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Amphipathic $\alpha$-Helix Mediates the Heterodimerization of Soluble Guanylyl Cyclase

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Short title: Heterodimerization of Soluble GC

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ABSTRACT - Soluble guanylyl cyclase (soluble GC) is an enzyme consisting of α and β subunits and catalyzes the conversion of GTP to cGMP. The formation of heterodimer (α₁/β₁ or α₂/β₁) is essential for the catalytic activity. Each subunit has been shown to comprise three functionally different parts: a C-terminal catalytic domain, a central dimerization domain, and an N-terminal regulatory domain. The central dimerization domain in the β₁ subunit, which contains an N-terminal binding site (NBS) and a C-terminal binding site (CBS), have been postulated to be responsible for the formation of α/β heterodimers. In this study, we analyzed heterodimerization by pull down assay using the affinity between histidine tag and Ni²⁺ Sepharose after co-expression of various N- and C-terminally truncated α₁ mutants (FLAG tag) and β₁ wild type (histidine tag) in the baculovirus/Sf9 system, demonstrating that the CBS-like sequence of the α₁ subunit is critical for the formation heterodimer with the β₁ subunit and the NBS-like sequence of the α₁ subunit is essential for the formation of enzymatically active heterodimer, although itself was not involved in heterodimerization. The analysis of the secondary structure of the α₁ subunit predicted the existence of an amphipathic α-helix in the residues 431-464. Experiments with site-directed α₁ subunit mutant proteins demonstrated that the amphipathicity of the α-helix is important for the formation of a correct active center in the dimer.

Key words: soluble guanylyl cyclase, cGMP, heterodimer, dimerization domain, recombinant protein, pull down assay, amphipathic α-helix, secondary structure
INTRODUCTION

Both the soluble and membrane forms of guanylyl cyclases (GCs) catalyze the conversion of GTP to cGMP, which is a ubiquitous second messenger in intracellular signaling cascades and responsible for a wide variety of physiological responses (Drewett and Garbers, 1994; Garbers and Lowe, 1994; Garbers et al., 1994). Soluble GC is a heterodimer consisting of α and β subunits, and contains a prosthetic ferrous heme group to which nitric oxide (NO) binds with high affinity. Formation of an NO-heme complex and a subsequent conformational change of the heterodimer are responsible for the up to 200-fold increases in the catalytic rate (Gerzer et al., 1981; Humbert et al., 1990; Stone and Marletta, 1994). In mammals, cDNAs for four soluble GC subunits (α₁, α₂, β₁, and β₂) have been isolated from various tissues (Koesling et al., 1988; Koesling et al., 1990; Yuen et al., 1990; Harteneck et al., 1991). Both the α₁/β₁ and α₂/β₁ heterodimers have been shown to be enzymatically active when formed in an in vitro expression system (Russwurm et al., 1991). A single expression of either one of the subunits has been shown to yield an α₁/α₁- or a β₁/β₁-homodimer, although both homodimers were catalytically inactive (Zabel et al., 1999). In previous studies, we have isolated and characterized the cDNA and genomic DNA clones encoding the soluble GC subunits, OIGCS-α₁ and OIGCS-β₁, both of which are aligned in tandem on the genome of the medaka fish Oryzias latipes (Mikami et al., 1998, Mikami et al., 1999). We recently demonstrated that the medaka fish contains another type of a soluble GC subunit gene, OIGCS-α₂, which locates on different linkage group of OIGCS-α₁ and OIGCS-β₁ (Yao et al., 2003).

Each subunit of soluble GC can be divided functionally into three parts: a C-terminal catalytic domain, a central region commonly referred to as the dimerization
domain, and an N-terminal regulatory domain participating in heme-binding (Koesling, 1999). The His$^{105}$ of the $\beta_1$ subunit has been identified as the proximal heme ligand site (Wedel et al., 1994; Zhao et al., 1998; Foerster et al., 1996). The C-terminal catalytic domain is highly conserved among various soluble GC subunits and is assigned as being responsible for catalysis based on homology to the related domain of adenyl cyclase (Koesling, 1999). Dimerization has been attributed to the central region of the soluble GC subunits mainly based on studies with the peptide receptor/membrane GCs (Wilson and Chinkers, 1996). Recently, it has been demonstrated that the dimerization region of the $\beta_1$-subunit consists of 205 amino acid residues over the regulatory and central regions and contains a discontinuous site of 41 amino acid residues (N-terminal binding site: NBS) and 30 amino acid residues (C-terminal binding site: CBS), respectively, facilitating binding of the $\beta_1$ subunit to the $\alpha_1$ subunit (Zhou et al., 2004). On the other hand, the function of the N-terminal part of the $\alpha_1$ subunit has been remained to be solved, although there are several papers to demonstrate that the N-terminal part of the $\alpha_1$ subunit is critical for heme-binding (Wedel et al., 1995) and that deletion of the N-terminal 259 amino acids from the $\alpha_1$ subunit had no effect on the properties of the enzyme at all, and the amino acids 259-364 of the $\alpha_1$ subunit represent an important functional domain for the transduction of the NO-activation signal (Koglin and Behrends, 2003). Although soluble GC has not been crystallized yet for X-ray diffraction analysis, it has been predicted that its catalytic domain is resemble to that of the class III adenyl cyclases (Tucker et al., 1998; Sunahara et al., 1998). Structural analysis of the catalytic core of the adenyl cyclases demonstrated an antiparallel orientation of the two catalytic domains ($C_1$ and $C_2$) with two pockets, each being formed by both the catalytic domains (Zhang et al., 1997; Tesmer et al., 1997).
To investigate the dimerization region of the $\alpha_1$ subunit, we generated several recombinant soluble GC subunits in an expression system with Sf9 cells and performed pull down assay using the affinity between histidine tag and Ni$^{2+}$ Sepharose. Here, we report that the CBS-like sequence of OIGCS-$\alpha_1$ is involved in the formation of heterodimer with OIGCS-$\beta_1$ and that the amphipathic $\alpha$-helix of OIGCS-$\alpha_1$ is critical for the formation of heterodimer with OIGCS-$\beta_1$. We also describe that the NBS-like sequence of OIGCS-$\alpha_1$ plays an important role in the formation of an enzymatically active heterodimer, although it is not involved in the formation of heterodimer with OIGCS-$\beta_1$.

**MATERIALS AND METHODS**

**Construction of plasmid for histidine-tagged OIGCS-$\beta_1$-full length protein (FL$\text{HT}$)**

Total RNA was isolated from the adult brain of *O. latipes* by the acid/guanidinium/thiocyanate/phenol/chloroform extraction method (Chomczynski and Sacchi, 1987). Total RNA (5 $\mu$g) was used as the template to synthesize the first strand cDNA using an oligo(dT) primer and Super Script II preamplification system for the first-strand cDNA synthesis (Gibco BRL, Tokyo, Japan) according to the manufacturer’s protocol. To obtain a C-terminal histidine-tagged OIGCS-$\beta_1$-full length protein (OIGCS-$\beta_1$-FL$\text{HT}$), we designed the following primers: 5’-GTG GAT CCA TGT ATG GTT TTG TGA AT-3’ and 5’-CGC GTC GAC TTA GTG ATG GTG ATG TGC CTT GTC AGC GTC GCT GC-3’. PCR was performed using *Ex Taq* polymerase (TaKaRa, Otsu, Japan) according to manufacturer’s protocol with the following PCR conditions: 30 cycles of 30 sec at 94°C, 30 sec at 50°C, 4 min at 72°C, and a final extension of 5 min at 72°C. The cDNA fragment amplified by the
PCR was purified and subcloned into the plasmid vector pBluescript II KS(-) (Stratagene, La Jolla, California, USA). A BamHI/SalI insert from the OlGCS-β_i clone in pBluescript II KS(-) vector was ligated into BamHI/SalI cut pFASTBAC1 vector (Invitrogen Japan K.K., Tokyo, Japan).

**Construction of plasmids for FLAG-tagged OlGCS-α_i-full length protein (FL_FLAG) and its truncated mutant proteins**

To obtain a C-terminal FLAG-tagged OlGCS-α_i-full length protein (OlGCS-α_i-FL_FLAG), several N-terminal and C-terminal deletion mutant proteins of OlGCS-α_i, we performed PCR using an OlGCS-α_i clone in pBluescript II KS(-) as a template. PCR was performed using Pfu polymerase (TaKaRa) according to the manufacturer’s protocol with various primers (Table 1). The reaction parameters used for PCR were as follows: 30 cycles of 30 sec at 94°C, 30 sec at each annealing temperature shown in Table 1, and 2 min at 72°C, with a final extension of 5 min at 72°C. Each PCR product was purified and digested by SpeI and XhoI. Resulting SpeI/XhoI cut PCR product was subcloned into pFASTBAC1 vector.

**Construction of plasmids for C-terminal FLAG-tagged site-directed OlGCS-α_i mutant proteins**

Mutagenesis of OlGCS-α_i was performed using the following primers: 5’-GCC CAG GAT GGC_AAG AAG AAG CG-3’ for L434K, 5’-GGC AGC CAA GGA AAA CGC TCA CC-3’ for L445K, 5’-CGC TCA CCA AGC GAA GGA GGA GG-3’ for L453K, and 5’-GAT CTT_AAG TTT TCC ATT TTC CC-3’ for L463K. As a template for the mutagenesis, we used the pFASTBAC1 vector containing C-terminal FLAG-tagged intact OlGCS-α_i-full length sequence. The mutagenesis was performed
by the “site-directed and semi-random mutagenesis” procedure (Sawano and Miyawaki, 2000).

**Generation of recombinant baculovirus**

All clones were checked for correctness of the sequence by DNA sequencing. The sequence was determined by the dideoxy chain termination procedure (Sanger et al., 1992) with an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Osaka, Japan) and analyzed with GENETYX-MAC/version 7.2.0 software (Software Development, Tokyo, Japan). Recombinant baculovirus of the respective clone was generated according to a BAC-TO-BAC Baculovirus Expression System (Invitrogen Japan K. K.).

**Sf9 cell culture, expression of recombinant soluble GC subunit constructs and the mutants, and cytosol preparation**

Sf9 cells were cultured in SF-900 II serum-free medium supplemented with 50 µg/ml streptomycin, 50 U/ml penicillin, and 20% fetal bovine serum (FBS). Spinner cultures were grown to a cell density of 3.0 x 10^6 cells/ml and then diluted to 2.0 x 10^6 cells/ml in a tissue culture flask for infection. Ten ml of the culture medium containing Sf9 cells (2.0 x 10^6 cells/ml) were infected by each virus stock of OlGCS-α1 or co-infected with OlGCS-β1-FL_{HT}-containing virus (ratio 1:1), and continued to culture for 4 days at 37°C. All subsequent experiments were carried out at 4°C. Cells were harvested by centrifugation at 1,000 g for 5 min. The resulting pellet was washed in 1 ml of ice-cold phosphate-buffered saline (PBS), pH 6.2, and then resuspended in 500 µl of an ice-cold lysis buffer for pull down assay (50 mM phosphate buffer, pH 7.2 containing 100 mM NaCl, 10% glycerol, and 0.1% protease inhibitor mixture in DMSO solution) (Wako, Osaka, Japan) or a lysis buffer for assay of GC activity (20 mM
Tris-HCl, pH 6.8, 90 mM NaCl, 10 % glycerol, and 0.1% protease inhibitor mixture-dimethylsulfoxide (DMSO) solution). The suspension was sonicated and then centrifuged at 15,000 g for 20 min, and the resulting supernatant fraction was collected and used for further experiments.

**Determination of protein concentration, Western blotting, and pull down assay**

Protein concentration was determined by the BCA method using BCA protein assay reagent (PIERCE, Rockford, Illinois, USA). Before each pull down assay, the similar expression level of the protein in each lysate was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The cell lysate was subjected to SDS-PAGE on a 10% polyacrylamide gel and transferred to a PVDF membrane. The membrane was blocked with 2% dry milk in TPBS (PBS, pH 7.4, containing 0.1% Tween 20) for 15 min at room temperature, rinsed, and incubated overnight at 4°C with anti-His-Tag antibody (MBL, Nagoya, Japan) or ANTI-FLAG M2 monoclonal antibody-alkaline phosphatase conjugate (SIGMA, Saint Louis, Missouri, USA) in TPBS containing 0.2% dry milk. Subsequently, the anti-His-Tag antibody-treated membrane was incubated with secondary antibody for 1 h at room temperature. Immunoreacted proteins were detected using the CDP-Star, ready-to-use (Roche, Tokyo, Japan) or Western blotting detection reagents (Amersham). The density of each band was determined by scanned image using Scion Image Beta 4.0.2 (Scion corporation, Frederick, Maryland, USA). The lysis buffer for pull down assay containing 500 µg protein was incubated overnight at 4°C with 150 µl of chelating Sepharose (Amersham Pharmacia Biotech, Tokyo, Japan), which was preincubated in a total volume of 600 µl containing 75 µl of 0.2 M NiCl₂ for 5 min according to the manufacture’s protocol. The sample was washed 3 times with 750 µl of the ice-cold
lysis buffer for pull down assay, and then washed 3 times with 300 µl of the ice-cold lysis buffer for pull down assay containing 15 mM imidazole. Proteins were eluted with the ice-cold lysis buffer for pull down assay containing 150 mM imidazole. Eluted proteins were subjected to SDS-PAGE and Western blotting as described above.

**Assay of GC activity**

Before the assay of GC activity, the similar expression level of the protein in each cytosolic fraction was confirmed by SDS-PAGE and Western blotting. GC activity of the cytosolic fraction (50 µg protein/assay tube) was determined in a total volume of 250 µl by incubation for 15 min at 37°C in an assay solution (50 mM Tris-HCl, pH 7.5, 4 mM MgCl₂, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 7.5 mM creatine phosphate, 25 U/ml creatine phosphokinase, and 1 mM GTP) with or without 1 mM sodium nitroprusside dihydrate (SNP). The reaction was stopped by boiling for 3 min, and then each assay tube was centrifugated at 15,000 g for 10 min. cGMP in the supernatant was determined by cGMP enzyme immunoassay system (Amersham Biosciences, Tokyo, Japan).

**RESULTS AND DISCUSSION**

**Identification of N-terminal binding site (NBS) and C-terminal binding site (CBS) of OIGCS-α₁**

To assign the putative NBS- and CBS-like sequences of OIGCS-α₁, the amino acid sequence of OIGCS-α₁ was aligned with that of rat soluble GC-α₁. Sequence identities of the NBS-like sequence of OIGCS-α₁ at positions 280-321 and the CBS-like
sequence of OlGCS-α₁ at position 448-477 to that of the corresponding rat soluble GC-α₁ were 45.2 and 90%, respectively. To define the binding ability of the NBS-like sequence of OlGCS-α₁ or the CBS-like sequence of OlGCS-α₁ to OlGCS-β₁, we generated FLAG-tagged N-terminal deletion mutants or C-terminal deletion mutants of OlGCS-α₁ (Fig. 1), and either one was expressed or co-expressed with the OlGCS-β₁-FL-HT in Sf9 cells.

To examine the binding ability of the NBS-like sequence to OlGCS-β₁-FL-HT, we performed pull down assay (Fig. 2A). OlGCS-α₁-FL-FLAG, OlGCS-α₁[280-678]FLAG or OlGCS-α₁[322-678]FLAG did not bind to Ni²⁺ Sepharose when each construct was expressed without OlGCS-β₁-FL-HT. Both OlGCS-α₁[280-678]FLAG and OlGCS-α₁[322-678]FLAG fully retained their ability to bind to OlGCS-β₁-FL-HT, judging from the result that each OlGCS-α₁ N-terminal deletion protein was detected in the Ni²⁺ Sepharose-binding fraction. This suggests that the NBS-like sequence of OlGCS-α₁ (280-321) is not involved in the heterodimerization with OlGCS-β₁.

We then examined the binding ability of the C-terminal deletion mutant proteins of OlGCS-α₁ to OlGCS-β₁. Co-expression of OlGCS-α₁[1-448]FLAG or OlGCS-α₁[1-477]FLAG with OlGCS-β₁-FL-HT was followed by pull down assay and Western blotting. As shown in Fig. 2B, OlGCS-α₁[1-448]FLAG did not exhibit the binding ability to OlGCS-β₁-FL-HT, and no protein band was detected in the Ni²⁺ Sepharose-binding fraction of α₁[1-448]FLAG/β₁-FL-HT by anti-FLAG antibody. Conversely, OlGCS-α₁[1-477]FLAG bound to OlGCS-β₁-FL-HT, suggesting that the CBS-like sequence of OlGCS-α₁ contains the structural elements mediating heterodimerization with OlGCS-β₁-FL-HT.

To examine the GC activity of the N-terminal deletion mutant proteins of OlGCS-α₁, OlGCS-α₁-FL-FLAG, OlGCS-α₁[280-678]FLAG or OlGCS-α₁[322-678]FLAG was
co-expressed with \textit{Olgcs-\(\beta_1\)-FL\textsubscript{HT}} in Sf9 cells and the GC activity of the each cytosolic fraction was measured. As shown in Fig. 3, \(\alpha_1\text{-[280-678]}\text{FLAG/}\beta_1\text{-FL\textsubscript{HT}}\) showed the basal and SNP-stimulated GC activity at almost the same extent as that of the control soluble GC (\(\alpha_1\text{-FL\text{FLAG/}\beta_1\text{-FL\textsubscript{HT}}})\), although \(\alpha_1\text{-[322-678]}\text{FLAG/}\beta_1\text{-FL\textsubscript{HT}}\) did not exhibit GC activity with and without SNP. Therefore, we presume that the NBS-like sequence of OIGCS-\(\alpha_1\) (amino acids 280-321) plays an important role to form the correct active center with the counterpart. This is in good agreement with the results that the \(\beta_1\) subunit lacking amino acids at position 204-303 (containing NBS: amino acids positions 204-244) failed to show the GC activity when co-expressed with the \(\alpha_1\) subunit, although it exhibits the binding ability to the \(\alpha_1\) subunit at a reduced level (Zhou \textit{et al.}, 2004). In a recent study, it was reported that an N-terminal binding site of bovine \(\alpha_1\) subunit (amino acids position 61-128) plays major important role in heterodimerization (Wagner \textit{et al.}, 2005). However, in this study, we did not observed such ability in the amino acids position 61-128. In the present study with the pull down assay and the assay of GC activity, we obtained the results to show that OIGCS-\(\alpha_1\text{[280-678]}\text{FLAG}\) could form heterodimer with OIGCS-\(\beta_1\text{-FL\textsubscript{HT}}\).

**Identification of amphipathic \(\alpha\)-helix mediating heterodimerization between OIGCS-\(\alpha_1\) and OIGCS-\(\beta_1\)**

To obtain further understanding on the nature of the CBS-like sequence of OIGCS-\(\alpha_1\) for heterodimerization and GC activity, we carried out prediction of the secondary structure of OIGCS-\(\alpha_1\) by the PredictProtein server (http://www.embl-heidelberg.de/predictprotein/) and found that OIGCS-\(\alpha_1\text{[431-464]}\) is able to form an amphipathic \(\alpha\)-helix structure (Fig. 4). It has been reported that amphipathic \(\alpha\)-helix often mediates protein-protein interactions (Carr \textit{et al}, 1991).
Therefore, we examined whether the amphipathic α-helix in the α₁-subunit could mediate heterodimerization of both subunits of soluble GC after introduction of site-directed mutation to the α₁-subunit. To disturb the amphipathicity of the α-helix, we focused the four Leu residues (Leu⁴³⁴, Leu⁴⁴⁵, Leu⁴⁵², and Leu⁴⁶³) as shown in dotted boxes in Fig. 4B, all of which were predicted to be located intensively on the one side of the α-helix. Various site-directed mutant proteins were generated as described in Materials and Methods. According to the prediction of the secondary structure of the α₁ subunit by the PredictProtein server, the α-helix structure should be maintained even after these four Leu residues were converted to Lys residues leading to loss of amphipathicity. OlGCS-α₁-L₄₃₄K_FLAG, L₄₄₅K_FLAG, L₄₆₃K_FLAG, L₄₃₄,₄₄₅,₄₆₃K_FLAG, or L₄₃₄,₄₄₅,₄₅₂,₄₆₃K_Flag was co-expressed with OlGCS-β₁-FL_HT in Sf9 cells and the GC activity of the each cytosolic fraction was measured with or without SNP. As shown in Fig. 5, α₁-L₄₃₄K_FLAG/β₁-FL_HT or α₁-L₄₄₅K_FLAG/β₁-FL_HT showed the basal and SNP-stimulated GC activities at almost the same extent as those of α₁-FL_FLAG/β₁-FL_HT, but α₁-L₄₆₃K_FLAG/β₁-FL_HT, L₄₃₄,₄₄₅,₄₆₃K_FLAG/β₁-FL_HT or L₄₃₄,₄₄₅,₄₅₂,₄₆₃K_FLAG/β₁-FL_HT did not show the basal GC activity nor the SNP-stimulatable GC activity. To investigate the binding ability of each site-directed mutant protein to OlGCS-β₁-FL_HT, we performed pull down assay. As shown in Fig. 6, the binding ability of OlGCS-α₁-L₄₃₄,₄₄₅,₄₆₃K_FLAG or OlGCS-α₁-L₄₃₄,₄₄₅,₄₅₂,₄₆₃K_FLAG to OlGCS-β₁-FL_HT was remarkably reduced, although α₁-L₄₃₄K_FLAG or α₁-L₄₄₅K_FLAG maintained its binding ability to OlGCS-β₁-FL_HT. These results suggest that the amphipathicity in the putative α-helix region of OlGCS-α₁ is important to form heterodimer with OlGCS-β₁.

The amphipathicity of the α-helix in OlGCS-α₁[1-448]_FLAG would be incomplete because of lacking the C-terminal half of the putative amphipathic α-helix (see Fig 1).
Therefore, we presume that it is one of the major reasons why OiGCS-α1[1-448]FLAG did not exhibit the binding ability to OiGCS-β1. Surprisingly, OiGCS-α1-L463KFLAG also maintained its binding ability to OiGCS-β1-FLHT, although the α1-L463KFLAG/β1-FLHT did not exhibit any GC activity, suggesting that the formation of heterodimer of OiGCS-α1 with OiGCS-β1 is not sufficient for exhibiting the GC activity, probably due to inadequate heterodimerization which leads to formation of inadequate active center. As shown in Fig. 4A, Leu463 is conserved among soluble GC-α1 subunits of various species and located in the C-terminal end of the amphipathic α-helix. For enzymatically active heterodimer formation, it seems to be essential that the correct active center between the catalytic domains of α and β subunit is formed. Conversion of Leu463 to Lys in OiGCS-α1 might lead the detachment of the hydrophobic bond in the C-terminal end of the amphipathic α-helix, although the rest of the amphipathic α-helix maintains the hydrophobic bonds. The detachment of this region appears to lead to the incorrect active center formation between the both subunit catalytic domains. This might be the case that L463KFLAG/β1-FLHT did not show any GC activity.

As shown in Fig. 4A, the amino acid sequence in this region varies among soluble GC α1 subunits of various species and the changes in the amino acid sequence did not affect the formation of the putative amphipathic α-helix, suggesting that the amphipathic α-helix plays a critical role in the heterodimerization of soluble GC α1 subunit with soluble GC β1 subunit. In addition, the analysis of the secondary structure of OiGCS-β1 by the PredictProtein server predicted that OiGCS-β1[367-395] forms an amphipathic α-helix, and similar analysis of OiGCS-α2 also resulted in prediction that OiGCS-α2[535-555] forms an amphipathic α-helix. These strongly suggest that the amphipathic α-helix mediates the heterodimerization of soluble GC α1 subunit with soluble GC β1 subunits.
ACKNOWLEDGEMENTS

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Legends to Figures

Fig. 1. Schematic representation of various O1GCS-α₁ constructs.
The NBS-like sequence (280-321) is represented by the hatched boxes and the CBS-like sequence is defined by dotted boxes. Numbers denote the relative positions of amino acids. A bold line indicates the predicted amphipathic α-helix region.

Fig. 2. Pull down assay on the NBS-like or the CBS-like sequence.
Sf9 cells were infected by baculovirus containing an O1GCS-α₁-FLFLAG, its N-terminal deletion mutants (A) or its C-terminal deletion mutants (B) with or without O1GCS-β₁-FLHT. Almost equal amount of O1GCS-β₁-FLHT was detected in the Ni²⁺ Sepharose-binding fraction by Western blotting (WB) using anti-HT antibody (top). The Ni²⁺ Sepharose-binding fraction was analyzed by Western blotting with anti-FLAG antibody (upper middle). To confirm the protein expression levels in the lysate, Western blotting was performed using anti-HT antibody or anti-FLAG antibody (lower middle and bottom, respectively). Similar results were obtained in two independent experiments.

Fig. 3. The GC activity of N-terminal deletion mutant proteins.
The GC activity in the lysate prepared from respective infected Sf9 cells was assayed with (black columns) or without (white columns) SNP. The activity represent means ± S.E.M. obtained from three independent experiments.

Fig. 4. The sequence and helical wheel diagram of the amphipathic α-helix of O1GCS-α₁.
(A) The amino acid sequences of the putative amphipathic $\alpha$-helix of various soluble GC $\alpha_1$ subunits were aligned. Identical residues are marked by asterisks and the hydrophobic residues were shaded. Numbers denote the relative positions of the amino acids. (B) Hydrophobic amino acid residues are boxed and the Leu residues modified by site-directed mutagenesis are covered with dotted boxes.

Fig. 5. The GC activity of site-directed mutant proteins. The GC activity in the lysate prepared from respective infected Sf9 cells was assayed with (black columns) or without (white columns) SNP. The activity represents means $\pm$ S.E.M. obtained from three independent experiments.

Fig. 6. Pull down assay of site-directed mutant proteins. Sf9 cells were infected by baculovirus containing $OlGCS-\alpha_1$-FL$_{FLAG}$ or its site-directed mutants with $OlGCS-\beta_1$-FL$_{HT}$. The fractions containing the Ni$^{2+}$ Sepharose-binding activity were analyzed by Western blotting with anti-FLAG antibody. The density of each band of the Ni$^{2+}$ Sepharose-binding proteins reacted with anti-FLAG antibody was estimated using Scion image. The data are expressed by fold density relative to the $\alpha_1$-FL$_{FLAG}$/\beta$_1$-FL$_{HT}$ as 1 and represent means $\pm$ S.E.M. obtained from three independent experiments. *$P < 0.05$ for the comparison with $\alpha_1$-L434K$_{FLAG}$/\beta$_1$-FL$_{HT}$ or $\alpha_1$-L445K$_{FLAG}$/\beta$_1$-FL$_{HT}$.
Table 1  Primers and the annealing temperature used for construction of plasmids for OIGCS-α₁-FL<sub>FLAG</sub> and its truncated mutants

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<td>a1endFLAG*XhoI</td>
<td>5′-CTCGAGTCACTTGTACATCGTCCCTGTTGTAAGGTTTGACA-3'</td>
</tr>
<tr>
<td>a1[280-678]FLAG</td>
<td>44°C</td>
</tr>
<tr>
<td>a1-dN279</td>
<td>5′-ACTAGTATGACCTCTGCTGGAACGCTCCCC-3'</td>
</tr>
<tr>
<td>a1endFLAG*XhoI</td>
<td>5′-CTCGAGTCACTTGTACATCGTCCCTGTTGTAAGGTTTGACA-3'</td>
</tr>
<tr>
<td>a1[322-678]FLAG</td>
<td></td>
</tr>
<tr>
<td>a1-dN321</td>
<td>5′-ACTAGTATGGGACTTAGAGGTCTCCCAAC-3'</td>
</tr>
<tr>
<td>a1endFLAG*XhoI</td>
<td>5′-CTCGAGTCACTTGTACATCGTCCCTGTTGTAAGGTTTGACA-3'</td>
</tr>
<tr>
<td>a1[1-477]</td>
<td></td>
</tr>
<tr>
<td>a1start-SpeI</td>
<td>5′-ACTAGTATGTTCTGCGGCAAGTTGAA-3'</td>
</tr>
<tr>
<td>a1-dC477</td>
<td>5′-CTCGAGTCACTTGTACATCGTCCCTGTTGTAAGGTTTGACA-3'</td>
</tr>
<tr>
<td>a1[1-448]</td>
<td>54°C</td>
</tr>
<tr>
<td>a1start-SpeI</td>
<td>5′-ACTAGTATGTTCTGCGGCAAGTTGAA-3'</td>
</tr>
<tr>
<td>a1-dC448</td>
<td>5′-CTCGAGTCACTTGTACATCGTCCCTGTTGTAAGGTTTGACA-3'</td>
</tr>
</tbody>
</table>

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Fig. 1

\( \alpha_1\text{-FL}_{\text{FLAG}} \)

\( \alpha_1[280-678]_{\text{FLAG}} \)

\( \alpha_1[322-678]_{\text{FLAG}} \)

\( \alpha_1[1-477]_{\text{FLAG}} \)

\( \alpha_1[1-448]_{\text{FLAG}} \)
Fig. 2

(A)  

<table>
<thead>
<tr>
<th></th>
<th>(\alpha_1)-FL _FLAG</th>
<th>(\alpha_1[280-678])-FL _FLAG</th>
<th>(\alpha_1[322-678])-FL _FLAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\beta_1)-FL _HT</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>(\beta_1)-FL _HT</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ni(^{2+}) Sepharose</td>
<td>\text{WB: anti-HT}</td>
<td>\text{WB: anti-HT}</td>
<td>\text{WB: anti-HT}</td>
</tr>
<tr>
<td>Ni(^{2+}) Sepharose</td>
<td>\text{WB: anti-FLAG}</td>
<td>\text{WB: anti-FLAG}</td>
<td>\text{WB: anti-FLAG}</td>
</tr>
<tr>
<td>lysate</td>
<td></td>
<td>\text{WB: anti-HT}</td>
<td>\text{WB: anti-HT}</td>
</tr>
<tr>
<td>lysate</td>
<td></td>
<td>\text{WB: anti-FLAG}</td>
<td>\text{WB: anti-FLAG}</td>
</tr>
</tbody>
</table>

(B)  

<table>
<thead>
<tr>
<th></th>
<th>(\alpha_1[1-477])-FL _FLAG</th>
<th>(\alpha_1[1-448])-FL _FLAG</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\beta_1)-FL _HT</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>(\beta_1)-FL _HT</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Ni(^{2+}) Sepharose</td>
<td>\text{WB: anti-HT}</td>
<td>\text{WB: anti-HT}</td>
</tr>
<tr>
<td>lysate</td>
<td>\text{WB: anti-HT}</td>
<td>\text{WB: anti-HT}</td>
</tr>
<tr>
<td>lysate</td>
<td>\text{WB: anti-FLAG}</td>
<td>\text{WB: anti-FLAG}</td>
</tr>
</tbody>
</table>
cGMP (pmol/mg protein/min)

- α₁-FL
- α₁[280-678]
- α₁[322-678]
- N/C

Fig. 3
mouse  421 QDGLKKRLGKLATLEHAQALEEEKKRTVDLLC 455
rat    420 QDGLKKRLGKLATLEHAQALEEEKKKTVDLLC 454
human  421 QDGLKKRLGKLATLEQAHQALEEEKKKTVDLLC 455
medaka 431 QDGLKKRLGGAKAALENAQHQQALEEEKKKTVDLLF 464
Drosophila 406 QDGLRRRMDKIKNSSIEEANSAVTKERKKNNSLIH 440
Manduca 422 QDGLRRRMDKLKNSIEEASKAVDKERKKNNSLIH 456

****  *  * *   * *  *   *    * **
Fig. 5

- $\alpha_{1-FL}$
- $\alpha_{1-L434K}$
- $\alpha_{1-L445K}$
- $\alpha_{1-L463K}$
- $\alpha_{1-L434,445,463K}$
- $\alpha_{1-L434,445,452,463K}$
- N/C

cGMP (pmol/mg protein/min)
Fig. 6

Ni²⁺ sepharose
WB: anti-FLAG

Relative Density

α₁-FLAG/β₁-FLAG
α₁-L434K-FLAG/β₁-FLAG
α₁-L445K-FLAG/β₁-FLAG
α₁-L463K-FLAG/β₁-FLAG
α₁-L434,445,463K-FLAG/β₁-FLAG
α₁-L434,445,452,463K-FLAG/β₁-FLAG

*