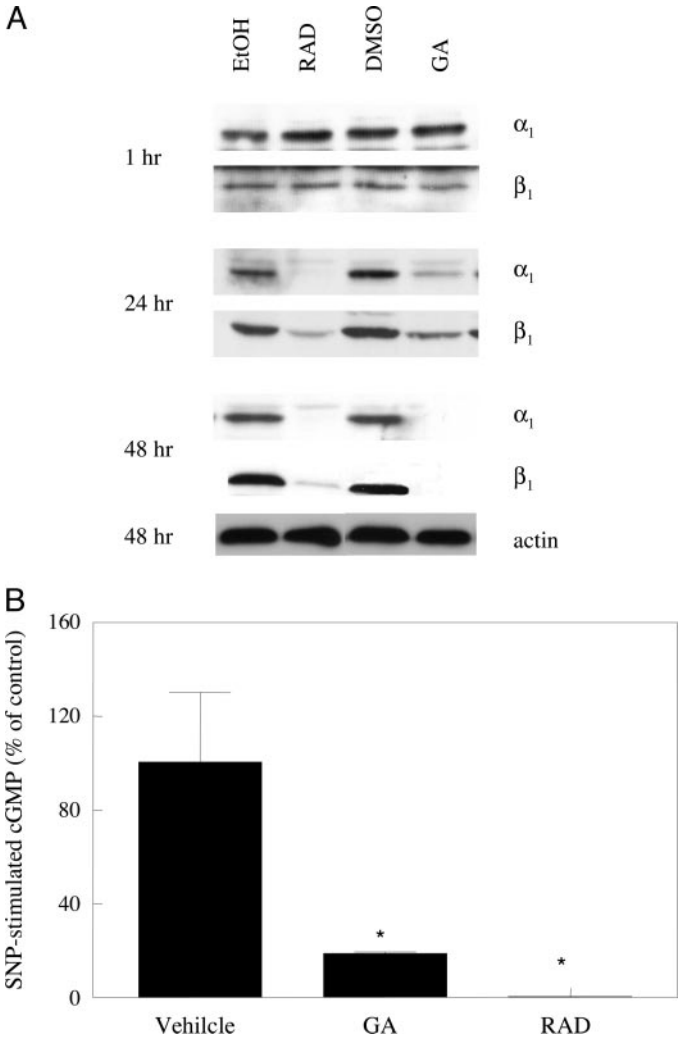

Fig. 7. Inhibition of hsp90 down-regulates sGC. A, rat aortic smooth muscle cells were treated for the indicated time with RAD (20 μM), GA (1 μg/ml), or the corresponding vehicle. Cell lysates were then prepared, subjected to SDS-PAGE, and blotted with sGC subunit antibodies. Blots are representatives of experiments repeated at least twice with similar results. Actin was used as a loading control. B, rat aortic smooth muscle cells were incubated with RAD (20 μM), GA (1 μg/ml), or vehicle for 24 h. After the end of the incubation period, cells were washed with Hanks' balanced salt solution and incubated in the presence of 10 μM sodium nitroprusside for 15 min. Throughout the 15-min period, cultures were exposed to the phosphodiesterase inhibitor IBMX (1 mM). Means ± S.E.M., $n = 4$ wells, ⋆, $p < 0.05$ from vehicle.

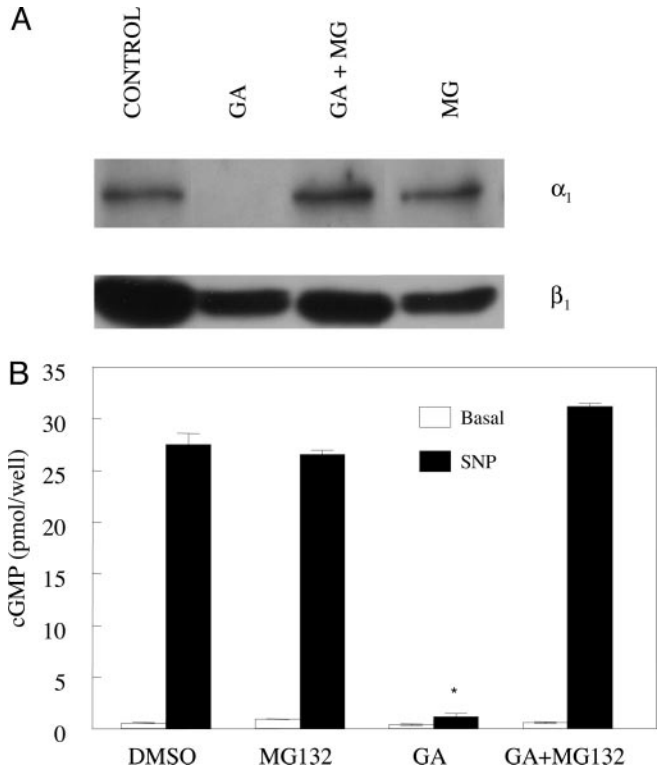

Fig. 8. Proteasome inhibition blocks the geldanamycin-induced down-regulation of sGC. A, rat aortic smooth muscle cells were pretreated with 10 μM concentrations of the proteasome inhibitor MG132 for 2 h and then incubated for an additional 24 h with vehicle (DMSO) or GA (1 μg/ml). Cell lysates were then prepared, subjected to SDS-PAGE, and blotted with sGC subunit antibodies (A). Blots are representatives of experiments repeated twice. B, cGMP accumulation was measured in the absence (basal) or presence of an NO donor (SNP, 10 μM), as described above. Means ± S.E.M., $n = 4$ wells, ⋆, $p < 0.05$ from vehicle.

the residues that mediate the hsp90/ β_1 interaction in the β_1 mutant proteins is enough to secure strong binding to hsp90.

All naturally occurring sGC heterodimers identified to date contain a β_1 subunit (Hobbs, 1997; Russwurm et al., 1998). The α_1/β_1 is the predominant form of sGC exhibiting higher expression than the α_2/β_1 in all tissues studied, with the exception of the brain, in which the two sGC forms are equally expressed (Mergia et al., 2003). To determine whether the interaction between individually expressed sGC subunits and hsp90 differs from that of the native heterodimeric enzyme, we cotransfected cells with a deletion mutant of β_1 that does not bind hsp90 and full-length α_1. Under these conditions, heterodimers were formed, but they did not interact with hsp90, suggesting that although both sGC subunits have the ability to bind hsp90 when individually expressed, binding of the sGC heterodimer involves only the β_1 subunit.

Protein-protein interactions can alter both the activity and subcellular localization of the interacting partners. In the case of sGC, CCTη interacts with β_1-inhibiting NO-stimulated sGC activity (Hanafy et al., 2004), whereas interaction of sGC with hsp70 increases the cGMP-forming ability of the cyclase (Balashova et al., 2005). Subcellular localization of sGC is also regulated by protein-protein interactions. The α_2/β_1 isoform of sGC is recruited to synaptic membranes after binding of its C terminus to postsynaptic density 95 (Russwurm et al., 2001). Moreover, Zabel et al. (2002) showed that similarly to the α_2/β_1, up to 20% of the α_1/β_1 isoform can be found in the membrane fraction of tissues (Zabel et al., 2002), offering additional proof that sGC is not, as originally believed, entirely cytosolic. Use of detergents like Triton X-100 during tissue homogenization is accompanied by the loss of sGC from the membrane fraction. The frequent inclusion of detergents in purification buffers might explain why investigators previously failed to observe the presence of membrane-associated sGC. Moreover, the labile nature of the sGC association with the plasma membrane suggests that this occurs through a protein-protein interaction. It is interesting that the distribution of sGC between membrane and cytosolic fraction differs with cell type: 80% of sGC in endothelial cells is membrane-associated, and only 20% is in a freely soluble cytosolic form, whereas these percentages are reversed in vascular smooth muscle (Venema et al., 2003). One could speculate that the presence of eNOS in the endothelium recruits sGC to the plasma membrane through their mutual interaction with hsp90, whereas in the eNOS-negative smooth muscle cells, sGC remains mostly in the cytosol.

The physiological importance of hsp90/sGC interaction is still a matter of investigation and debate. Pretreatment of human umbilical vein endothelial cells with GA did not affect SNP-induced cGMP accumulation, and incubation of rat aortic rings with the same hsp90 inhibitor did not alter the vasodilatory response to nitroglycerin (Garcia-Cardena et al., 1998). Moreover, GA did not modify the vasodilatory action of SNP in perfused rat mesenteric vessels (Shah et al., 1999). Taken together, these data suggest that interruption of the hsp90/sGC heterocomplex formation in an acute fashion ($\leq$1 h) does not affect the ability of sGC to respond to NO donors. It should be noted that in all of the cases mentioned above, incubation of cells or tissues with GA reduced their ability to produce NO. Yet two other studies have yielded different results. GA and radicicol reduced the vasodilation brought about by DETA NONOate in mouse arterioles (Ou et al., 2004); this effect, however, was only evident at high and not at low concentrations of the NO donor. Moreover, pretreatment of bovine aortic endothelial cells with GA reduced the SNP-stimulated cGMP accumulation, whereas treatment of anesthetized rats with GA inhibited the SNP-induced reduction of both systolic and diastolic blood pressure (Venema et al., 2003). These latter findings argue in favor of the hypothesis that the hsp90/sGC interaction improves the responsiveness of sGC to NO donors. In the present study, we explored the impact of long-term hsp90 inhibition on the function of sGC. Incubation of rat aortic smooth muscle cells with two different hsp90 inhibitors (GA or radicicol) for 24 to 48 h drastically reduced the levels of α_1 and β_1, suggesting that blockade of the hsp90/sGC interaction destabilizes both subunits of the enzyme. The possibility that reactive oxygen species released from GA (Dikalov et al., 2002), rather than hsp90 inhibition, is responsible for the reduction in sGC levels can be ruled out because radicicol exhibits a similar effect. Treatment of cells with hsp90 inhibitors has also been shown to affect the levels of other hsp90 client proteins. For example, disrupting the hsp90/nNOS interaction promotes ubiquitination and proteolytic degradation of the latter protein (Bender et al., 1999). As expected, the reduction in α_1 and β_1 levels was accompanied by an inhibition of NO-stimulated cGMP formation. To further investigate the mechanism of GA-induced inhibition of sGC subunit levels, cells were incubated with the proteasome inhibitor MG132. Such treatment restored α_1 and β_1 levels and NO responsiveness, suggesting that restricting the hsp90/sGC heterocomplex formation targets sGC for proteasomal degradation, presumably as a mechanism of removal of misfolded proteins.

In summary, we have shown that when individually expressed, both sGC subunits bind hsp90, but only the β_1 associates with hsp90 in the context of the native heterodimeric form of the enzyme. We also have shown that the M domain of hsp90 and the regulatory domain of β_1 participate in this interaction. The function of hsp90/sGC heterocomplex may differ with cell type: in smooth muscle and in other cell types in which the two proteins are coexpressed, it might be important for sGC stability and/or processing allowing for high levels of sGC expression. In cells that express eNOS or nNOS along with sGC, hsp90, in addition to preserving sGC levels, would facilitate the autocrine actions of NO by bringing together the NO source and its target; the proximity of NOS with sGC could preserve critical NO functions during oxidative stress.

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