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# Interaction between hsp90 and soluble guanylyl cyclase: physiological significance and mapping of the domains mediating binding

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**Running Title**: sGC and hsp90 interaction

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Abbrevitions: endothelial nitric oxide synthase (eNOS); geldanamycin (GA); glutathione S-transferase (GST); Hanks' balanced salt solution (HBSS); Heat shock protein 90 (hsp90); isobutylmethylxanthine (IBMX); nitric oxide (NO); radicicol (RAD); rat smooth muscle cells (RASMC); sodium nitroprusside (SNP); soluble guanylyl cyclase (sGC

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## ABSTRACT

Heat shock protein 90 (hsp90) regulates stability and function of many client proteins including members of the NO-cGMP signaling pathway. Soluble guanylyl cyclase (sGC), a nitric oxide (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 $\beta_1$ in the heterodimeric form of the enzyme. Characterization of the region of hsp90 required to bind each subunit in immunoprecipitation experiments, revealed that residues 310-456 of hsp90 interact with the sGC subunits. The region of $\beta_1$ responsible for binding to hsp90β was mapped using *in vitro* binding assays and immunoprecipitation experiments and found to lie in the regulatory domain. The physiological importance of the hsp90/sGC interaction was investigated by treating rat smooth muscle cells (RASMC) with the hsp90 inhibitors radicicol (RAD) and geldanamycin (GA) and determining both sGC activity and protein levels. Long-term (24 or 48hr) inhibition of hsp90 resulted in a strong decrease of both $\alpha_1$ and $\beta_1$ protein levels, as well as sGC activity. Moreover, incubation of smooth muscle cells with the proteasome inhibitor MG132 blocked the GA-induced downregulation of sGC. We conclude that the N-terminal region of the $\beta_1$ 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 enzyme by affecting the protein levels of the two subunits.

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Heat shock protein 90 (hsp90) is one of the most abundant cytosolic proteins in eukaryotes, amounting to 1-2% of the total soluble protein even under resting conditions. Two isoforms of hsp90 exist: hsp90α is inducible by heat shock and other stressful stimuli, while hsp90β is constitutively expressed(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 co-chaperones, the identity of which depends on the protein being folded(Buchner, 1999; Pratt, 1998; 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 with hsp90 in order 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(Fontana et al., 2002; Pratt and Toft, 1997). On the other hand, the C-terminal domain binds proteins with

tetratricopeptide repeats (for example immunophillin) and facilitates homodimer formation(Sreedhar et al., 2004).

Soluble guanylyl cyclase (sGC) is the best studied receptor for the labile signaling molecule nitric oxide (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 $\alpha_1\beta_1$ that is expressed in all tissues studied so far(Budworth et al., 1999). sGC subunits are divided in three domains: an N-terminal domain, that 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(Foerster et al., 1996; Wedel et al., 1995; Wedel et al., 1994). The central domain contains the information needed for subunit dimerization(Zhou Z, 2004). Finally, residues that are important for substrate recognition and catalytic activity are distributed on the C-terminal domains of the $\alpha_1$ and $\beta_1$ subunits(Sunahara et al., 1998).

Recently, we reported that 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, as the hsp90/$\beta_1$ heterocomplex is resistant to high salt concentrations(Venema et al., 2003). In the present study we set out to 1) determine the regions of the two proteins that participate in the interaction and 2) evaluate the effect of hsp90 inhibition on sGC function. We observed that the regulatory domain of $\beta_1$ in the $\alpha_1/\beta_1$ sGC heterodimer

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mediates binding to hsp90 and that this interaction facilitates NO-signalling since it preserves high levels of sGC expression.

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## MATERIAL & METHODS

*Materials* — Dulbecco's Modified Eagle Medium (DMEM) and fetal calf serum (FCS) were obtained from GIBCO-BRL (Paisley, UK). Cell culture plastic ware was from Greiner (Frickenhausen, Germany); BL21-codon plus strain of *E. coli* from Stratagene (La Jolla, CA, USA); monoclonal anti-V5 antibody, platinum *Pfx* DNA polymerase and pcDNA3.1 Directional TOPO Expression kit from Invitrogen (Paisley, UK); dNTPs were purchased from 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, USA); SuperSignal West Pico chemiluminescent substrate from Pierce (Rockford, IL, USA); *DC* Protein assay kit, Tween 20 and other immunoblotting reagents from BioRad (Munich, Germany); jetPEI transfection reagent from Polyplus-transfection (Illkirch, France); protein G-agarose beads and nitrocellulose membrane Hybond ECL from Amersham Biosciences (Vienna, Austria). Radicicol was purchased from Tocris Cookson (Avonmouth, UK). The anti-HA was obtained from Boehgringer-Mannheim (Mannheim-Germany); the anti-hsp90 was from Stressgen (Victoria, BC Canada) and the anti-$\beta_1$ was from Cayman Chemicals (Ann Arbor MI); MG132 was from Calbiochem-Novabiochem (Schwalbach, Germany). All other reagents including agarose beads coupled to glutathione or $Ni^{+2}$, antibodies to myc, FLAG and $\alpha_1$, penicillin, streptomycin, isobutylmethylxanthine (IBMX), sodium nitroprusside (SNP), bovine serum albumin, phenylmethylsulfonyl fluoride (PMSF), aprotinin, EGTA, EDTA, and pepstatin were from Sigma (St. Louis, MO, USA).

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*Construction of Expression Plasmids for sGC Subunit Mutants and transfections* — The cDNAs for rat $\alpha_1$ and $\beta_1$ 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 PCR and cloned into the pcDNA3.1/V5-His TOPO vector using standard methodology(Zhou Z, 2004). The $\beta_1$ N-terminally truncated mutants were tagged with V5/$His_6$. All cDNA constructs used in this study were sequenced prior to use. African green monkey kidney COSm6 cells were cultured in DMEM supplemented with 10% FCS. Cells plated in 6-well plates at a density of 2 x $10^5$ cells per well were grown overnight. They were transfected with appropriate plasmids using the jetPEI transfection reagent according to the manufacturers' instructions, applying a total of 3 µg DNA and 6 µl of jetPEI per well. For co-transfection 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 µg/ml aprotinin, 10 µg/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 determined. Cell lysates containing 100 µg of protein were incubated overnight at 4 ºC 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 transferred to nitrocellulose membranes. The membranes were blocked with 5% dry milk in TBS-T (10 mM Tris, pH 7.5, 100 mM NaCl, 0.1%

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Tween-20) for 1 h at room temperature, rinsed and incubated overnight at 4 ºC with primary antibody in TBS-T. Subsequently, 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 sGC$\beta_1$ were created by subcloning the full-length rat cDNA into the GST-fusion protein-cloning vector pGEX-Kg. Deletion constructs for GST-$\beta_1$ were produced by PCR and cloning 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% NP40, 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 20min at 4°C under constant rotation. The samples were centrifuged for 30min (12,000xg) at 4°C. Lysates (1mg of proteins) were then incubated overnight at 4°C with beads containing 300pmol of the GST- $\beta_1$ constructs in a total volume of 500µl. After binding the beads were washed (5-8 times) with wash buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5mM $MgCl_2$. To determine whether eNOS and sGC binding to hsp90 is mutually exclusive, GST- $\beta_1$ (5 µg) was mixed with purified hsp90 (0.25µg) in the presence or absence of eNOS (1µg). Hsp90 was pre-incubated with eNOS for 2 hrs at 4º C then combined with the GST-β1 and incubated for an additional 2 hrs at 4º C in binding buffer (50 mM Tris-Cl, pH 7.4, 20% glycerol). The beads were washed 5 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 biniding to hsp90 in smooth muscle cells.* Cells lysates (20μg) were immunoblotted (IB) for hsp90 or sGC. Another 100μg of cell lysate were immunoprecipitated (IP) with an hsp90 Ab and blotted for either hsp90 or sGC. After transferring on the same membrane, membranes were cut and exposed to sGC and hsp90 Abs 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 (20μg/100μg). 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 3-7) were grown in 24 multi-wel clusters with DMEM containing 10% FCS, 100U/ml penicillin, 100mg/ml streptomycin and 2mM glutamine. Cells were treated for 24hr with 20μM radicicol (RAD) or 1μg/ml geldanamycin (GA) or both 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 EtOH. In the experiments performed to assess the involvement of the proteasome in GA-induced downregulation of sGC, RASMC were pretreated for 30 min with 10μM MG132 prior to being exposed to the hsp90 inhibitor for 24hr. After the treatments, cells were washed with Hanks' balanced salt solution (HBSS) and incubated in HBSS in the presence of 1 mM of the phosphodiesterase inhibitor IBMX for 15 min with or

without 10μM 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 EIA low pH kit according to the manufacturers' instructions.

*Data analysis and statistics*. Results are presented as means± SEM of the number of observations. Statistical comparisons between groups were made using the one-way ANOVA followed by a host-hoc test. Statistical differences were considered significant when $p<0.05$.

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## RESULTS

*Mapping of the hsp90 domain that binds to sGC subunits*

In order to confirm the previously described interaction between hsp90 and sGC, we utilized native or transfected cultured cells, as well as tissue. Immunoprecipitation of sGC $\beta_1$ from rat aortic smooth muscle cells, transfected COSm6 cells and rat lung indicated that endogenous hsp90 exists in heterocomplexes with sGC under all 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 co-transfected cells with FLAG-tagged truncation mutants of hsp90 and myc-tagged full-length $\beta_1$. Anti-myc immunoprecipitates were immunoblotted with an anti-FLAG antibody. In spite of similar levels of expression for the various forms of hsp90, only the longer variants 1-456 and 1-530, but not the shortest variant 1-309 or the C-terminal fragment 530-724, 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 analysing them with an anti-$\beta_1$ (supplemental on-line data, Fig.S1). To test if the $\alpha_1$ subunit has the potential to directly bind to hsp90, we used the same approach that was employed for $\beta_1$, i.e. co-transfected cells with FLAG-tagged hsp90 and myc-$\alpha_1$ (in the absence of the $\beta_1$ subunit). In these experiments, we observed the presence of the hsp90 M domain-containing fragments in $\alpha_1$ 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. As sGC subunits interact with the same domain of hsp90 that eNOS interacts with, we sought to determine whether $\beta_1$ and eNOS binding to hsp90 is mutually exclusive. Data from these experiments

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suggest that GST-$\beta_1$ retains the ability to interact with hsp90/eNOS complexes (Fig.3).

*Mapping of the $\beta_1$ 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 $\beta_1$ 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 binding of this heat shock protein to GST- $\beta_1$, GST- $\beta_1$ [1-408] and GST-$\beta_1$ [67-377], but not to GST-$\beta_1$ [1-100], suggesting that $\beta_1$ 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 co-transfected with full-length HA-tagged hsp90 and full-length $\beta_1$ or mutants of $\beta_1$ lacking parts of the dimerization region, tagged with V5/$His_6$. Using $Ni^{+2}$ conjugated agarose beads we precipitated the $\beta_1$ 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 co-precipitate with hsp90 (Fig.4B), indicating that the dimerization region of $\beta_1$ does not participate in hsp90/sCG heterocomplex formation. To more precisely define the area in the $\beta_1$ regulatory domain that binds to hsp90, we progressively deleted the first 61, 78, 140 or 160 residues of $\beta_1$ (Fig.5A). Analysis of these N-terminally truncated mutants in co-transfection immunoprecipitation assays revealed that deletion of residues 63-78 reduced binding compared to 62-619 $\beta_1$ and that additional deletion of amino acids 79-140 did not further reduce binding to hsp90. A shorter N-terminally truncated mutant (161-619)

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displayed marked reduction of hsp90 binding and could only be clearly seen in overexposed film. Finally, the 204-619 mutant of $\beta_1$ did not exhibit any hsp90 binding ability. Internal deletion of residues 111-140, 141-159, 191-210 or 141-210 in the context of full-length $\beta_1$ did not affect binding to hsp90 (Fig.5B & C), while deleting residues 63-210 reduced, but did not abolish, binding of $\beta_1$ to hsp90 (data not shown). These results taken together suggest that multiple residues dispersed throughout the regulatory domain participate in the interaction of $\beta_1$ and hsp90.

*Biding 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. Based on the calculations described in the materials and methods section about 20% of sGC can be found in complex with hsp90. To evaluate whether the presence of the $\alpha_1$ subunit affects $\beta_1$ binding to hsp90, we co-transfected full-length myc-tagged $\alpha_1$ with the $\beta_1$ 204-619 that lacks the ability to bind hsp90 (Fig.6A). As previously shown, this $\beta_1$ mutant binds to $\alpha_1$; however, under these conditions the sGC heterodimer did not exhibit hsp90 binding, suggesting that native sGC heterodimer interacts with hsp90 only through the $\beta_1$ subunit. In addition, when full-length $\beta_1$ was co-expressed with $\alpha_1$ 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 geldanamycin (GA, 1μg/ml) and radicicol (RAD, 20μM) and assessed sGC subunit

protein levels, as well as cGMP accumulation. When cells were treated for up to 1hr with either GA or RAD protein levels were not altered. On the other hand, long-term treatment (24 and 48hrs) of the cells with the hsp90 inhibitors resulted in a profound decrease of both $\alpha_1$ and $\beta_1$ 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 (Fig7B). Interestingly, pretreatment of smooth muscle cells with the proteasome inhibitor MG132 prevented the GA-induced reduction in $\alpha_1$ and $\beta_1$ (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 prior to GA exposure, also restored their responsiveness to nitric oxide (Fig.8B).

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## 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 remodelling(Moncada et al., 1991; Papapetropoulos et al., 1997; Rudic et al., 1998; Ziche et al., 1994). Components of this pathway include 1) the NO synthases (NOS) that produce NO; 2) sGC, that acts as the NO receptor; and 3) effector molecules like cGMP-dependent protein kinases, channels and phosphodiesterases(Andreopoulos and Papapetropoulos, 2000; Forstermann et al., 1994; 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 nNOS 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). Additionally, 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., recently showed that sGC co-immunoprecipitates with heat shock protein 90 (hsp90) in vascular cells(Venema et al., 2003). Moreover, this interaction is regulated by endothelial cell activators, such as bradykinin and VEGF, both of which promote recruitment of sGC to the hsp90/eNOS complex in a geldanamycin-sensitive manner. In the present study we have confirmed the hsp90/sGC interaction in native tissue (lung) and cultured cells, and utilized a heterologous expression system to structurally characterize hsp90 binding to sGC. We have found that hsp90/sGC

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heterocomplex formation is mediated by the M region of hsp90, the same region that mediates binding of hsp90 to eNOS. Although it is unknown if 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 an 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 $\beta_1$ subunit has the ability to interact with purified hsp90 (Venema et al., 2003), while in the present study both sGC subunits were shown to bind hsp90 when expressed individually. This discrepancy could be accounted for if binding of $\alpha_1$ to hsp90 requires an accessory protein. Alternatively the differences observed could be attributed to the fact that in the *in vitro* binding assays the $\alpha_1$ used was a GST fusion and not the native subunit.

To better characterize the region of the $\beta_1$ subunit that 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 $\beta_1$ expressed as a GST fusion bound hsp90. Moreover, we observed that the catalytic domain of $\beta_1$ is not required for the interaction of the two proteins, as a C-terminally truncated version of $\beta_1$ lacking this domain also bound hsp90. Interestingly, the 1-408 truncation form of $\beta_1$ seemed to have higher hsp90-binding affinity; if this does not represent an in-vitro-binding artefact, it could perhaps be due to the fact that in the $\beta_1$ mutant lacking the catalytic domain the hsp90-binding region is more accessible to the chaperone.

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The central part of sGC is involved in the formation of heterodimers, which is a pre-requisite for the exhibition of catalytic activity(Buechler et al., 1991). We identified a sequence segment spanning positions 204 to 408 that mediates binding of $\beta_1$ to $\alpha_1$(Zhou Z, 2004); within this region two distinct segments contribute to $\alpha_1$ binding, an N-terminal site (NBS; residues 204-244) and a C-terminal site (CBS; residues 379-408). To determine if either of these areas of $\beta_1$ participates in heterocomplex formation with hsp90 we used truncated variants of $\beta_1$ carrying deletions of the NBC and/or CBS. All three N-teminally truncated $\beta_1$ mutants used ([204-619], [304-619] and [380-619]) showed no hsp90-binding ability. In line with this observation, deletion of residues 204-303 or 346-408 in the context of full-length $\beta_1$ did not alter hsp90 binding (data not shown), further suggesting that the dimerization region is not important for the hsp90/$\beta_1$ interaction. Although the dimerization region was not necessary for the hsp90/$\beta_1$ 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/$\beta_1$ interaction, we used additional $\beta_1$ mutants that lacked parts of the regulatory domain ($\beta_1$ 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 that spreads throughout the first 200 amino acids of the regulatory domain participates in $\beta_1$ binding to hsp90 and that preservation of some the residues that mediate the hsp90/ $\beta_1$ interaction in the $\beta_1$ mutant proteins, is enough to secure strong binding to hsp90.

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All naturally occurring sGC heterodimers identified to date contain a $\beta_1$ subunit(Hobbs, 1997; Russwurm et al., 1998). The $\alpha_1/\beta_1$ is the predominant form of sGC exhibiting higher expression than the $\alpha_2/\beta_1$ in all tissues studied, with the exception of the brain where the two sGC forms are equally expressed(Mergia et al., 2003). In order to determine if the interaction between individually expressed sGC subunits and hsp90 differs from that of the native heterodimeric enzyme, we co-transfected cells with a deletion mutant of $\beta_1$ that does not bind hsp90 and full-length $\alpha_1$. 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 $\beta_1$ subunit.

Protein-protein interactions can alter both the activity and subcellular localization of the interacting partners. In the case of sGC, CCTη interacts with $\beta_1$ 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 $\alpha_2/\beta_1$ isoform of sGC is recruited to synaptic membranes following binding of its C-terminus to post-synaptic density-95 (Russwurm et al., 2001). Moreover, Zabel et al showed that similarly to the $\alpha_2/\beta_1$, up to 20% of the $\alpha_1/\beta_1$ isoform can be found in the membrane fraction of tissues(Zabel et al., 2002), offering additional proof that soluble GC is not, as originally thought, entirely cytosolic. Use of detergents like Triton-X-100 during tissue homogenization is accompanied by loss of sGC from the membrane fraction(Zabel et al., 2002). 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 would suggest that this occurs through a protein-protein interaction. Interestingly, 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, while 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). This data taken together suggest that interruption of the hsp90/sGC heterocomplex formation in an acute fashion ($\leq$1hr) does not affect the ability of sGC to respond to NO donors. It should be noted that in all of the above-mentioned cases 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, though, was only evident at high, but not low concentrations of the NO donor. Moreover, pretreatment of bovine aortic endothelial cells with GA reduced the SNP-stimulated cGMP accumulation, while 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 would 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 24-48hr drastically reduced the levels of $\alpha_1$ and $\beta_1$, suggesting that blockade of the hsp90/sGC interaction destabilises 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 as 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 $\alpha_1$ and $\beta_1$ 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 $\alpha_1$ and $\beta_1$ levels, as well as NO responsiveness, suggesting that restricting 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 $\beta_1$ associates with hsp90 in the context of the native heterodimeric form of the enzyme. We have also shown that the M domain of hsp90 and the regulatory domain of $\beta_1$ participate in this interaction. The function of hsp90/sGC heterocomplex may differ with cell type: in smooth muscle, as well as other cell types

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where the two proteins are co-expressed, 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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# FIGURE LEGENDS

**FIG. 1 Evidence for sGC binding to hsp90 in cells and tissue.** 300-400μg of protein extracts from rat aortic smooth muscle cells (RASMC; lane 1 in A), COSm6 cells transfected with $\alpha_1$ and $\beta_1$ (COSm6 $\alpha_1/\beta_1$; Lane 1 in B) or rat lung (lane 1 in C) were immunoprecipitated using anti-$\beta_1$ (1μg) and blotted for either hsp90 or the $\alpha_1$ sGC subunit. Protein G beads (lane 2 in A, B, and C) or rabbit IgG (lane 3 in C) were used as controls for the immunoprecipitation. Blots shown are representative of experiments repeated at least twice with identical results.

**FIG. 2. Individually expressed sGC subunits bind to the M domain of hsp90.** A. COS cells were co-transfected with the cDNAs encoding myc-tagged full-length $\beta_1$ and various deletion mutants of hsp90. Immunoprecipitation (IP) was performed using a myc antibody and the precipitates analyzed by SDS-PAGE and Western blotting (WB) with an antibody to either $\beta_1$ or FLAG. Equal expression of $\beta_1$ and of the hsp90 deletion mutants was monitored in the lysates. B. Experimental set up is as in A only cells were transfected with cDNAs encoding myc-tagged full-length $\alpha_1$ 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.

**FIG. 3. Binding of eNOS and sGC to hsp90 is not mutually exclusive.** A GST-fusion protein of the full-length $\beta_1$ was incubated with purified hsp90 or hsp90 and eNOS. After incubation at $4^0$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

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hsp90. Blots shown are representative of experiments repeated at least twice with identical results.

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**FIG. 4. The regulatory domain of $\beta_1$ mediates the interaction with hsp90.** A. GST fusion proteins of the $\beta_1$ subunit were expressed in E.coli and purified; 300pmol of each protein was incubated with COSm6 lysate (1mg) overnight at $4^0$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 blotted with an hsp90 antibody. B. Cells were co-transfected with the cDNAs encoding HA-tagged full-length hsp90 and full-length or N-terminally deleted forms of $\beta_1$ tagged with V5/$His_6$. $Ni^{2+}$-agarose-bound $His_6$-tagged $\beta_1$ or total cell lysates were subjected to SDS-PAGE and blotted with anti-HA or anti-V5. Retained $\beta_1$ sequences are bracketed. Blots shown are representative of experiments repeated three times with identical results.

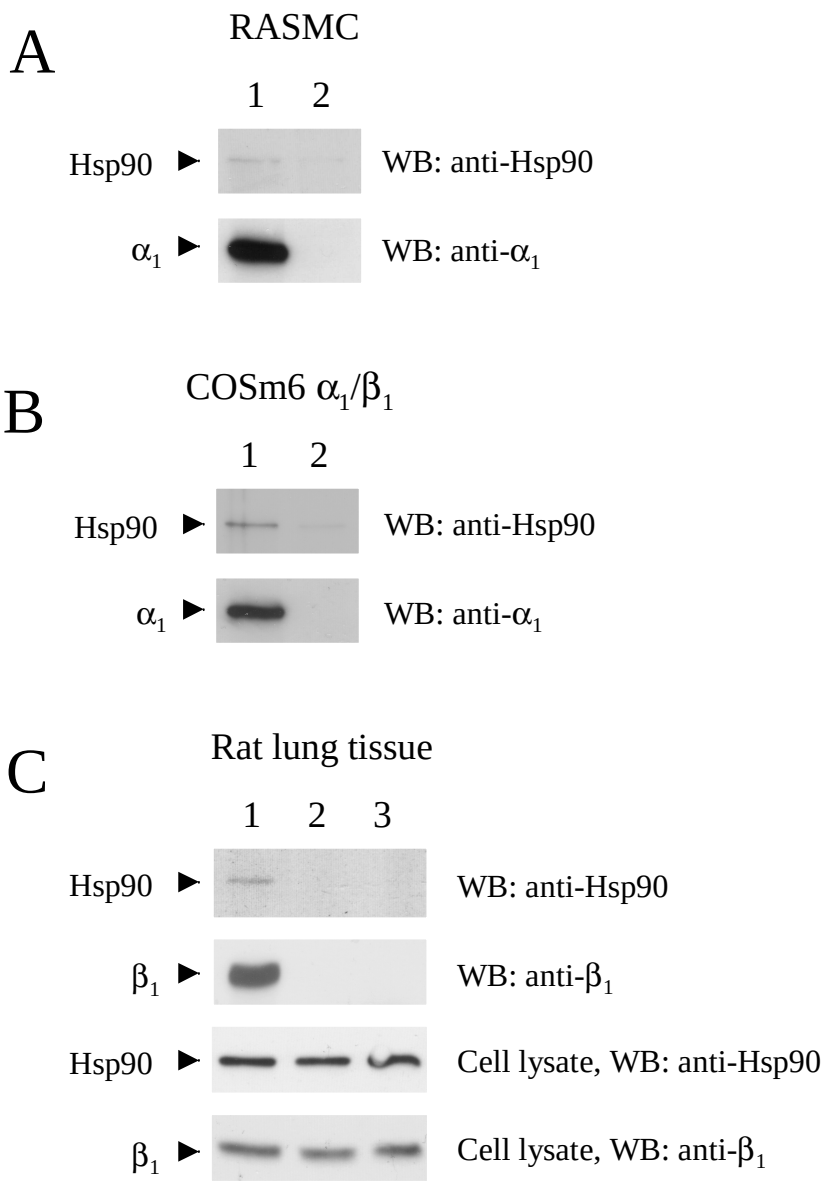
**FIG. 5. A discontinuous region within the regulatory domain of $\beta_1$ is responsible for sGC/hsp90 binding.** COS cells were co-transfected with cDNAs encoding the indicated mutant of β1 and full-length HA-tagged hsp90. $Ni^{2+}$-agarose-bound $His_6$-tagged $\beta_1$ or total cell lysates were 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 the prefix Δ.

**FIG.6. The sGC heterodimer binds hsp90 through the $\beta_1$ subunit.** A. COS cells were co-transfected with the cDNAs encoding HA-tagged hsp90, the 204-619 truncation mutant of $\beta_1$ (tagged with V5/His) in the presence or absence of full-length $\alpha_1$. The $\beta_1$ was precipitated using a $Ni^{2+}$ column and precipites were blotted with anti-

HA, V5 or myc. B. Same as in A only full-length $\beta_1$ was used instead of the truncation mutant. Blots shown are representative of experiments repeated at least twice with identical results.

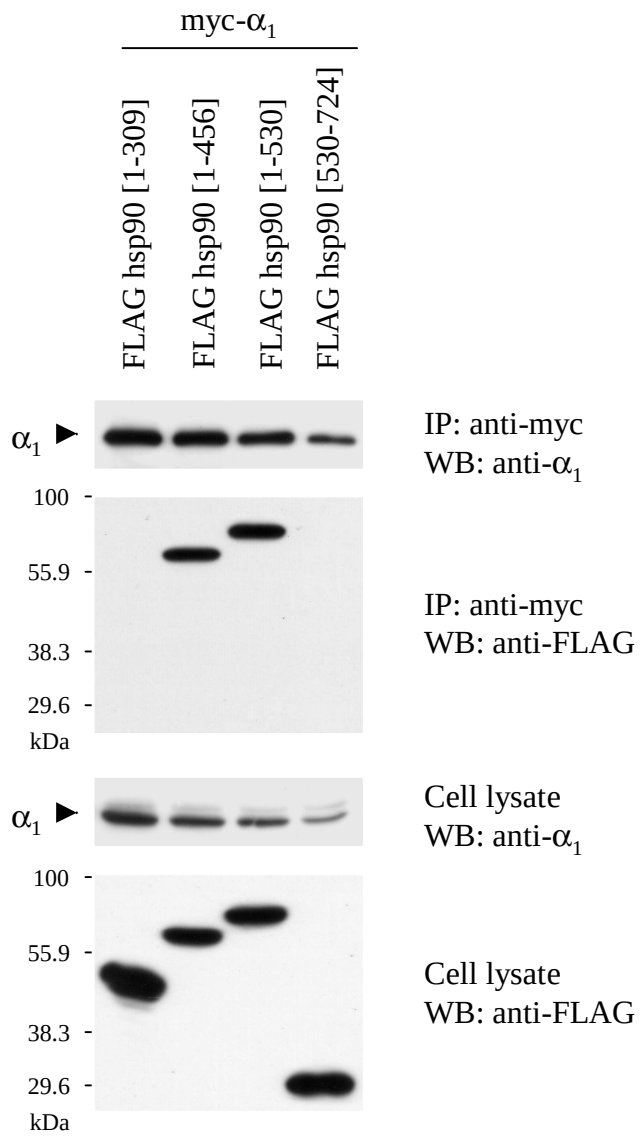
**FIG. 7. Inhibition of hsp90 downregulates sGC.** A. Rat aortic smooth muscle cells were treated for the indicated time with radicicol (RAD; 20μM), geldanamycin (GA; 1μg/ml), or the corresponding vehicle. Cell lysates were then prepared, subjected to SDS-PAGE and blotted with sGC subunit antibodies. 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 radicicol (RAD; 20μM), geldanamycin (GA; 1μg/ml), or vehicle for 24hr. After the end of the incubation period cells were washed with Hanks' balanced salt solution and incubated in the presence of 10μM sodium nitroprusside for 15min. Throughout the 15min period cultures were exposed to the phosphodiesterase inhibitor IBMX (1mM). Means ± SEM, n=4 wells $^{*}p<0.05$ from vehicle.

**FIG. 8. Proteasome inhibition blocks the geldanamycin-induced downregulation of sGC.** A. Rat aortic smooth muscle cells were pretreated with 10μM of the proteasome inhibitor MG132 for 2hr and then incubated for an additional 24hr with vehicle (DMSO) or geldanamycin (GA; 1μg/ml). Cell lysates were then prepared, subjected to SDS-PAGE and blotted with sGC subunit antibodies (A). Blots are representatives of experiments repeated twice. In B, cGMP accumulation was measured in the absence (basal) or presence or a NO donor (SNP, 10μM), as described above. Means ± SEM, n=4 wells $^{*}p<0.05$ from vehicle.




MOLPHARM/2005/012682Fig 1

MOLPHARM/2005/012682Fig 2A




MOLPHARM/2005/012682Fig 2B

MOLPHARM/2005/012682Fig 3

MOLPHARM/2005/012682Fig 4A

MOLPHARM/2005/012682Fig 4B

MOLPHARM/2005/012682Fig 5A

MOLPHARM/2005/012682Fig 5B

MOLPHARM/2005/012682Fig 5C

MOLPHARM/2005/012682Fig 6

MOLPHARM/2005/012682Fig 7A

MOLPHARM/2005/012682Fig 7B

MOLPHARM/2005/012682Fig 8A

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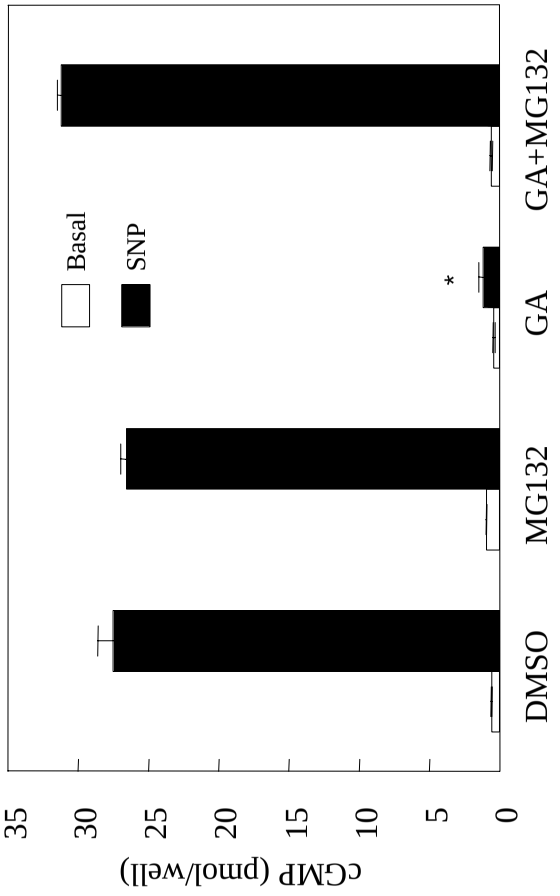


MOLPHARM/2005/012682Fig 8B