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The G-protein regulator LGN modulates the activity of the NO receptor soluble guanylate cyclase

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Abstract

sGC (soluble guanylate cyclase) is the main mediator of NO signalling. Biochemical and physiological studies suggest that, besides NO, *in vivo* regulation of sGC involves direct interaction with other proteins. Using yeast two-hybrid screening, we identified that the multidomain LGN (Leu-Gly-Asn repeat-enriched protein) interacts with both $\alpha 1$ and $\beta 1$ sGC subunits. LGN and sGC co-localized in the cell cytoplasm, and the LGN–sGC complex was co-immunoprecipitated from cells expressing both proteins and from native tissues. Their interaction requires the N-terminal tetratricopeptide repeats of LGN, but does not require the N-terminal portions of $\alpha 1$ or $\beta 1$ sGC subunits. Overexpression of LGN decreases the activity of cellular sGC, whereas knockdown of LGN mRNA and protein correlated with increased sGC activity. Although purified LGN interacts directly with purified sGC, the inhibitory effect *in vitro* is observed only after supplementation of cell lysate to the reaction. Although resting sGC and sGC activated by the stimulator BAY41-2272 have very similar LGN-IC₅₀ values to the NO-stimulated sGC, they have a much higher Hill coefficient, suggesting co-operative binding with respect to LGN in the low-activated state of sGC. AGS3 (activator of G-protein signalling 3), the closest LGN homologue, also inhibits sGC. The interaction of sGC with these scaffolding proteins may expand the cross-talk between NO/cGMP signalling and other cellular pathways and tailor sGC function to specific tissues or signals.

Keywords

activator of G-protein signalling 3 (AGS3); cGMP; Leu-Gly-Asn repeat-enriched protein (LGN); nitric oxide (NO); soluble guanylate cyclase (sGC)

INTRODUCTION

NO is a potent pleiotropic signalling molecule. Many of its physiological effects are mediated by the NO-sensitive sGC (soluble guanylate cyclase) (also called GTP pyrophosphatase). These effects include regulation of smooth muscle cell relaxation, neurotransmission, platelet function, mitochondrial biogenesis and cell proliferation [1,2]. sGC converts GTP into the second messenger cGMP, resulting in activation of downstream

cGMP effectors [3]. Mammalian sGC is an obligate heterodimer composed of one α and one β subunit. Although two separate genes for each subunit ($\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$) are found in mammals, only the ubiquitously expressed $\alpha 1\beta 1$ and the less abundant $\alpha 2\beta 1$ heterodimers have been detected *in vivo* [4]. The C-terminal half of both subunits are necessary and sufficient to form a functional catalytic site with low cGMP-forming activity [5]. The binding of NO to the ferrous haem group located in the N-terminal domain of the $\beta 1$ subunit activates cGMP catalysis several-hundred-fold [6]. Such a strong activation results in a very rapid increase in intracellular cGMP [7], which is under tight regulation. The equilibrium between the rate of cGMP synthesis by sGC and degradation by cyclic nucleotide phosphodiesterases governs the dynamics of cGMP levels in cells in response to NO stimuli.

Accumulating evidence suggests that the cellular environment alters some aspect of sGC function, compared with the purified protein. For example, the sensitivity of sGC to NO and NO donors seems to be much higher in the cellular environment than *in vitro* [7,8]. The effects of cellular factors on sGC properties extend to sGC deactivation as well. *In vitro* studies demonstrated that, in the presence of the NO scavenger oxyhaemoglobin and the GTP substrate, the sGC–NO complex decomposes with a half-life of the order of several seconds [9]; this decomposition correlates well with the loss of high cGMP-forming activity [10]. In cerebellar cells, however, the deactivation rate of sGC is 25-fold higher [11]. These comparisons strongly suggest that cellular factors modulate the process of sGC activation and deactivation.

A number of cellular proteins have been found to interact with sGC and influence its function. The association of sGC with HSP (heat-shock protein) 90 [12,13] or HSP70 [14] promotes NO-dependent sGC activation, whereas the interaction with CCT η (chaperonin-containing T-complex polypeptide 1, η subunit) [15] decreases NO-induced sGC functions. Other proteins affect cellular localization of sGC isoforms [16], promote the formation of multiprotein complexes [13] or may contribute to the process of sGC trafficking in the cell [17]. The opposite also occurs, as a recent report described that the sGC $\alpha 1$ subunit alone affects transcriptional activity of p53 in prostate cells [18].

In the present study, we characterize LGN [Leu-Gly-Asn repeat-enriched protein; also called GPSM2 (G-protein signalling modulator-2) and AGS3 (activator of G-protein signalling 3)-like], an inhibitor of GDP dissociation from $G\alpha$ proteins [19], as one of the protein modulators of cellular sGC. We identified LGN as both an $\alpha 1$ and $\beta 1$ sGC interactor through yeast two-hybrid screening. The LGN–sGC interaction involves the sGC catalytic domain and the N-terminal region of LGN. LGN protein inhibits sGC activity, but this effect requires additional cellular factors. AGS3, an LGN homologue, also inhibits sGC. We propose that, through interaction of sGC with AGS3-like proteins, the NO/cGMP network interfaces with a multiplicity of signalling pathways, providing a means to modulate the NO/cGMP pathway and/or adapt the cells to rapid changes of intracellular cGMP.

EXPERIMENTAL

Yeast two-hybrid screening

The full-length cDNA of the human $\alpha 1$ subunit or $\beta 1$ subunit of sGC were cloned in-frame with the GAL4 DNA-binding domain of the pGBKT7 bait plasmid (Clontech) to generate pGB- α GAL4DB or pGB- β GAL4DB plasmids respectively. These plasmids were transfected into AH109 yeast cells (Clontech). A human brain cDNA library cloned in-frame with the GAL4 activation domain of the pACT2 vector was transfected into yeast containing the pGB- α GAL4DB or pGB- β GAL4DB plasmids. The transfectants were selected on agar lacking tryptophan, leucine, histidine and adenine and supplemented with 5 mM 3-aminotriazole. After 3 weeks of incubation at 30°C, positive clones greater than 2 mm in

diameter were transferred on to fresh quadruple-dropout agar and used for β -galactosidase filter-lift assays (Clontech) to minimize the number of false positives. The pACT2 plasmid containing putative sGC-interacting proteins was amplified in TOP10 cells (Invitrogen) and retested for growth on quadruple-dropout agar plate using the β -galactosidase assay in the AH109 strain lacking any bait plasmid and AH109 with the pGBKT7 vector as bait. The plasmids from the clones that passed these tests were amplified and sequenced to determine the identity of putative sGC-interactors.

Expression and sGC purification

The expression and purification of human recombinant sGC from an Sf9/baculovirus system was performed as described previously [20].

Cloning of full-length AGS3, LGN and LGN variants

Full-length ORFs (open reading frames) of LGN and AGS3 carrying N-terminal hexahistidine tags were synthesized at GenScript and cloned into pGEX3 (GE Healthcare) and pET28a (EMD Biosciences) vectors respectively. The plasmids expressing the truncated LGN-GoLoco protein were obtained by inserting a stop codon after the Leu⁴³⁸ codon of LGN. To generate the plasmid expressing the truncated LGN-TPR (tetra-tricopeptide repeat) protein, a HindIII site was inserted after the Phe⁴⁴⁶ codon and used to perform an in-frame deletion of the N-terminal region between residues 22 and 446. For expression of mammalian cell LGN, the ORF was cloned into the pCDNA3.1 vector. The N-terminal hexahistidine tag was removed or replaced by a FLAG tag through PCR mutagenesis to obtain the pCDNA3.1-LGN and pCDNA3.1-FLAG-LGN plasmids respectively.

Expression and purification of AGS3, LGN and LGN variants

To obtain recombinant full-length or truncated variants of AGS3 and LGN, pGEX-LGN, pGEX-LGN-TPR, pGEX-LGN-GoLoco or pET-28a-AGS3 plasmids were transformed into the BL21-DE3 strain of *Escherichia coli*. Cultures (1–2 litres at a D_{600} of 0.6) were induced with 0.25 mM IPTG (isopropyl β -D-thiogalactopyranoside) and incubated at 22°C overnight. The induced culture was then harvested by centrifugation and the cell pellet was resuspended in 40 mM TEA (triethanolamine), pH 7.5, with 2 mM PMSF and a protease inhibitor cocktail (5 mg/ml pepstatin A, leupeptin, aprotinin and chymostatin). Lysozyme (100 μ g/ml) was added to the cell suspension and kept on a rocking platform for 30 min at 4°C. The resulting cell suspension was sonicated four times for 2 min with a 10 s interval between pulses. The lysate was centrifuged at 20000 g for 30 min at 4°C. The resulting supernatant was loaded on to a pre-equilibrated 15 ml column packed with PerfectPro Ni-NTA (Ni²⁺ -nitrilotriacetate) Superflow (Fisher Scientific). The column was washed with 40 mM imidazole, pH 7.5, and 200 mM NaCl, and the protein was eluted with increasing concentrations of imidazole, pH 7.5. The purified protein was divided into aliquots (50 μ l), quantified and stored at –80°C after adding 20% (v/v) glycerol.

Cell culture and transient transfection

MDA468, MDA453 and COS-7 cells (A.T.C.C., Manassas, VA, U.S.A.) were routinely cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium/F12K medium supplemented with 10% (v/v) FBS (fetal bovine serum), 0.1 mM MEM (minimal essential medium) non-essential amino acids, a penicillin/streptomycin mixture (50 units/ml and 50 μ g/ml respectively), 10 mM Hepes, pH 7.4, 1 mM sodium pyruvate and 2 mM L-glutamine (all from Invitrogen), and maintained at 37°C and 5% CO₂. For transient transfections, the cell were seeded on a 100-mm-diameter cell-culture dish at a density of 5×10^5 cells/cm² and cultured until 80% confluency. Lipofectamine™ with Plus reagent (Invitrogen) was used according to the manufacturer's protocol for transfection of pCDNA3.1 plasmids expressing

sGC $\alpha 1$ and $\beta 1$ subunits and LGN protein. To prepare lysates, the cells were collected gently using a cell scraper, washed twice with PBS, resuspended in 40 mM TEA, pH 7.4, containing a protease inhibitor mixture (Roche), and disrupted by sonication as detailed below. The lysates were centrifuged at 15000 *g* for 30 min to prepare the cleared supernatant fractions, which were used for Western blotting, immunoprecipitation or activity measurements.

Co-immunoprecipitation and pull-downs

BE2 cells transfected with pCDNA3.1-FLAG-LGN plasmid or control pCDNA3.1 vector were collected from confluent 100-mm-diameter culture dishes, washed twice with PBS and resuspended in 500 μ l of PBS containing protease inhibitor mixture. For co-immunoprecipitation of sGC-LGN complex from tissues, ~0.5 cm³ pieces of rat lung and brain (cerebral cortex) were homogenized using a tightly fit tissue homogenizer in 500 μ l of PBS with protease inhibitors. The samples were then disrupted by sonication using the VCX130 disruptor (Sonic and Materials) for a total of 40 s in 2 s pulses at ~4 W. The homogenates were cleared by a 30 min centrifugation at 15000 *g* and the supernatants were incubated with 10 μ g (cell lysates) or 25 μ g (tissue extracts) of polyclonal anti- $\beta 1$ sGC antibodies [21] overnight at 4°C (cell lysates) or for 2 h at room temperature (25°C) (tissue extracts). The cell lysates were then combined with 100 μ l of pre-equilibrated Protein A/G-agarose beads (Santa Cruz Biotechnology), whereas the tissue homogenates were combined with Protein G-magnetic beads (Fisher Scientific), and further incubated for 1.5 h. The supernatant was then removed and the beads were washed three times with 40 mM TEA, 200 mM NaCl and 1% Nonidet P40, pH 7.4. The bound proteins were eluted by boiling in 100 μ l of Laemmli buffer. The samples were loaded on an SDS/PAGE gel (8%) and the Western blot was probed for sGC and LGN as indicated below. For pull-down experiments, partially purified full-length or truncated versions of sGC (1 μ g) and LGN (10 μ g) were pre-incubated for 20 min at room temperature, diluted in 500 μ l of PBS with protease inhibitors, incubated with anti- $\beta 1$ antibodies and processed as described above.

Western blotting

Protein samples were resolved by SDS/PAGE (8% gels) and transferred to methanol-activated PVDF membranes. After blocking, the membranes were treated with custom anti- $\alpha 1$ and anti- $\beta 1$ sGC polyclonal antibodies [21] at 1:2000 dilution, a monoclonal anti-FLAG antibody (clone M2, Sigma) at 1:5000 dilution or an anti-LGN goat polyclonal antibody (Abcam) at 1:10000 dilution. Secondary horseradish peroxidase-conjugated antibodies (Sigma) were used at 1:5000 (anti-rabbit) and 1:10000 (anti-mouse) dilutions. Protein bands were visualized by enhanced chemiluminescence (ECL Plus, GE Healthcare).

Immunofluorescence

MDA468 cells were cultured on gelatin-coated chamber slides (Nalgene Nunc from Fisher Scientific) and incubated at 37°C and 5% CO₂ until 50% confluency. The cells were washed in PBS, fixed in 3.7% (w/v) paraformaldehyde for 10 min and permeabilized in 0.1% Triton X-100 (Sigma) for 10 min at room temperature. The slides were blocked with 5% (v/v) FBS for 1 h and then incubated for 1 h at room temperature with primary antibodies raised against the C-terminal peptide of the $\beta 1$ sGC (1:500) and/or anti-LGN antibodies (1:750) diluted in 5% (v/v) FBS. The signal was developed with secondary antibodies labelled with Alexa Fluor® 488 (1:2000) or Alexa Fluor® 594 (1:2000) respectively for sGC and LGN detection. Nuclear staining was achieved by staining with 1 μ M Sytox Orange solution (Life Technologies) for 10 min. To visualize mitochondria, antibodies raised against COX IV protein (Cell Signaling Technology) were used. For visualization of the endoplasmic reticulum, antibodies raised against the ERP29 (endoplasmic reticulum protein 29) marker

were used, whereas for Golgi, anti-RCAS1 (receptor-binding cancer antigen expressed on SiSo cells) antibodies were used. Digital confocal images were acquired using a Leica confocal microscopy system and processed with ImageJ software.

siRNA (small interfering RNA) knockdown

For LGN knockdown experiments, 10^7 MDA468 cells plated on a 100-mm-diameter culture dish were transfected with 10 nM LGN siRNA (sc106999, Santa Cruz Biotechnology) using Lipofectamine™ RNAiMAX reagent according to the manufacturer's instructions (Invitrogen). Scrambled-sequence siRNA was used as a control. The cells were collected 48 h post-transfection and were processed for RNA isolation, Western blotting and sGC assay. Changes in *LGN* mRNA expression were assessed using real-time qPCR (quantitative PCR), whereas changes in protein level were monitored using anti-LGN antibodies.

RNA isolation, cDNA preparation and real-time qPCR

RNA isolation was performed using TRIzol® reagent as per the manufacturer's instructions (Invitrogen). Total RNA (5 µg) was used for cDNA synthesis using a random hexamer primer and SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's protocol. The amount of *LGN* transcript was quantified by real-time PCR and normalized to the level of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) transcript using LGN- and GAPDH-specific TaqMan assays respectively (Applied Biosystems).

sGC assay

Indicated amounts of purified LGN and sGC proteins were incubated for 10 min at room temperature with 20 µl of control buffer (2.5 mg/ml albumin, 50 mM TEA and 100 µM EGTA) or 50 µg of COS-7 cell lysate. Following the incubation, the sample was mixed with 40 µl of reaction buffer [125 mM TEA, 250 µM EGTA, 2.5 mM IBMX (isobutylmethylxanthine), 0.5 mg/ml albumin, 2.5 mM cGMP, 0.125 mg/ml creatine kinase, 12.5 mM creatine phosphate and 7.5 mM MgCl₂] and 40 µl of substrate buffer (500 µM GTP/0.08 µCi of [α -³²P]GTP) and transferred to 37°C. When necessary, BAY41-2272 was added to the reaction buffer to a final concentration of 5 µM. To test the activation by NO, DEA-NO [2-(*N,N*-diethylamino)diazenolate-2-oxide, diethylammonium salt] donor was added into the substrate buffer at a final concentration of 100 µM. After 10 min (basal or BAY41-2272 assays) or 2 min (NO assay), the reaction was stopped by zinc acetate and sodium carbonate and processed as described previously [22] to quantify the amount of cGMP generated. To determine sGC activity in MD468, MDA453 or COS-7 cells, a 20 min assay was performed using 20 µl of lysate out of 400 µl of lysate obtained from 2×10^7 cells.

Statistical analysis

Results are expressed as means \pm S.D., unless indicated otherwise. Student's *t* test was used for statistical comparison between groups. $P < 0.05$ was considered statistically significant. Non-linear regression analysis used to calculate EC₅₀ was performed using GraphPad Prism (GraphPad Software).

RESULTS

Yeast two-hybrid screening

To identify the putative protein(s) that interacts with human sGC, we performed two independent yeast two-hybrid screens using full-length human sGC $\alpha 1$ or sGC $\beta 1$ as baits. A two-hybrid cDNA library (Clontech) containing 1.2×10^7 cDNAs from human brain was used for this screen. Each screen is the result of three transfections performed with

independent preparations of yeast competent cells. In the primary screen on quadruple-dropout selective medium, we found 152 clones for $\alpha 1$ bait and 368 clones for $\beta 1$ bait. Of these clones, 58 for $\alpha 1$ and 27 for $\beta 1$ were also positive in the second round of screening with the β -galactosidase lift assay. Sequencing of the amplified target plasmids revealed that they represent 26 proteins for the $\alpha 1$ screen and seven proteins for the $\beta 1$ screen. One identified target, LGN, was present twice in the $\alpha 1$ screen and four times in the $\beta 1$ screens. The specificity of interaction was then confirmed by transfecting the plasmid carrying the fusion between the Gal4-activating domain and the LGN gene-activation domain into AH109 yeast with a control plasmid carrying the Gal4-binding domain without bait and into yeast with no plasmid. In both cases, no growth on selective medium was observed, arguing for specificity of the identified interaction. These data suggest that LGN (also referred to as GPSM2 or AGS3-like), a known GDP-dissociation inhibitor regulating the function of G_{α} proteins of heterotrimeric G-proteins, may also interact with $\alpha 1\beta 1$ sGC in mammalian systems.

Co-localization and co-immunoprecipitation of sGC and LGN

We next evaluated whether the sGC–LGN interaction occurs not only in the yeast system, but in mammalian cells as well. To test whether sGC and LGN co-exist in the same cellular compartment, we co-stained fixed MDA468 cells, which express functional sGC [23], with antibodies against $\beta 1$ -sGC and LGN. Confocal microscopy determined that both sGC (green fluorescence) and LGN (red fluorescence) were distributed throughout the cytoplasm (Figure 1A). Although anti-LGN antibodies also strongly stained the nucleus (as shown by the purple nuclear signal in the merged image), the yellow signal obtained by merging the sGC and LGN stainings highlighted only the extranuclear region of the cell (Figure 1A, bottom middle and right-hand panels). The confocal orthogonal sections of the cells (Figure 1A, bottom right-hand panel) demonstrated that the merged yellow signal exists throughout the cytoplasm. Since sGC staining appeared to be somewhat granular, we investigated whether there is any significant co-localization of sGC with major cellular organelles, i.e. Golgi, mitochondria and endoplasmic reticulum (Supplementary Figure S1 at <http://www.BiochemJ.org/bj/446/bj4460445add.htm>). Although the staining of sGC and the organelles were confined, as expected, to the cytoplasm, the confocal orthogonal sectioning of the cells in the regions highlighted by organelle markers showed no detectable association of sGC with any of the tested organelles (Supplementary Figure S1).

We next determined whether the sGC–LGN complex may be detected by co-immunoprecipitation. For this purpose, we transfected BE2 neuroblastoma cells, which endogenously express functional $\alpha 1\beta 1$ sGC, with a plasmid expressing LGN tagged with the FLAG peptide (pCDNA3.1-FLAG-LGN). Using polyclonal antibodies raised against the C-terminus of the $\beta 1$ sGC, we immunoprecipitated sGC from the lysate of BE2/pCDNA-FLAG-LGN. We found that FLAG–LGN co-precipitated along with sGC (Figure 1B), demonstrating the formation of a complex between sGC and LGN. This complex was detected not only in the cells overexpressing LGN, but also in native tissues. Homogenates from rat lung and cerebral cortex, two tissues with the highest level of sGC expression, were used to determine whether LGN co-precipitates with sGC. Our analysis demonstrated that a significant amount of LGN protein was detected in immunoprecipitates obtained with anti-sGC serum, but not with pre-immune serum (Figure 1C), confirming the existence of the sGC–LGN complex in native tissues.

Mapping the regions of sGC and LGN interaction

To characterize the nature of the interaction between sGC and LGN, we mapped the regions of both proteins necessary for the formation of the sGC–LGN complex. As depicted in Figure 2(A), LGN protein has seven TPRs clustered in the N-terminal half of the protein

(TPR domain) and four GoLoco motifs clustered in the C-terminal half (GoLoco domain). We used *E. coli* to express and then partially purify the hexahistidine-tagged full-length LGN protein and the truncated LGN versions lacking the TPR or the GoLoco domains (Supplementary Figure S2 at <http://www.BiochemJ.org/bj/446/bj4460445add.htm>). We also used the baculovirus/Sf9 expression system to generate and partially purify the full-length $\alpha1\beta1$ sGC and the truncated α CAT (α -catenin)/ β CAT (β -catenin) protein, which lacks the N-terminal regulatory regions of sGC (Supplementary Figure S2). When the pull-down experiments were performed with the full-length LGN and $\alpha1\beta1$ or α CAT/ β CAT sGC, we found that LGN co-precipitated with both sGC variants. These data indicate that the N-terminal regions of both sGC subunits are not required for the formation of the LGN–sGC complex (Figure 2B). Alternatively, we used the full-length sGC and evaluated which of the truncated LGN proteins co-precipitate. As shown in Figure 2(C), the C-terminal half of LGN, which contains the GoLoco motifs, failed to co-precipitate with sGC, whereas the N-terminal TPR domain was detected in the eluate. These data indicate that the TPR domain of LGN is sufficient to bind sGC.

Changes in LGN expression affect sGC activity

We then tested how changes in the level of LGN affect the function of sGC. Human MDA468 adenocarcinoma cells, which express endogenous sGC, were transiently transfected with the pCDNA3.1-LGN plasmid or the control pCDNA3.1 plasmid. After 72 h, the activity of sGC in the supernatant obtained by centrifugation at 15000 g was measured under basal conditions or in the presence of 100 μ M of the NO donor DEA-NO or a combination of DEA-NO and 5 μ M of the sGC allosteric regulator BAY41-2272. As expected, DEA-NO substantially increased sGC activity, whereas the addition of BAY41-2272 potentiated this effect. We found that the cGMP-forming activity of stimulated sGC in cells transfected with LGN-expressing plasmid was substantially lower than in control cells (Figure 3A). The extent of the LGN-dependent decrease was the same, regardless of whether the stimulation was by NO donor alone or in combination with BAY41-2272. We also found that this inhibition depended on the amount of LGN plasmid, and that this *LGN* gene-dose effect was similar for DEA-NO- and DEA-NO/BAY41-2272-induced sGC. The decrease in sGC activity was not caused by changes in the level of sGC protein (Figure 3A, inset), indicating that LGN affects sGC function rather than its abundance. Similar results were obtained with human MDA468 breast cancer cells and with COS-7 cells co-transfected with sGC and LGN plasmids (Supplementary Figure S3 at <http://www.BiochemJ.org/bj/446/bj4460445add.htm>).

We also investigated whether decreasing the expression of LGN affects sGC activity. MDA468 cells were transfected with LGN-targeted siRNA to knock down LGN. After 48 h, the amount of *LGN* transcript was reduced almost 3.5-fold (Figure 3B), which correlated well with the decrease in the level of LGN protein (Figure 3C). Although the level of sGC protein was not affected by LGN-siRNA, the activity of sGC in the lysates of cells transfected with LGN-siRNA was higher than in cells treated with scrambled siRNA (Figure 3D), consistent with the inhibitory effect of LGN observed in Figure 3(A).

Recombinant LGN alone does not affect purified sGC

As a next step, we investigated how the purified LGN protein (Figure 4A) affects the activity of purified sGC. The cGMP-forming activity of sGC pre-incubated for 10 min with the LGN preparation was determined (Figure 4B). The results indicate that basal, BAY41-2272- and DEA-NO-treated sGC was not inhibited by LGN in this *in vitro* system. In fact, a slight, but statistically significant, increase in activity was observed for the NO-stimulated samples. These data indicate that LGN alone is not sufficient to cause the inhibition of sGC activity, which is in contrast with the inhibition observed in cells

transfected by LGN (Figure 3A). The presence of an LGN inhibitory effect in the cellular lysates (Figure 3A) and the absence of inhibition in experiments with purified components (Figure 4B) led us to conclude that LGN-dependent inhibition of sGC requires additional factors present in the cellular lysates.

Supplementation of cell lysate promotes LGN inhibition of sGC

To test this hypothesis, we tested the effect of recombinant LGN on purified sGC after supplementation with 150 μg of cleared COS-7 lysate (15000 g supernatant). Pre-incubating LGN with purified sGC in the presence of the COS-7 lysate resulted in inhibition of basal, BAY41-2272 and DEA-NO activities of sGC (Figure 4C). Heat-inactivated COS-7 lysate, however, did not promote LGN-dependent inhibition. Consistent with a previous report [14] in the absence of LGN, COS-7 lysate alone had a slight stimulatory effect on sGC activity (results not shown), which also disappeared in heat-inactivated samples. These data indicate that some heat-labile cellular factor(s) is provided by the COS-7 lysate and is needed for LGN-dependent inhibition of sGC.

To better understand the nature of this inhibition, we investigated whether the extent of sGC inhibition correlates with the amount of LGN used. Different amounts of LGN were pre-incubated with purified sGC in the presence of the COS-7 cell lysate and the effect on basal, BAY41-2272- and DEA-NO-stimulated sGC was determined. As shown in Figure 5, the LGN-dependent inhibition was dose-dependent for all of the tested conditions. Although the apparent IC_{50} was comparable (95.2 compared with 87.5 compared with 65.3 $\mu\text{g}/\text{ml}$ for basal, BAY41-2272 and DEA-NO activities respectively), a significant difference was observed in the shape of the dose–response curve. Whereas the inhibitory curve for DEA-NO had a Hill coefficient of 1.65 (Figure 5A), the slope of the curves for the basal and BAY41-2272-stimulated sGC were much steeper, with estimated Hill coefficients of 9.2 and 7.9 respectively (Figures 5B and 5C). These data suggest a co-operative association of LGN or inhibitory factors for the low-activity states of sGC.

AGS3, a homologue of LGN, alters sGC activity

AGS3, also known as GPSM1, shares 66% similarity with LGN (Figure 6A), also known as GPSM2 or AGS3-like protein. Both proteins belong to a group of GDIs (guanine-nucleotide-dissociation inhibitors), which modulate the signalling of heterotrimeric G-proteins. To determine whether AGS3 also has an inhibitory effect on sGC activity, we expressed and purified a hexahistidine-tagged full-length AGS3 protein. In the absence of cell lysate, we observed no effects of recombinant AGS3 on sGC activity (results not shown). However, when the sGC/AGS3 mixture was supplemented with 150 μg of COS-7 lysate, a significant inhibition of BAY41-2272- and DEA-NO-activated sGC was observed (Figure 6B), whereas the effect on basal activity was minor. These data indicate that the AGS3 protein has similar properties with respect to the modulation of sGC function, but it may favour the activated state of sGC.

DISCUSSION

Previous studies demonstrated that sGC may form complexes with a number of proteins, affecting sGC localization [16], influencing intracellular trafficking of sGC [17] or modulating sGC activity [13–15]. In the present study, we identified the protein LGN as an sGC-interacting partner modulating the activity of cellular sGC.

Using two independent yeast two-hybrid screens with full-length $\alpha 1$ and $\beta 1$ sGC subunits as baits, we found a number of possible sGC-interacting partners. Among these proteins, we found CCT η , HSP70 and HSP90, previously characterized as sGC interactors [13–15].

However, we did not find the AGAP1 [Arf (ADP-ribosylation factor) GAP (GTPase-activating protein) with GTP-binding-protein-like, ankyrin repeats and PH (pleckstrin homology) domains] protein, which was also identified as an sGC-interacting protein [17]. This is probably due to a much lower level of *AGAP1* transcript represented in the brain cDNA library used in the present study than in the placental cDNA library used in the previous study that identified AGAP1 [17]. Interestingly, although the number of screened clones was very similar for $\alpha 1$ and $\beta 1$ subunits, we identified a significantly higher number of $\alpha 1$ interacting partners than $\beta 1$ interactors. Both sGC subunits have extensive similarity in the C-terminal regions, but are less similar in the N-terminal portion. Although the N-terminal region of the $\beta 1$ subunit harbours the haem-binding domain and is indispensable for sGC function as an NO receptor, previous studies by us [21] and others [24] demonstrated that the deletion of the N-terminal segment of the $\alpha 1$ subunit does not affect the NO-dependent activation of sGC. It is possible that one of the functions of this apparently dispensable region of sGC is interaction with other proteins.

This report characterizes the interaction and functional effects of the LGN protein that was identified in both $\alpha 1$ and $\beta 1$ screens. LGN is a member of the family of receptor-independent AGS proteins, also known as GPSMs. These proteins are distinguished by possessing one or more GPR (G-protein regulator) motifs, also known as GoLoco motifs. This GoLoco motif mediates the interaction with the G_{α} subunit of heterodimeric G-proteins and stabilizes the GDP-bound conformation of G_{α} , acting like a GDI with nanomolar binding affinities. Two members of this family, AGS3 and LGN, possess seven TPRs, which are known to modulate protein-protein interaction. The LGN protein has ten Leu-Gly-Asn repeats, from which the LGN designation is derived. LGN was shown to interact with $G_{\alpha i}$, $G_{\alpha o}$ and transducin ($G_{\alpha t}$) through its GoLoco domain (reviewed in [25]).

The present study demonstrated that, in MDA468 cells, sGC and LGN co-localize throughout the cytoplasm (Figure 1A). This staining is consistent with predominantly cytosolic staining of sGC in cultured cells [26] and cells of the rat hippocampus [15,26], as well as with cellular localization of LGN and its homologue AGS3 during interphase in a number of cell lines [27,28]. We demonstrated a stable intracellular interaction between sGC and LGN by co-immunoprecipitating both proteins from LGN-transfected BE2 cells and from brain and lung homogeneates (Figure 1).

Using co-precipitation of purified full-length and truncated variants of sGC and LGN in various combinations, we mapped the regions of both proteins required for the interaction (Figure 2). We found that the N-terminal regions of both sGC subunits are dispensable for the formation of the complex with LGN. This mapping of the interaction to the region of extensive homology between $\alpha 1$ and $\beta 1$ subunits is consistent with the fact that LGN was identified in both $\alpha 1$ and $\beta 1$ yeast two-hybrid screens. We also demonstrated that the TPR-containing region of LGN is sufficient for the interaction with sGC, suggesting that the interaction with sGC is provided by the TPRs of LGN. The TPR-containing domain of LGN was demonstrated to bind a number of proteins, including NuMA (nuclear mitotic apparatus) [29], ha-Ras [30], the mammalian homologue of the *Drosophila* Insc protein and Par6 [31].

In the present study, we demonstrated that LGN not only directly interacts with sGC, but also affects sGC activity. Overexpression of LGN in cell lines with endogenous or ectopic sGC expression resulted in lower sGC activity in cell lysates (Figure 3 and Supplementary Figure S3). Moreover, siRNA-mediated knockdown of the LGN transcript resulted in higher sGC activity (Figure 3). Taken together, these data demonstrate that LGN is a negative modulator of sGC. However, our experiments demonstrate that, despite a direct interaction between sGC and LGN, no sGC inhibition is observed when purified sGC and LGN are used. However, the LGN-mediated inhibitory effect is restored after the samples are

reconstituted with cell lysate (Figure 4). These results led us to conclude that additional cellular factors are required for the observed attenuation of sGC activity. Analysis of the LGN dose-dependent inhibition of sGC indicates that the LGN IC₅₀ is very similar between basal and stimulated sGC (~65–95 µg/ml) and corresponds to an IC₅₀ of 0.8–1.1 µM (Figure 5), which is comparable with the potency of the haem-targeting sGC inhibitor ODQ. Despite the similar values for the apparent LGN IC₅₀, the differences in the Hill coefficient of the dose–response curves (~9 for basal, ~8 for BAY41-2272 and ~2 for DEA-NO) suggest a strong co-operativity in the processes leading to inhibition of un-induced and BAY41-2272-stimulated sGC. Moreover, DEA-NO-activated sGC was more sensitive to lower doses of LGN, as the apparent LGN IC₂₀ for NO-activated sGC is 36 µg/ml, whereas for basal and BAY41-2272-treated sGC it is substantially higher at 71 and 79 µg/ml respectively. These differences in the inhibition suggest that the NO-activated enzyme is more susceptible to the effects of LGN due to changes in the nature of the sGC–LGN interaction, which probably results from the conformational changes of sGC caused by NO activation.

LGN shares a 59% overall protein sequence similarity with AGS3 protein [25]. Both proteins have a similar domain composition (seven TPR motifs and four GoLoco motifs) and similar binding selectivity for G_α subunits. The results of the present study demonstrate that, like LGN, recombinant AGS3 inhibits activated sGC in the presence of cellular lysates (Figure 6). However, unlike LGN, AGS3-dependent inhibition was observed only with activated sGC, suggesting a preference for the activated conformation of sGC. Previously reported characterization of mice lacking AGS3 revealed that AGS3^{-/-} animals have a decreased MAP (mean arterial pressure) and a prolonged drop in MAP in response to the administration of the NO donor SNP (sodium nitroprusside), as compared with wild-type mice [32]. The authors concluded that vascular control mechanisms involving heterodimeric G-protein signalling are affected because of AGS3 deficiency. However, considering the inhibition of activated sGC by AGS3 demonstrated in the present study, the effect of AGS3 deficiency on blood pressure may also reflect an increased sGC activity due to diminished sGC deactivation mediated by AGS3.

In several instances, it was shown that LGN simultaneously binds different proteins to its TPR and GoLoco regions. For example, FRET (fluorescence resonance energy transfer) studies demonstrated that binding of NuMA factor to the second TPR motif of LGN is preceded by the binding of G_α protein to the GoLoco motif [29]. In this respect, LGN functions as a scaffolding protein that brings together G-protein signalling and other cellular processes. Since each GoLoco motif may bind one G_α protein, up to four G_α proteins may be simultaneously bound to LGN or AGS3. Moreover, for some TPR-repeat containing proteins, it was demonstrated that only one repeat is sufficient to mediate protein–protein interactions [33]. Both LGN and AGS3 have seven such repeats and thus multiple interactions are possible. The region between the TPR and GoLoco domains also has been shown to interact with proteins. For example, several members of the PSD95 (postsynaptic density 95) family of proteins, such as PSD95, SAP (synapse-associated protein) 102 and SAP97, or the LKB1 serine/threonine kinase interact with LGN in this region [34]. Thus LGN and AGS3 may serve as scaffolds for the formation of protein complexes containing multiple protein components, including sGC. The observed co-operative nature of sGC inhibition by LGN may be the result of several different components being associated into a final composite complex. Thus, through interaction with LGN (or AGS3), sGC may be interfacing with a wide range of cellular processes or may be the focus of modulation by different cellular stimuli and factors. Future studies that will systematically assess the effect of known LGN interactors on sGC function will uncover the additional cellular factor(s) needed for sGC inhibition.

In summary, we identified LGN as a protein that interacts with sGC. This interaction is mediated by the TRP-containing domain of LGN and the C-terminal half of sGC. The interaction attenuates sGC activity, but is dependent on additional cellular factor(s). AGS3, the closest homologue of LGN, also inhibits sGC activity when combined with purified sGC in the presence of cell lysates. Interaction of sGC with these scaffolding proteins opens the possibility of the formation of multiprotein complexes tailored to specific tissues or signals and has the potential of expanding the cross-talk between NO/cGMP signalling and other cellular pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

AGAP1	Arf (ADP-ribosylation factor) GAP (GTPase-activating protein) with GTP-binding-protein-like, ankyrin repeats and PH (pleckstrin homology) domains
AGS3	activator of G-protein signalling 3
αCAT	α -catenin
βCAT	β -catenin
CCTη	chaperonin-containing T-complex polypeptide 1, η subunit
DEA-NO	2-(<i>N,N</i> -diethylamino)diazene-2-oxide, diethylammonium salt
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GDI	guanine-nucleotide-dissociation inhibitor
GPSM2	G-protein signalling modulator 2
HSP	heat-shock protein
LGN	Leu-Gly-Asn repeat-enriched protein
MAP	mean arterial pressure
NuMA	nuclear mitotic apparatus
ORF	open reading frame
PSD95	postsynaptic density 95
qPCR	quantitative PCR
SAP	synapse-associated protein
sGC	soluble guanylate cyclase

siRNA	small interfering RNA
TEA	triethanolamine
TPR	tetratricopeptide repeat

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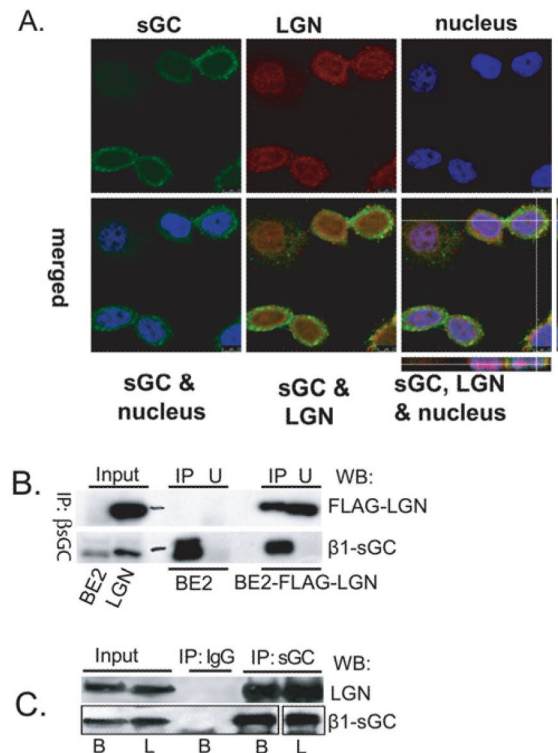


Figure 1. Overlapping co-localization and co-immunoprecipitation of sGC and LGN

(A) MDA468 cells, which endogenously express sGC, were grown in chamber slides to 30–50% confluency, fixed with 3.7% (w/v) paraformaldehyde and incubated with primary antibodies followed by incubation with fluorescent conjugated secondary antibodies as described in the Experimental section. Immunostaining of sGC and LGN and Sytox Orange staining of the nucleus are shown in the top panels. Different combinations of the merged signals are shown in the bottom panels. Confocal orthogonal sections with merged signals are shown in the bottom right-hand panel. (B) BE2 neuroblastoma cells were transfected with pCDNA3.1 plasmid expressing FLAG-tagged LGN. sGC was immunoprecipitated with polyclonal antibodies raised against the C-terminus of $\beta 1$. The immunoprecipitates (IP) and unbound samples (U) were probed by Western blotting (WB) with monoclonal anti- $\beta 1$ sGC and anti-FLAG antibodies. The data are representative of three independent immunoprecipitations, which showed similar results. (C) sGC from rat lung (L) and brain (B) tissue homogenates was precipitated using polyclonal anti- $\beta 1$ sGC antibodies immobilized on Protein G–magnetic beads (IP:sGC). Magnetic beads pre-incubated with pre-immune rabbit serum were used as a control (IP:IgG). Tissue homogenates (100 μ g) and the immunoprecipitated samples were separated by SDS/PAGE and examined separately by Western blotting with anti-LGN and anti- $\beta 1$ sGC antibodies. The results shown are from contiguous gels and are representative of three other immunoprecipitations performed with homogenates from three different animals.

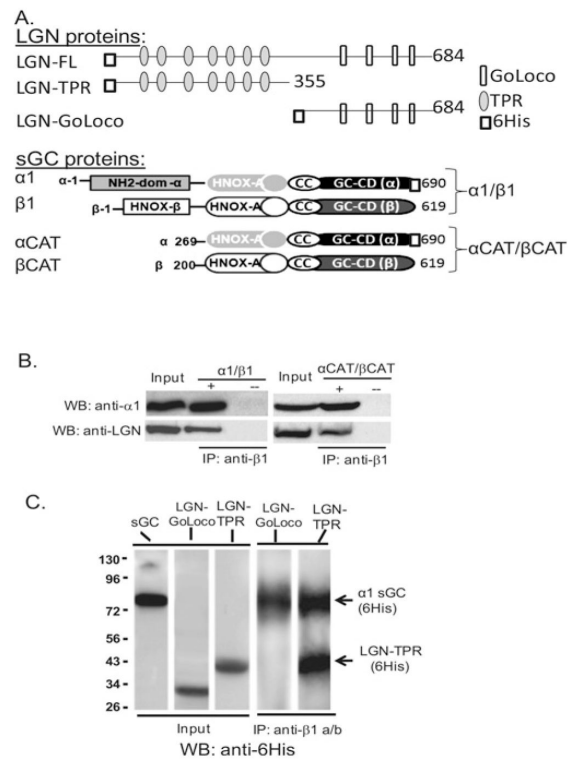


Figure 2. Mapping the regions of the sGC–LGN interaction

(A) Schematic representation of the recombinant full-length (FL) and truncated LGN and sGC used to map the regions of interaction. CC, coiled-coil; CD, catalytic domain; dom, domain; HNOX, haem/NO/oxygen-binding domain. (B) Full-length LGN co-precipitates with both full-length (left-hand panel) and truncated (right-hand panel) sGC variants, but is not pulled down in the absence of sGC protein. (C) The N-terminal region of LGN (TPR domain) co-precipitates with sGC, whereas the C-terminal region of LGN (GoLoco domain) does not. Anti-β1 antibodies (10 μg) were used for immunoprecipitation (IP), whereas anti-hexahistidine (6His) antibodies were used for Western blotting (WB). Molecular mass in kDa is shown on the left-hand side. Data in (B and C) are representative of three independent experiments with similar results.

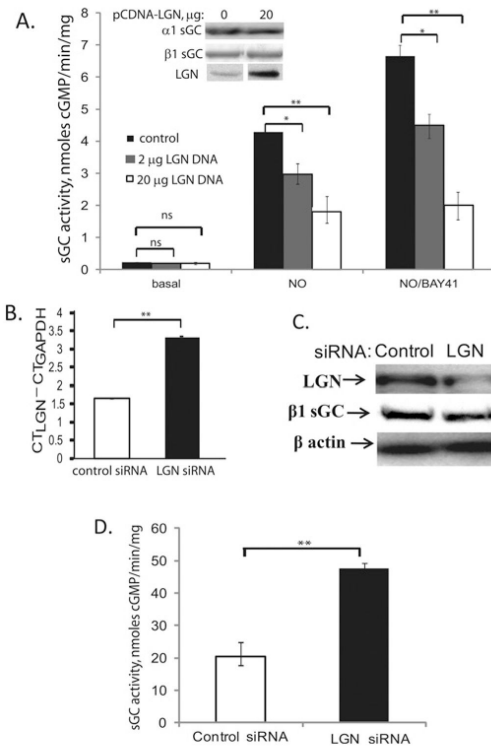


Figure 3. Changes in LGN expression affects sGC activity in cell lysates

(A) MDA468 cells (2×10^6) were transfected with the indicated amount of pCDNA 3.1-LGN construct or 2 µg of control pCDNA3.1 vector. sGC activity stimulated by NO (100 µM DEA-NO) or NO and BAY41-2722 (5 µM) was compared in lysates from both groups.

Activity data are means \pm S.D. for three independent transfections performed in triplicate.

(B and C) MDA468 cells were transfected with control scrambled siRNA (control) or LGN-targeted siRNA (siRNA LGN). qRT-PCR analysis (B) indicates that the relative abundance of *LGN* mRNA is decreased 48 h after transfection. *LGN* mRNA levels are normalized to the level of *GAPDH* mRNA. Average $\Delta C_T \pm$ S.D. from three biological triplicates is shown and indicates a $69 \pm 5\%$ decrease in *LGN* transcript. (C) Western blotting confirms that the level of LGN protein diminishes in response to LGN-targeted siRNA, but not control siRNA, whereas the level of sGC protein tested by anti-β1 antibodies does not.

Similar results were observed in all three experiments. (D) sGC activity stimulated by NO (100 µM DEA-NO) was higher in lysates from cells treated with LGN siRNA. Activity data are means \pm S.D. for three independent transfections performed in triplicate. ns, not significant; * $P < 0.05$; ** $P < 0.01$.

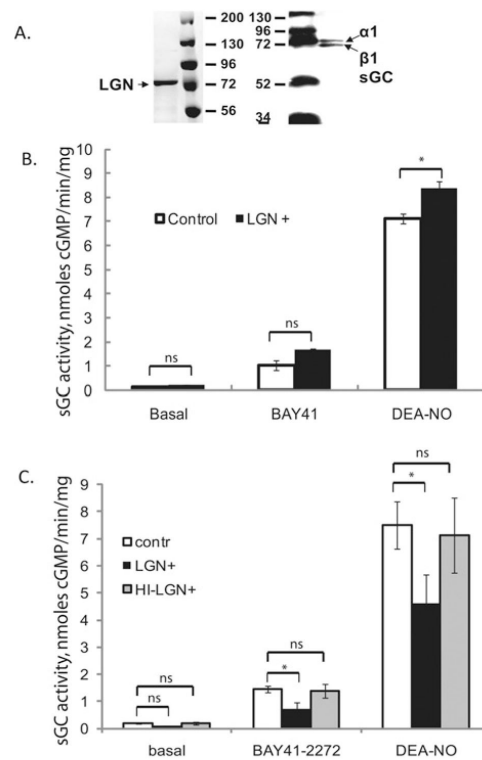


Figure 4. Cell lysate supplementation is required for inhibition of sGC by LGN

(A) Coomassie Blue staining of purified LGN (left-hand panel) and silver staining of purified sGC (right-hand panel) preparations. Molecular mass is shown in kDa. (B) Recombinant sGC was incubated with BSA (control) or purified LGN (LGN +) for 15 min prior to determining the basal, BAY41-2272 (BAY41) and DEA-NO activities of sGC. Data are means \pm S.D. for four independent measurements performed in triplicate. (C) Recombinant sGC was incubated with BSA [control (contr)], purified LGN (LGN +) or heat-inactivated LGN (HI-LGN +) for 15 min in the presence of 150 μ g of COS-7 lysate prior to determining the basal, BAY41-2272 and DEA-NO activities of sGC. Data are means \pm S.D. for three independent measurements performed in triplicate. ns, not significant; * $P < 0.05$.

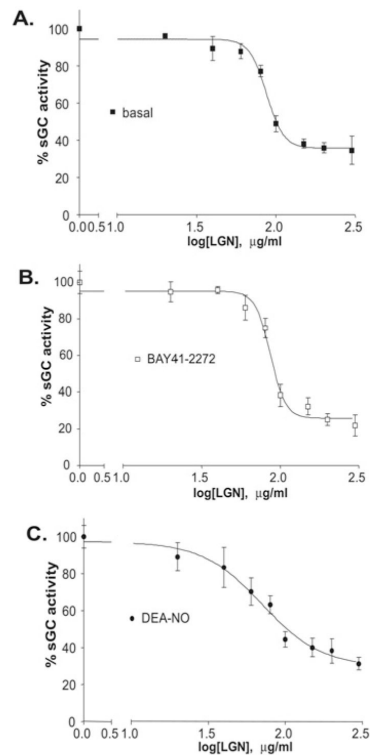


Figure 5. Dose-dependent effect of LGN on sGC activity

Recombinant sGC was incubated for 15 min with different amounts of purified LGN in the presence of 150 μ g of COS-7 lysate prior to determining sGC activity under basal conditions (A), or in the presence of 5 μ M BAY41-2272 (B) or 100 μ M DEA-NO (C). Data are means \pm S.D. for four independent experiments ($n = 10$), which were performed with three independent preparations of sGC and two preparations of LGN.

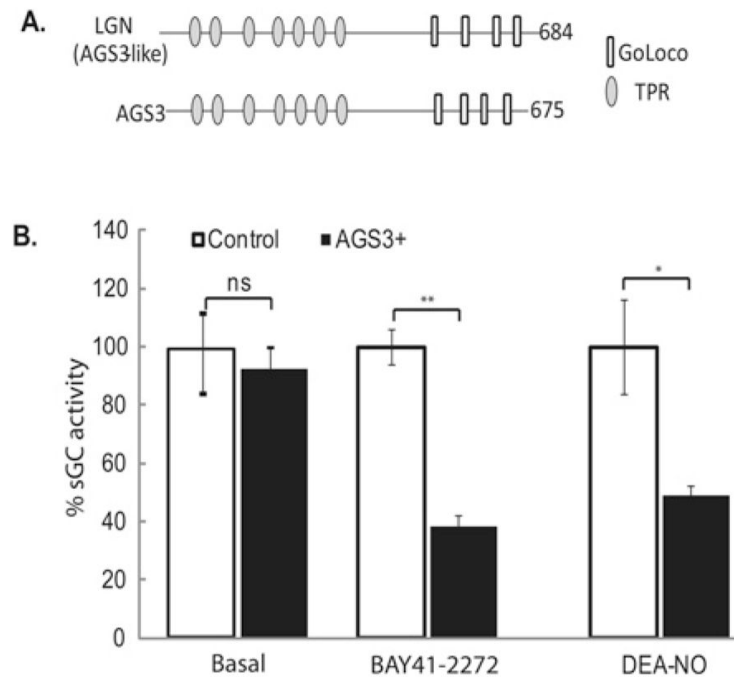


Figure 6. Effect of AGS3 on sGC activity

(A) Schematic representation of the motif composition of LGN (AGS3-like) and AGS3 proteins, which share 59% homology. (B) Recombinant sGC was incubated for 15 min with purified AGS3 (AGS3 +) or BSA (control) in the presence of 150 μ g COS-7 lysate prior to determining sGC activity in the presence of 5 μ M BAY41-2272 or 100 μ M DEA-NO. Data are means \pm S.D. for three independent experiments ($n = 7$). ns, not significant; * $P < 0.05$; ** $P < 0.01$.