Homodimerization of Soluble Guanylyl Cyclase Subunits

DIMERIZATION ANALYSIS USING A GLUTATHIONE S-TRANSFERASE AFFINITY TAG

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Soluble guanylyl cyclase (sGC) is an αβ-heterodimeric hemoprotein that, upon interaction with the intercellular messenger molecule NO, generates cGMP. Although the related family of particulate guanylyl cyclases (pGCs) forms active homodimeric complexes, it is not known whether homodimerization of sGC subunits occurs. We report here the expression in Sf9 cells of glutathione S-transferase-tagged recombinant human sGCα1 and β1 subunits, applying a novel and rapid purification method based on GSH-Sepharose affinity chromatography. Surprisingly, in intact Sf9 cells, both homodimeric GSTα/α and GSTβ/β complexes were formed that were catalytically inactive. Upon coexpression of the respective complementary subunits, GSTα/β or GSTβ/α heterodimers were preferentially formed, whereas homodimers were still detectable. When subunits were mixed after expression, e.g. GSTβ and β or GSTα and β, no dimerization was observed. In conclusion, our data suggest the previously unrecognized possibility of a physiological equilibrium between homo- and heterodimeric sGC complexes.

Nitric oxide plays an important role as an intercellular messenger in a great variety of physiological processes (1, 2). To mediate these effects, NO binds to and regulates several proteinaceous and nonproteinaceous cellular targets. The presently best characterized signal-transducing receptor for NO is the heme-containing enzyme soluble guanylyl cyclase (sGC) (3–5). Upon binding of NO to the prosthetic heme group, sGC catalyzes the conversion of GTP to cGMP, which in turn regulates various effector proteins, such as protein kinases, phosphodiesterases, and ion channels (6, 7).

Native sGC purifies as a heterodimeric complex composed of a larger α (~80 kDa) and a smaller β subunit (~70 kDa) (3, 8).

The N-terminal domains of both subunits are essential for the stimulation of the enzyme by NO (9, 10), whereas heme-binding occurs solely in the β subunit (11–13). Both the α and β subunit contain a C-terminal cyclase homology domain (CHD), which, in analogy to adenyllyl cyclases and pGCs, constitutes a bipartite catalytic center by the association of the α and β C-terminal domains (4, 14).

Whereas active pGCs are formed by homodimerization, i.e. association of identical CHDs (14), sGC activity depends on heterodimerization. Only the coexpression of α and β subunit cDNAs in heterologous expression systems constitutes active sGC (15–17), whereas separate expression of α or β subunits yields neither NO-sensitive nor basally active enzyme. Moreover, sGC activity could not be restored by mixing of the expressed subunits (15).

The central parts of sGCs and β (9) share extensive homologies with each other and with a 43-amino acid sequence in pGC that is essential for homodimerization (18). This prompted us to speculate whether homodimer formation of sGCα or sGCβ may also occur. It is, however, unknown whether α/α or β/β homodimers can assemble intracellularly or whether separately expressed sGC subunits stay monomeric in the absence of a complementary subunit. This would prevent the formation of a “two-CHD” catalytic center and thereby explain the lack of cGMP formation.

Using glutathione S-transferase (GST)-tagged recombinant human sGC subunits expressed in a baculovirus expression system, we here present a single-step purification method for recombinant human sGC (rhsGC) and its subunits, which enabled us to analyze the oligomerization behavior of the sGC subunits. Here we demonstrate for the first time the formation of homodimeric yet inactive sGC complexes. The possible physiological implications for regulation of sGC activity in intact cells are discussed.

EXPERIMENTAL PROCEDURES

Materials—GSH was purchased from Roche Molecular Biochemicals; cell culture materials were from Life Technologies, Inc. (Eggenstein, Germany). All other chemicals were of the highest purity grade available and obtained from either Sigma Chemicals (Deisenhofen, Germany) or Merck AG (Darmstadt, Germany). Water was deionized to 18 MΩ cm (Milli-Q; Millipore, Eschborn, Germany).

Baculovirus Construction—cDNAs comprising the complete coding sequences for hsGCα and β subunits (17, 19) were cloned into the pAcG2T baculovirus transfer vector (Pharmingen, San Diego, CA), which allows the expression of GST-sGC fusion proteins and provides a thrombin cleavage site for proteolytic removal of the GST tag. In-frame cloning, BamHI sites (underlined below) were introduced by polymerase chain reaction immediately upstream of the translational start sites. Fragments were amplified with the primer pairs 5’-AAAA-GATTCATGTTCTGCACGAAGCTC-3’ (bp 524–541) and 5’-ATTAT-GGAAAGCAAGGAGG-3’ (bp 1249–1232) for α1 and 5’-AAAAAGATCC-ATGTAAGATTTGTGGAAT-3’ (bp 89–106) and 5’-ATGTCGTAGTTC-TGGGTACC-3’ (bp 711–692) for β1. Products were cut with BamHI/ BsAI (α1 or BamHI/KpnI (β1) and ligated with Rsai/EcoRI (bp 1193–3015, α1) or KpnI/EcoRI (bp 692–2444, β1) cDNA fragments to the BamHI/EcoRI-claved vector. Recombinant GST-hsGCα1 and GST-hsGCβ1 baculoviruses were isolated as described for hsGCα1 and hgGCβ1 baculoviruses (17).

Sf9 Cell Culture and Production of rhsGC—Sf9 cells were cultured as described (17). For expression of nontagged rhsGC subunits, spinner cultures (2 x 10^10 cells ml^-1) were infected with recombinant baculoviruses coding for hsGCα1 or hsGCβ1 (17) at a multiplicity of infection (m.o.i.) of 5 plaque-forming units/cell. This high m.o.i. ensured that nearly all cells (> 99%) were infected with the viruses encoding the
Expression and heterodimerization of GST-tagged and nontagged rhsGCα1 and β1 subunits. Crude supernatant fractions of Sf9 cells expressing GST-tagged rhsGCα1 (GSTα), GST-tagged rhsGCβ1 (GSTβ), nontagged rhsGCα1 (α), and/or β1 (β) were subjected to GSH-Sepharose affinity chromatography (see “Experimental Procedures”). Load and eluate fractions were analyzed by Western blot, which was developed simultaneously with α1- and β1-specific antibodies (see “Experimental Procedures”). A, GSTα and GSTβ specifically bind to GSH-Sepharose. GSTα, GSTβ, α, and β were expressed separately. Sf9, crude supernatant fraction of noninfected Sf9 cells. B, heterodimerization of GST-tagged rhsGC. GSTα and β (GSTβ and α) were coexpressed (lanes 1–4) or mixed after separate expression and incubated for 15 min at room temperature prior to GSH-Sepharose binding (lanes 5–8). L, load; E, GSH eluate.

nontagged subunits, which is necessary in the triple expression experiment shown in Fig. 2D (see “Results”). Infections with GST-hsGCα and GST-hsGCβ viruses (see above) were performed at an m.o.i. of 0.5 plaque-forming units/cell, because expression levels of the GST-tagged rhsGC subunits were substantially higher (data not shown). Infection of cells with a baculovirus coding for GST (kind gift from C. Weber, Institut für Medizinische Strahlenkunde und Zellforschung, Würzburg) were performed at a m.o.i. of 2.5 plaque-forming units/cell. Cells were harvested 72 h post infection, and all subsequent procedures were performed at 4 °C. Cells were lysed for 15 min on ice in hypotonic lysis buffer (25 mM triethanolamine, pH 7.8, 1 mM EDTA, 5 mM dithiothreitol, 1 mM leupeptin, 0.5 μg/ml 1 mM soybean trypsin inhibitor), and crude supernatant and particulate fractions were separated by centrifugation (20,000 × g) for 15 min at 4 °C. Supernatant fractions were brought to a final concentration of 10% (v/v) glycerol and kept at −20 °C, a final concentration of 10% (v/v) glycerol was used. Protein concentrations were determined according to Bradford (20), using bovine serum albumin as a standard.

GST-Sepharose Affinity Chromatography—Glutathione-Sepharose 4B (Amersham Pharmacia Biotech, Freiburg, Germany) was equilibrated with lysis buffer (see above) containing 75 mM NaCl, incubated with crude supernatant fractions of rhsGC-containing Sf9 cells for 1 h at 25 °C in a rotation mixer, and washed two or three times with lysis buffer containing 75 mM NaCl. For GST elution, GSH-Sepharose was incubated with 5 mM GSH in 50 mM Tris-HCl, pH 8.0, for 5 min at 25 °C. Fractions were brought to a final concentration of 10% (v/v) glycerol and kept at −20 °C.

Size Exclusion Chromatography—Crude rhsGC-containing Sf9 supernatant fractions were subjected to fast protein liquid chromatography on a Superose 6 column (Amersham Pharmacia Biotech) at a flow rate of 0.2 ml min−1 in 50 mM Tris-HCl, pH 6.7, 300 mM NaCl, 1 mM EDTA and 1 mM dithiothreitol. Aliquots of each fraction were assayed for rhsGC-immunoreactive protein by Western blot. Signals were quantitated by flatbed scanning and densitometry using the NIH Image software (Division of Computer Research and Technology, National Institutes of Health, Bethesda, MD). The column was calibrated with standard proteins (Sigma) of known Stck’s radii: thyroglobulin (8.5 nm), apoferritin (6.1 nm), alcohol dehydrogenase (4.55 nm), bovine serum albumin (3.55 nm), and carbonic anhydrase (2.01 nm).

sGC Activity Assay—sGC activity was measured as the formation of cGMP at 37 °C for 10 min in a total volume of 100 μl containing 50 mM triethanolamine HCl, pH 7.4, 3 mM GSH, 1 mM 3-isobutyl-1-methylxanthine, 5 mM creatine phosphate, 0.25 mg ml−1 creatine kinase, 500 μM GTP, and either 3 mM MgCl2 or 3 mM MnCl2. Reactions were started by adding the enzyme-containing fraction and immediately thereafter the sGC activator sodium nitroprusside (100 μM). The cGMP content was determined by an enzyme-linked immunosassay (Biotrend, Cologne, Germany). Results are expressed as the means ± S.E. of at least three experiments.

Western Blot—Immunodetection of nontagged and GST-tagged rhsGC subunits was performed as described previously (17), using polyclonal antibodies raised against peptide sequences that correspond to hGGuCr1 (amino acids 634–647) and hGGuCp1 (amino acids 590–614), which were affinity-purified against the respective peptides. Blots were developed using the ECL detection system (Amersham Pharmacia Biotech) according to the manufacturer’s protocol.

RESULTS AND DISCUSSION

To examine sGC subunit association, we established a rapid and efficient method to purify rhsGC and its subunits. Fusion proteins composed of a GST affinity tag (25 kDa), and the recombinant human sGCα1 and β1 subunits (rhsGCα1, 79.5 kDa, and rhsGCβ1, 65, 5 kDa (17)) were constructed and expressed in the baculovirus/Sf9 cell system. As shown in Fig. 1A, the recombinant GST-rhsGCα1 and GST-rhsGCβ1 (GSTα, GSTβ) fusion proteins migrated with the expected apparent molecular masses of 105 and 94 kDa, bound to GSH-Sepharose, and were specifically eluted with GSH (lanes 2–5). In contrast, the nontagged rhsGCα1 (α) and rhsGCβ1 (β) subunits did not bind to the GSH beads (lanes 6–9). In addition to the full-length recombinant proteins, some sGC-immunoreactive degradation products were recognized in crude Sf9 lysates (lanes 2, 4, and 6), which did not appear in lysates from noninfected Sf9 cells (lane 1).

As shown in Fig. 1B, the GST-tagged sGC subunits retained their ability to heterodimerize with the respective complementary nontagged subunit. When coexpressed in Sf9 cells, nontagged β co-eluted with GSTα and vice versa (lanes 1–4), indicating a direct physical interaction and demonstrating that addition of the GST tag did apparently not interfere with the dimerization function. Whereas coexpression of GSTα and β subunits yielded active and NO-sensitive sGC in crude Sf9 cell supernatant fractions, only basal sGC activity was detected in the load or eluate fractions derived from mixed expression (lanes 5–8; Table I). As shown in lane 9, the GSTαβ N terminus interfered with NO stimulation, probably because of the close proximity of the GST to the heme-binding site (11–13). GSTαβ activity in the presence of Mg2+ was very similar to that of nontagged rhsGC expressed in the same system (17). In the presence of Mn2+, basal sGC activity was increased, whereas NO-stimulated activity was decreased (Table I), as reported for native (21) and recombinant sGC (9). Specific basal sGC activity was dramatically increased in the GSH eluate fraction (Table I), similar to sGCαβ prepared by multi-step purification procedures (8, 22–24). Moreover, NO sensitivity of purified GSTαβ was preserved, because the enzyme was activated 30-fold by 100 μM sodium nitroprusside (Table I).

Heterodimerization of both GSTαβ and GSTβα complexes was dependent on coexpression of the respective subunits (Fig. 1B, lanes 5–8). Accordingly, no basal or NO-stimulated sGC activity was detected in the load or eluate fractions derived from mixed expression (lanes 5–8; Table I). As shown in lane 9, the GSTαβ N terminus interfered with NO stimulation, probably because of the close proximity of the GST to the heme-binding site (11–13). GSTαβ activity in the presence of Mg2+ was very similar to that of nontagged rhsGC expressed in the same system (17). In the presence of Mn2+, basal sGC activity was increased, whereas NO-stimulated activity was decreased (Table I), as reported for native (21) and recombinant sGC (9). Specific basal sGC activity was dramatically increased in the GSH eluate fraction (Table I), similar to sGCαβ prepared by multi-step purification procedures (8, 22–24). Moreover, NO sensitivity of purified GSTαβ was preserved, because the enzyme was activated 30-fold by 100 μM sodium nitroprusside (Table I).
Similarly, the nontagged \( \beta \) and purified from rat lung were determined in crude Sf9 cell supernatant fractions in the presence of 3 mM Mg\(^{2+} \) or 3 mM Mn\(^{2+} \). sGC activities are given as mean ± S.E. of at least three experiments performed in duplicate. The corresponding stimulation factors are provided (fold), NA, not applicable.

| sGC activity | Basal | SNP |
|--------------|-------|-----|
|               | \( \text{nmol cGMP mg}^{-1} \text{min}^{-1} \) | \( \text{nmol cGMP mg}^{-1} \text{min}^{-1} \) |
| GST\( \alpha/\beta \) | 1.3 ± 0.3 | 30.1 ± 5.1 |
| GST\( \beta/\alpha \) | 0.5 ± 0.1 | 0.5 ± 0.1 |
| GST\( \alpha/\alpha \) | <0.03 | <0.03 |
| GST\( \beta/\beta \) | <0.03 | <0.03 |

From separately expressed and mixed GST\( \alpha \) and \( \beta \) (or GST\( \beta \) and \( \alpha \)) subunits (data not shown), which was in agreement with previous findings on nontagged \( \alpha \) and \( \beta \) subunits (15).

When GST\( \alpha/\alpha \) and GST\( \beta/\beta \) were coexpressed in Sf9 cells, none of the crude cell lysates contained any detectable sGC activity (i.e., <30 pmol cGMP mg\(^{-1} \text{min}^{-1} \)), Table I), neither with Mg\(^{2+} \) nor Mn\(^{2+} \). To investigate whether sGC subunits homo-oligomerized, fractions were analyzed by Western blot. Interestingly, nontagged \( \beta \) efficiently copurified with GST\( \beta \), demonstrating a direct physical interaction between sGC subunits (Fig. 2A, lanes 3 and 4). This \( \beta/\beta \) homodimerization was dependent on coexpression of both subunits (lanes 7 and 8).

Similarly, the nontagged \( \alpha \) subunit copurified with GST\( \alpha \). However, binding of GST\( \alpha \) to the GSH-Sepharose was weakened when nontagged \( \alpha \) was coexpressed (Fig. 2A, lanes 1 and 2), requiring longer exposure times during Western blot analysis (Fig. 2B, upper panel). In contrast, nontagged \( \alpha \), which was expressed separately and mixed with GST\( \alpha \), had no effect on binding of GST\( \alpha \) to the beads (Fig. 2A, lanes 5 and 6). This demonstrated that nontagged \( \alpha \) interacted with GST\( \alpha \) upon coexpression, leading to an interference with binding to GSH-Sepharose, possibly because of steric hindrance. Coelution of \( \alpha \) and \( \beta \) with the respective GST-tagged subunit did not result from unspecific binding to the GSH-Sepharose, because the wash steps prior to elution did not contain any nontagged sGC (Fig. 2B). Likewise, \( \alpha \) and \( \beta \) did not coelute with GST alone (Fig. 2C). Therefore, coelution of \( \alpha \) with GST\( \alpha \) and of \( \beta \) with GST\( \beta \) represent specific protein-protein interactions of identical rhsGC subunits.

To analyze whether single rhsGC subunits form dimeric (as does \( \alpha/\beta \)) or multimeric complexes, crude Sf9 supernatant fractions containing rhsGC were subjected to size exclusion chromatography (Fig. 3). To exclude any potential artifacts caused by the GST tag, nontagged subunits were applied in this set of experiments. As shown in Fig. 3A, coexpressed rhsGC\( \alpha \) and \( \beta \) subunits perfectly co-eluted upon size exclusion chromatography, with a Stoke’s radius of 5.3 nm (Fig. 3A, inset). A similar Stoke’s radius (4.8 nm) was reported for heterodimeric sGC purified from rat lung (3). Importantly, rhsGC\( \alpha \) and rhsGC\( \beta \), when expressed separately, showed an elution pattern very similar to heterodimeric sGC (Fig. 3B), suggesting that both subunits indeed exist as homodimers. However, a fraction of rhsGC\( \beta \) (>20%) formed aggregates under these conditions (Fig. 3B, fractions 2–6). The peaks of rhsGC\( \alpha \) and rhsGC\( \beta \) were slightly separated, reflecting apparent differences in the Stoke’s radii between the \( \alpha/\alpha \) and \( \beta/\beta \) homodimers (Fig. 3B).

To further investigate whether sGC homodimer formation can occur also in the presence of complementary subunits, Sf9 cells were cotransfected with three viruses coding for GST\( \alpha \), \( \alpha \), and \( \beta \) (or GST\( \beta \), \( \alpha \), and \( \beta \)). Viruses coding for \( \alpha \) and \( \beta \) were applied at a high m.o.i. to ensure that nearly all cells (>99%) expressing GST\( \alpha \) (or GST\( \beta \)) were simultaneously infected with both \( \alpha \) and \( \beta \) viruses. As shown in Fig. 2D, heterodimer (i.e., GST\( \beta/\alpha \) or GST\( \alpha/\beta \), formation is preferred under these conditions in insect cells. However, about 10% of the recombinant protein formed GST\( \alpha/\alpha \) or GST\( \beta/\beta \) complexes even in the presence of the respective complementary subunits. This demonstrated the existence of an equilibrium between homo- and heterodimeric sGC.

Based on these data, sGC\( \alpha \) and \( \beta \) subunits are in fact capable of forming homodimeric complexes in intact Sf9 cells. It has to be clarified whether classical purification methods efficiently remove homodimeric sGC from crude preparations of recombi-
tions of Sf9 cells coexpressing rhsGCα1erodimeric rhsGC was 5.3 nm.
against the respective elution fraction. The Stoke’s radius of het-
etry. The from Western blot analysis of fractions were quantitated by densitom-
rhsGC
rhsGC
homodimeric sGC upon mixing of separately expressed sub-
glutamate. The signal presented here, only GST
b
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tion depend on co- or post-translational processes. It has
sciousness. An endogenous inhibitor of sGC has been de-
b
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in heterodimeric sGC complexes; \( \beta \), homodimeric rhsGCα1; \( \gamma \), homodimeric rhsGCβ1.
Soluble Guanylyl Cyclase Homodimers
units, which seems to be similar to that of heterodimeric sGC, argues against substantial affinity differences. On the other hand, sGC dimerization might be a regulated process in living cells. The existence of at least two different isoforms of each subunit (α1, α2, β1, and β2) led to the concept that sGC activity is regulated in vivo by alternative heterodimerization (4, 5). It is an intriguing possibility that regulation of sGC activity in vivo might involve not only alternative heterodimerization but also changes in the extent of homodimerization. Based on our data, it cannot be excluded that a further protein is associated with homodimeric sGC complexes, which may be involved in complex formation and therefore be a functional inhibitor of sGC activity. An endogenous inhibitor of sGC has been described in Ref. 25. Interestingly, both homo- and heterodimerization depend on co- or post-translational processes. It has been suggested that chaperone-mediated formation of hetero-
oligomeric protein complexes is involved in the regulation of signaling pathways (26). A related process may regulate sGC protein-protein interactions and thus NO/cGMP signaling.

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FIG. 3. Size exclusion chromatography. Crude supernatant fractions of Sf9 cells coexpressing rhsGCα1 and β1 (A) or rhsGCα1 or rhsGCβ1 (B) were subjected to size exclusion chromatography. Signals from Western blot analysis of fractions were quantitated by densitometry. The inset in A depicts Stoke’s radii of protein standards plotted against the respective elution fraction. The Stoke’s radius of heterodimeric rhsGC was 5.3 nm. ■, rhsGCα1 in heterodimeric sGC complexes; □, rhsGCβ1 in heterodimeric sGC complexes; ▲, homodimeric rhsGCα1; ○, homodimeric rhsGCβ1.