Functional Domains of Soluble Guanylyl Cyclase*

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Soluble guanylyl cyclase is a heterodimer consisting of an α and β subunit and stimulation occurs upon binding of NO to a prosthetic group. Little is known about the localization of catalytic and regulatory domains within the subunits of soluble guanylyl cyclase. We used deletion mutagenesis to identify the regions of α1 and β1 subunits that are responsible for cGMP production or NO-heme-mediated activation.

The amino terminus of the β1 subunit was necessary for NO stimulation since deletion of the 64 NH2-terminal amino acids resulted in a mutant with intact basal activity but complete loss of NO activation. The amino terminus of the α1 subunit also appeared to be essential for NO sensitivity since deletion of 131 NH2-terminal amino acids of α1 led to markedly reduced NO activation. These results suggest that NH2-terminal regions of α1 and β1 are involved in NO-heme-mediated signal transduction. The NH2 terminally truncated β1 subunit exerted a dominant negative effect exclusively on the NO-stimulated activity of the wild type enzyme, further underlining that the regulatory domain is located within the NH2 terminus of the enzyme. Aside from the structural implications, the mutant represents a powerful tool to investigate nitric oxide-sensitive signaling pathways. Coexpression of the COOH-terminal halves of α1 and β1 were sufficient for basal cGMP production while either of the halves expressed alone was inactive. Therefore the COOH-terminal regions appear to contain sufficient information for dimerization and basal enzymatic activity.

Thus, we provide the first evidence that the regulatory and catalytic properties of soluble guanylyl cyclase can be attributed to different regions of the subunits and that the catalytic domain can be functionally expressed separately from the NH2-terminal regulatory domain. Taken together with findings on the membrane bound enzyme form, guanylyl cyclases, appear to resemble fusion proteins where different regulatory domains have been joined with a common cGMP-forming segment.

Guanylyl cyclases (GTP pyrophosphate-lyase (cycycling); EC 4.6.1.2), the enzymes catalyzing the formation of cGMP, exist in membrane-bound and soluble forms. The membrane-bound enzymes are stimulated by different peptide hormones and belong to the group of receptor-linked enzymes with one membrane spanning region (1). Accordingly, their structure can be divided into three domains: an amino-terminal extracellular ligand-binding domain, an intracellular protein kinase-like domain, which has been proposed to function as a negative regulatory element (2), and the COOH-terminal catalytic region comprising about 250 amino acids responsible for the synthesis of cGMP, as shown by a deletion mutant of the membrane-bound guanylyl cyclase GC-A (3, 4). In contrast to the membrane-bound enzymes which exist as homodimers or higher ordered structures, soluble guanylyl cyclase consists of two different subunits designated α and β, which are both required for catalytic activity (5). Four subunits have been reported up to date (α1, α2, β1, and β2). The α1β1 heterodimer corresponds to the enzyme purified from bovine lung, whereas the α2 and β2 subunits have been identified by homology screening but have not yet been detected on the protein level. All subunits show some homologies over the whole length of the polypeptide chains; the strongest homologies are found in the COOH-terminal regions which are also shared with the membrane-bound guanylyl cyclases and adenylyl cyclases (6).

Soluble guanylyl cyclase is a heme protein with spectral properties indicative of a 5-coordinate ferrous heme with histidine as the axial ligand (7), and it is the heme moiety which serves as the receptor for nitric oxide (NO),1 the activator of the soluble enzyme (8). Recently, histidine 105 of the β1 subunit has been shown to be essential for the stimulation by NO since substitution by phenylalanine yielded an enzyme that was catalytically active but insensitive to NO (9).

It is unknown whether heme binding and catalytic activity can be attributed to distinct domains of soluble guanylyl cyclase. Here, we show that the COOH-terminal halves of the α1 and β1 subunits comprising the putative catalytic domain are sufficient for cGMP formation. Moreover, deletions of the poorly conserved NH2-termini of the β1 (β1-ΔN150) and α1 subunits (α1-ΔN150) led to severe impairment of NO stimulation, thus underlining the importance of the NH2-terminal regions of both subunits for heme binding and/or transduction of the stimulatory binding signal to the catalytic domain. The NH2-terminally truncated β1 subunit specifically blocks only the stimulated activity of α1β1 wild-type enzyme while leaving the non-stimulated activity unchanged. Therefore, this mutant offers a new and potentially powerful means by which to inhibit the NO stimulated but not basal catalytic activity of the soluble form of guanylyl cyclase.

EXPERIMENTAL PROCEDURES

NH2-terminal truncated mutants of α1 and β1 subunits were constructed by polymerase chain reaction (PCR). Fragments coding for shortened NH2-termini were amplified and used to substitute the re-

1. The abbreviations used are: NO, nitric oxide; GSNO, S-nitrosothiolamine; PCR, polymerase chain reaction; m.o.i., multiplicity of infection.
functional domains of soluble guanylyl cyclase.
Burk plot of the data revealed similar apparent 
the presence of increasing GTP concentrations. A Lineweaver-
acterized. To compare the kinetic properties of this mutant 
stimulated conditions (100
subunits (Fig. 3
B
activitywasnotduetoadecreaseinexpressionofthewildtype 
nation of the catalytic activity, we performed Western blots of 
3
mg
and 20% of the original activity (5200 pmol of cGMP 
Mn2
2), suggesting proper folding of the catalytic domain.

The use of Mn2
1
MGTPfor
3
mM Mg2
1
2
1
1
1
1
1
1
1

α

β

β

α

α

α

β

α

β

ND
ND
90
90
90
ND
ND
60
50
50
1
90
90
1
1
10
1
1
1

a

ND, activity not detectable.

a Fold stimulation cannot be given as basal activity was under the detection limit.

**TABLE I**

| Coexpressed subunits | Guanylyl cyclase activity | Stimulation | Guanylyl cyclase activity | Stimulation |
|-----------------------|---------------------------|-------------|---------------------------|-------------|
|                       | pmol cGMP \( \times \) min\(^{-1}\) \times mg\(^{-1}\) | -fold       | pmol cGMP \( \times \) min\(^{-1}\) \times mg\(^{-1}\) | -fold       |
| \( \alpha_1 \)         | 1 __________________ 691 | 50         | 4290         | 92          |
| \( \beta_1 \)          | 1 __________________ 619 | 40         | 40          | 1           |
| \( \beta_1\Delta N_{64} \) | 65 __________________ 619 | ND         | ND          | ND          |
| \( \alpha_1 \)         | 1 __________________ 691 | ND         | ND          | ND          |
| \( \beta_1\Delta N_{305} \) | 306 ________________ 619 | ND         | ND          | ND          |
| \( \alpha_1\Delta N_{131} \) | 306 ________________ 691 | ND         | ND          | ND          |
| \( \beta_1 \)          | 1 __________________ 619 | ND         | ND          | ND          |
| \( \alpha_1\Delta N_{366} \) | 367 ________________ 691 | ND         | ND          | ND          |
| \( \beta_1\Delta N_{305} \) | 306 ________________ 619 | ND         | ND          | ND          |

DISCUSSION

In the present study, we demonstrate that coexpression of the COOH-terminal halves of the \( \alpha_1 \) and \( \beta_1 \) subunits of soluble guanylyl cyclase, comprising the region conserved in all cyclases, yields an enzyme sufficient for the formation of cGMP but insensitive to NO. Preliminary results indicate that COOH-
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Fig. 3. Dominant-negative effect of the β1ΔN64 mutant on the stimulated activity of the wild type. Sf9 cells were coinfectd with viruses coding for the α1 (0.1 m.o.i.) and β1 subunits (1 m.o.i.) alone (WT) or in the presence of increasing amounts of the virus coding for the β1ΔN64 mutant (0.02, 0.04, 0.06, 0.08, and 0.1 m.o.i.) or with the viruses coding for the the α1 (0.1 m.o.i.) and the β1ΔN64 mutant (0.05 m.o.i.). A, cyclase activity was determined in the presence of 3 mM MnCl2 as described under "Experimental Procedures." B, shown are immunobLOTS of the same cytosolic protein preparations that were used for the determination of enzymatic activity. Cytosolic proteins (30 μg) were separated on SDS-polyacrylamide electrophoresis gels and blotted onto nitrocellulose, peptide antibodies against the COOH termini of the β1 and α1 subunits were used for detection of the wild type subunits and the β1ΔN64 mutant.

terminal truncated mutants are catalytically inactive, thus further underlining the importance of the catalytic consensus domain. Our results are in good agreement with reports on an NH2-terminal truncated GC-A receptor mutant exhibiting ligand-independent cGMP production. In the deletion mutant of the GC-A receptor, the cyclase catalytic domain is preceded by the so-called hinge region (47 amino acids) and a small portion (15 amino acids) of the carboxyl segment of the consensus protein kinase-like domain. These additional amino acids were shown to be essential for a properly folded catalytically active site, and it was suggested that these sequences may be required for dimerization (12). In a recent report, the 43 amino acids in front of the catalytic domain have indeed been shown to be required for dimerization (13). In analogy, the 80 additional amino acids NH2-terminal of the putative catalytic region present in the COOH-terminal halves of the α1 and β1 subunits may be required for proper folding or dimerization of the subunits. Dimerization of the subunits is a prerequisite for enzyme activity since expression of one subunit or a truncated mutant did not yield any cGMP forming activity.

The various adenylyl cyclases also contain two cyclase homology domains which in contrast to soluble guanylyl cyclase are localized on one polypeptide chain. Similarly as in soluble guanylyl cyclase, both domains present in adenylyl cyclases are required for catalysis as the separate expression of either region results in a loss of enzyme activity (14). Moreover, the ability of the NH2-terminally truncated GC-A receptor mutant to dimerize coincided with intact catalytic activity, further underlining the necessity of two catalytic consensus domains. The significance of the association of two different catalytic domains in soluble guanylyl cyclases and adenylyl cyclases as opposed to the existence of two identical catalytic domains in the membrane bound guanylyl cyclases, however, is unknown.

Besides the identification of the catalytic domain, we show that even the very low conserved NH2-terminal part of the β1 subunit (64 amino acids) is required for the stimulation by NO. Recently, we identified histidine 105 of the β1 subunit as a likely candidate to be the residue forming a linkage to the central iron atom of the prosthetic heme group, since substitution of this histidine 105 by phenylalanine yielded an NO-insensitive enzyme lacking the prosthetic heme group (9). Although histidine 105 of the β1 subunit is present in the mutant β1ΔN64, the loss of NO sensitivity suggests disturbance of either a configuration required for heme binding or lack of structures involved in the transduction of the stimulatory effect to the catalytic center. Deletion of the NH2-terminal part of the α1 subunit (131 amino acids) yielded an enzyme still activated by NO, although the non-responsiveness in the presence of MnCl2 suggests a severe impairment of NO stimulation. As further deletion of the α1 subunit also destroys NO activation of the enzyme, we conclude that the NH2-terminal regions of the α1 and β1 subunits are responsible for the regulation of the enzyme. Characterization of the purified mutants will reveal whether those are still able to bind heme or whether the mediation of the stimulatory signal is impaired. When coexpressed with the wild type enzyme, the NH2-terminally truncated β1 subunit inhibits only the stimulated activity but leaves the basal activity unchanged. The ability of the mutant to compete with the wild type β1 subunit for dimerizing with the α1 subunit further emphasizes the NH2-terminal location of the regulatory domain. The dominant-negative effect of the mutant on the stimulated activity will allow the selective inhibition of nitric oxide-dependent cGMP formation in intact cells. Hence, the mutant will be a helpful tool in forthcoming studies of the NO-cGMP signaling system and, in this regard, the retained ability of basal cGMP formation is probably beneficial when compared to the recently identified dominant-negative mutants that knock out guanylyl cyclase activity completely (16).

Our results show that the two defining features of soluble guanylyl cyclase, namely the catalytic activity and the regulation by NO, can be attributed to different regions on the subunits and that the catalytic domain can be expressed separately. Similar results exist for the bacterial protein FixL, a dimeric hemoprotein kinase whose enzymatic activity is reversibly blocked by oxygen binding to the heme. There the heme binding domain and the phosphotransferase activity were also attributed to the NH2-terminal and COOH-terminal regions, respectively; they still contained their heme-dependent oxygen-binding and catalytic properties when expressed separately (15). These findings suggest that during evolution, regulatory segments have been connected to distinct catalytic domains. In the case of guanylyl cyclases, a common cGMP-forming catalytic unit occurs under the control of different regulatory domains; in that respect, the regulatory heme-containing domain of the soluble enzyme appears to represent the equivalent to the ligand-binding and kinase-like domains of the membrane bound guanylyl cyclases.

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