Identification of a Novel Guanylyl Cyclase That Is Related to Receptor Guanylyl Cyclases, but Lacks Extracellular and Transmembrane Domains

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We have identified a novel guanylyl cyclase, named MsGC-I, that is expressed in the nervous system of Manduca sexta. MsGC-I shows highest sequence identity with receptor guanylyl cyclases throughout its catalytic and dimerization domains but does not contain the ligand-binding, transmembrane, or kinase-like domains characteristic of receptor guanylyl cyclases. In addition, MsGC-I contains a C-terminal extension of 149 amino acids that is not present in other receptor guanylyl cyclases. The sequence of MsGC-I contains no regions that show similarity to the regulatory domain of soluble guanylyl cyclases. Thus, MsGC-I appears to represent a member of a new class of guanylyl cyclases. We show that both a transcript and a protein of the sizes predicted from the MsGC-I cDNA are present in the nervous system of Manduca and that MsGC-I is expressed in a small population of neurons within the abdominal ganglia. When expressed in COS-7 cells, MsGC-I appears to exist as a soluble homodimer with high levels of basal guanylyl cyclase activity that is insensitive to stimulation by nitric oxide. Western blot analysis, however, shows that MsGC-I is localized to the particulate fraction of nervous system homogenates, suggesting that it may be membrane-associated in vivo.

The intracellular messenger 3',5'-cyclic guanosine monophosphate (cGMP) plays an important role in numerous physiological functions, including visual and chemosensory signal transduction, control of fluid and ion transport, smooth muscle relaxation, and the modulation of synaptic efficacy (1–4). The enzyme responsible for cGMP synthesis is guanylyl cyclase (GTP pyrophosphate-lyase (cycling); EC 4.6.1.2). Currently, guanylyl cyclases are classified as one of two distinct enzymatic forms, soluble or receptor, based upon their cellular distribution and structure. Soluble guanylyl cyclases are localized within the cell cytoplasm, where they function as heterodimers composed of α and β subunits. Each subunit consists of a regulatory domain, which contains sequences responsible for heme binding and heterodimer formation, and a catalytic domain (5). The soluble guanylyl cyclases contain an attached protoporphyrin-IX-type heme prosthetic group that is required for activation. The best characterized activator of soluble guanylyl cyclase is nitric oxide (NO)1 which binds to the iron within the attached heme moiety, resulting in a conformational change in the protein and increased enzymatic activity (6). Other factors such as carbon monoxide have also been shown to activate soluble guanylyl cyclase (7) by a similar mechanism.

Receptor guanylyl cyclases are transmembrane proteins thought to act primarily as homodimers although they have been shown to form trimers, tetramers, and other higher oligomer units (8). All known receptor guanylyl cyclases are glycoproteins containing five functional domains: an extracellular ligand-binding domain, a single transmembrane domain, a kinase-like regulatory domain, a dimerization domain, and a catalytic domain (9, 10). At present there are seven known mammalian receptor guanylyl cyclase isoforms, named GC-A through GC-G (3, 13), and recently a family of at least 26 different putative receptor guanylyl cyclase isoforms has been identified in Caenorhabditis elegans (3). Receptor guanylyl cyclases are generally activated through the binding of an extracellular peptide ligand, although the retinal guanylyl cyclases can be activated through a decline in intracellular calcium levels (11). In addition, ATP, which binds to a distinct site within the kinase-like domain (12), can also modulate their activity.

Recent reports have suggested the existence of additional forms of guanylyl cyclase that are cytoplasmically localized yet insensitive to NO. An unusual guanylyl cyclase, designated ksGC (kinase-like domain containing soluble guanylyl cyclase), has been cloned from rat kidney cells (14). From DNA sequence analysis, this clone appears to contain the kinase-like, dimerization and catalytic domains characteristic of receptor guanylyl cyclases but contains no ligand-binding or transmembrane domains. This indicates that ksGC is a cytoplasmically localized guanylyl cyclase that is insensitive to NO. These sequence-based predictions have not yet been tested, as the putative protein encoded by this cDNA is enzymatically inactive when expressed in heterologous cells. Another cytoplasmically localized NO-insensitive guanylyl cyclase activity has also recently been reported in the nervous system of lobsters, which can be separated from a less prevalent NO sensitive form by anion exchange high performance liquid chromatography (15).

Here we describe the cloning and characterization of a novel form of guanylyl cyclase, from the nervous system of the insect, Manduca sexta. This cyclase, named MsGC-I, shows highest

1 The abbreviations used are: NO, nitric oxide; cGMP, 3',5'-cyclic guanosine monophosphate; GST, glutathione S-transferase; SNP, sodium nitroprusside; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; kb, kilobases(s); GCAP, guanylyl cyclase activating protein.
sequence identity with the receptor guanylyl cyclase, GC-B, throughout its catalytic and dimerization domains, but does not contain the other domains associated with receptor guanylyl cyclases. The discovery and characterization of this novel guanylyl cyclase has the potential to expand the known mechanisms of cGMP regulation.

MATERIALS AND METHODS

Animals—The rearing and staging of M. sexta have been described previously (16).

Cloning of the MsGC-I cDNA Clone—MsGC-I was isolated using a degenerate oligonucleotide reverse transcriptase-polymerase chain reaction approach to identify guanylyl cyclases in the abdominal nervous system of M. sexta. Degenerate oligonucleotide primers were designed against the amino acid sequences DYYKVETI (CRRCAAARCCARTA-ICKNGGCA) and MPYCLFLG (GAYTTTAYAARRTGIACNAT) from the catalytic domain common to both soluble and receptor guanylyl cyclases. RNA isolation, reverse transcriptase-polymerase chain reaction, cDNA library construction and screening, sequencing, and Northern blot analysis were carried out using conventional procedures described previously (16).

COS-7 Cell Expression, Guanylyl Cyclase Activity Assay, and cGMP Measurement—The full-length open reading frame of MsGC-I was subcloned into the mammalian expression vector pGEX 4T-2 (Amersham Pharmacia Biotech). The resulting protein was produced in Escherichia coli using the T7 promoter. The resulting GST-fusion protein was purified by glutathione-Sepharose 4B affinity chromatography and transiently transfected into COS-7 cells (1:2,000 concentration to 100 mg/ml). The composition of the hybridization solution was also modified to: 5 x saline/sodium phosphate/EDTA, 50% formamide, 5% dextran sulfate, 1 x Denhardt’s solution, 500 mg/ml sonicated salmon sperm DNA, 250 mg/ml yeast tRNA. Tissue was pre-hybridized in this solution for 1-2 h at 50 °C, denatured probe was then added, and samples were incubated overnight at 50 °C. Wash steps following RNase treatment were modified by the addition of a wash in 1 x SSC at room temperature for 10 min, followed by three washes in 0.1 x SSC for 20 min each at 50 °C prior to blocking. Two percent cold-water fish gelatin was added to the blocking solution. Alkaline phosphatase-conjugated anti-digoxigenin antibody was used at 1:1,000 dilution and incubated overnight at 4 °C. Following the wash steps, the alkaline phosphatase-conjugated antibody was detected using 3 mg/ml 5-bromo-4-chloro-3-indolylphosphate substrate (Boehringer, Mannheim), washed, dried, and exposed to Kodak XAR-5 film. All procedures involving animals were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of California at Berkeley.

RESULTS

Cloning and Sequence Analysis of MsGC-I—We have used RT-PCR with degenerate oligonucleotides designed to a conserved region of the catalytic domain to identify guanylyl cyclases from the nervous system of M. sexta and have isolated fragments of eight different cyclases. Cloning and characterization of three of these, which belong to the soluble guanylyl cyclase class, have already been reported (16, 20). Here we report the cloning of a novel receptor-like guanylyl cyclase, named MsGC-I. Based on sequence analysis, MsGC-I appears to create a new class of guanylyl cyclase, which does not fit into either the soluble or receptor guanylyl cyclase class.

We have screened two independent cDNA libraries made from Manduca abdominal central nervous tissue and obtained five identical full-length copies of the MsGC-I clone. The full-length MsGC-I cDNA is 2,560-base pairs long and contains a 1,500-base pair open reading frame. The open reading frame begins with an initiator methionine at position 108 and ends with a stop codon at position 1,608 followed by a 3'-untranslated region and a poly(A) tail. One in-frame and several out-of-frame stop codons precede the start of the open reading frame. The full-length sequence of MsGC-I has been placed into GenBank under accession number AF073342.

The MsGC-I open reading frame translates into a predicted 500-amino acid protein that displays highest similarity to atrial natriuretic peptide receptor-B (GC-B) by BLAST analysis. As can be seen in this figure, MsGC-I shows high similarity (76-77% identity) to GC-B within the catalytic domain (GC-B, 840-1047; MsGC-I, 144-350), and putative dimerization domain (GC-B, 798-839; MsGC-I, 102-143). There are, however, only 101 amino acids preceding the start of the dimerization domain of MsGC-I, and these do not appear to contain a signal sequence or the ligand-binding, transmembrane, or kinase-like domains characteristic of receptor guanylyl cycla-
The MsGC-I protein appears to begin within the C-terminal region of the kinase-like domain of GC-B. Throughout this region MsGC-I shows only 12% identity with the GC-B kinase-like domain, and visual examination of the sequence reveals no consensus ATP binding site \((G-X_3-X_4-G; \text{Ref } 12)\). A novel sequence is also found at the C terminus of MsGC-I, which extends 149 amino acids beyond the end of the catalytic domain. This domain does not show significant homology to any proteins found within the NCBI database.

A phylogenetic tree analysis of receptor and soluble guanylyl cyclase catalytic domains groups the catalytic domain of MsGC-I with the catalytic domains of receptor guanylyl cyclases. When compared in a pairwise fashion, MsGC-I shows only 33% identity with the catalytic domain of soluble cyclases compared with 77% identity with GC-B. The N-terminal end of MsGC-I also shows no significant similarity with the sequences containing the heme-binding and heterodimerization regions of the soluble guanylyl cyclases (21, 22). Thus, sequence analysis suggests that MsGC-I is a novel guanylyl cyclase that is most similar to receptor cyclases within the catalytic and dimerization domains but does not contain sequences that would allow either membrane localization or stimulation by ligand binding.

**Northern Blot Analysis of MsGC-I Transcripts**—To confirm that the MsGC-I cDNA represented a full-length clone rather than a truncated version of a receptor guanylyl cyclase, we performed Northern blot analysis using a series of probes designed to hybridize to all portions of the MsGC-I cDNA. These results are shown in Fig. 2. All of the probes hybridized to a 2.5-kb transcript, the size predicted from the MsGC-I cDNA. An additional, longer transcript of 4 kb was also labeled by probes made to the highly conserved catalytic region (23, 25). These results demonstrate that a transcript of the predicted size for MsGC-I is made within the *Manduca* nervous system and suggest that MsGC-I is not a truncated version of a larger receptor guanylyl cyclase.

**Expression of MsGC-I in COS-7 Cells**—To examine the enzymatic properties of MsGC-I, we subcloned its open reading frame into the mammalian expression vector pcDNA3.1 and transiently transfected COS-7 cells with this construct. Cell extracts were examined for guanylyl cyclase activity in the presence of both Mg\(^{2+}\) and Mn\(^{2+}\) as guanylyl cyclases show different levels of activity in the presence of these two cations (23). No guanylyl cyclase activity could be detected in untransfected COS-7 cells, cells treated with LipofectAMINE alone, or cells transfected with the control pcDNA3.1 vector (data not shown).
MsGC-I or co-transfected with MsGC-I transiently transfected with either COS-7 cells were compared with cells cotransfected with MsGC-I accumulated a similar level of cGMP lyl cyclase activity of MsGC-I under these conditions: cells same conditions. It is interesting to note the high basal guany-
subunits clearly showed SNP-stimulated activity under these
conditions. To determine the molecular weight of MsGC-I proteins in COS-7 cells, we used gel filtration to separate the particulate and soluble fractions. In both cases, the supernatant was further separated by gel filtration, and fractions were assayed for guanylyl cyclase activity in the presence of 4 mM Mn²⁺. Molecular weights of the active fractions were calculated based on the elution times of known standards run under the same conditions (see inset).

Fig. 3. A, guanylyl cyclase activity in transfected COS-7 cells. COS-7 cells were transiently transfected with either MsGC-I or co-transfected with MsGC-α1 and MsGC-β1 in the expression vector pcDNA3.1. Enzyme activity was measured in the presence of either 4 mM Mg²⁺ or 4 mM Mn²⁺ as shown and in the presence (filled bars) or absence (open bars) of 100 μM SNP. Values reported are the mean ± S.E. of four to nine determinations. B, subcellular localization of MsGC-I in COS-7 cells. COS-7 cells were transiently transfected with MsGC-I, homogenized, and separated by centrifugation at 100,000 × g for 1 h at 4 °C. Guan-

Loss of heme, and thus loss of NO sensitivity, is a common phenomenon that occurs during purification of soluble guanylyl cyclase (22). To determine whether a similar phenomenon was responsible for the lack of NO stimulation of MsGC-I, we measured the NO sensitivity of MsGC-I in intact COS-7 cells. We exposed intact transfected COS-7 cells to SNP and then determined the level of cGMP within the cells. Again MsGC-I-transfected COS-7 cells showed no response to SNP (Table I), whereas COS-7 cells co-transfected with the M. sexta α1 and β1 subunits clearly showed SNP-stimulated activity under these same conditions. It is interesting to note the high basal guanylyl cyclase activity of MsGC-I under these conditions: cells transfected with MsGC-I accumulated a similar level of cGMP compared with cells cotransfected with MsGC-α1 and MsGC-β1 and then stimulated with SNP to determine the cellular localization of MsGC-I in these transfected COS-7 cells, we used ultracentrifugation to separate the homogenates before assaying the guanylyl cyclase activity. Fig. 3B shows that the majority of the activity is in the supernatant, suggesting that MsGC-I is cytoplasmically localized in this heterologous cell system.

MsGC-I contains a sequence with high similarity to a region in GC-A known to function as a dimerization domain (9). To determine whether MsGC-I functioned as a homodimer, we measured its apparent molecular weight using high performance liquid chromatography gel filtration. The results of this experiment are shown in Fig. 3C. The predicted size of the MsGC-I monomer protein, based on sequence analysis, is 55 kDa. The majority of the enzyme activity in transfected COS-7 cell homogenates eluted in fractions corresponding to the predicted sizes of both a dimer (M_r = 110,000) and a trimer (M_r = 165,000), with no activity detectable in fractions eluting at the predicted elution time for a monomer protein.

Western Blot Analysis of the MsGC-I Protein—To determine whether a protein of the predicted size is present in the nervous system of Manduca, we generated antisera to the C-terminal portion of the protein and carried out Western blot analysis. Fig. 4A shows the results of Western blot analysis on COS-7 cells that had been transfected with either MsGC-I, or one of the Manduca soluble guanylyl cyclase subunits (MsGC-α1, MsGC-β1, and MsGC-β3). Two bands were detected in COS-7 cells that had been transfected with MsGC-I. One of these bands was of the predicted size for the MsGC-I protein product, approximately 55 kDa, whereas the other band was smaller, appearing to be slightly less than 44 kDa. No immunoreactive bands were detected in any of the samples from COS-7 cells transfected with other cloned guanylyl cyclases nor when the antiserum was specific for MsGC-I. Band 1 showed a higher abundance of the smaller band was greatly enhanced while the abundance of the smaller band was the product of protease activity (data not shown). No bands were detected by incubation of the blot with pre-immune serum. Furthermore, pre-incubation of the antiserum with the

![Fig. 3](http://www.jbc.org/)

**Fig. 3.** A, guanylyl cyclase activity in transfected COS-7 cells. COS-7 cells were transiently transfected with either MsGC-I or co-transfected with MsGC-α1 and MsGC-β1 in the expression vector pcDNA3.1. Enzyme activity was measured in the presence of either 4 mM Mg²⁺ or 4 mM Mn²⁺ as shown and in the presence (filled bars) or absence (open bars) of 100 μM SNP. Values reported are the mean ± S.E. of four to nine determinations. B, subcellular localization of MsGC-I in COS-7 cells. COS-7 cells were transiently transfected with MsGC-I, homogenized, and separated by centrifugation at 100,000 × g for 1 h at 4 °C. Guanylyl cyclase assays were performed on the whole homogenate (Homog.), the supernatant (Sup.), and the pellet in the presence of 4 mM Mn²⁺. Values are the mean ± S.E. of four determinations. C, gel filtration of MsGC-I. COS-7 cells were transfected and homogenized, and the particulate and soluble fractions were separated. The supernatant was further separated by gel filtration, and fractions were assayed for guanylyl cyclase activity in the presence of 4 mM Mn²⁺. Molecular weights of the active fractions were calculated based on the elution times of known standards run under the same conditions (see inset).
COS-7 cells were transiently transfected and three days later were incubated in the presence or absence of 1 mM SNP for 5 min, harvested, and extracts assayed for cGMP content. cGMP content is expressed as fmol of cGMP/well (each well of a 24-well plate contained approximately 10⁵ cells) and represents the mean ± S.E. of six determinations. The experiment was duplicated and gave similar results.

TABLE I
Activity of MsGC-I in intact COS-7 cells

| Transfection | cGMP content | cGMP content (less control transfection) | Fold stimulation |
|--------------|--------------|------------------------------------------|-----------------|
| pCNA3.1      | 11.0 ± 0.4   | 193 ± 10                                 |                 |
| MsGC-I       | 347 ± 20     | 559 ± 54                                 |                 |
| MsGC-αa + MsGC-β1 | 15.7 ± 1.5 | 554 ± 38                                 |                 |
| MsGC-β1      | 336 ± 20     | 366 ± 54                                 | 1.09            |
| MsGC-β1      | 4.7 ± 1.5    | 340 ± 38                                 | 72.3            |

**DISCUSSION**

This paper describes the cloning and characterization of a novel guanylyl cyclase isoform, which we have named MsGC-I. Previously described guanylyl cyclases have been classified as either receptor or soluble, based on their intracellular localization and general structure. Receptor guanylyl cyclases are membrane bound and primarily activated by ligand binding. Soluble guanylyl cyclases are cytoplasmically localized and primarily activated by NO. MsGC-I does not fit into either of these classifications and thus may define a new class of guanylyl cyclase. The catalytic domain of MsGC-I appears most similar to those of receptor cyclases, specifically GC-B, but it does not contain a signal sequence or the ligand-binding, transmembrane and kinase-like domains of previously identified receptor guanylyl cyclases. In addition, MsGC-I shows no similarity to the regulatory domain of soluble guanylyl cyclases. It also contains a 149-amino acid extension beyond the catalytic domain that has no similarity to any protein in the data bases and has no known function.

The guanylyl cyclase most similar in domain structure to MsGC-I is ksGC, cloned from rat kidney cells (14). Sequence analysis of ksGC cDNA shows that it is most closely related to receptor guanylyl cyclases and contains catalytic, dimerization and kinase-like domains yet contains no ligand-binding or transmembrane domains. It has been suggested, however, that ksGC is a partial-length cDNA of longer guanylyl cyclase, specifically the recently described GC-G (13). This is based on both sequence similarity between the two cDNAs and the fact that ksGC has not been shown to produce a functionally active protein. MsGC-I, on the other hand, is clearly not a cloning artifact. The cDNA was independently isolated five times from two different cDNA libraries, and both a transcript and a protein of the predicted sizes for MsGC-I have been shown to be present in the Manduca nervous system using northern and Western blot analysis.
to cells transfected with the MsGC-I synthesized similar amounts of cGMP accumulation of cGMP in intact COS-7 cells that had been activity of MsGC-I within COS-7 cells, we also measured the Mg-GTP is the substrate used activity when Mn-GTP is provided as a substrate, it is likely substrate rather than Mg-GTP. This is a similar property to the functionally active guanylyl cyclase by expressing it in COS-7 cells. In COS-7 cells, MsGC-I shows high basal guanylyl cyclase activity, which is much higher when Mn-GTP is provided as a substrate, suggesting that this region in MsGC-I monomers, whereas most of the activity eluted at a position consistent with either homodimer or homotrimer formation. Although homodimerization of MsGC-I appears the most likely explanation of these results, it is also possible that it forms complexes with endogenous proteins in the COS-7 cells.

Ultracentrifugation of COS-7 cell homogenates shows that MsGC-I is located in the cytoplasm of these cells. Western blot analysis, however, shows that, in the nervous system of Manduca, MsGC-I is present in the particulate fraction suggesting association with membranes. Although there are two potential sites for fatty acylation within the MsGC-I sequence, they are not localized at either end of the protein, suggesting that neither represent true fatty acylation sites. In addition, fatty acyl-mediated protein localization has been demonstrated in heterologously expressed proteins in COS-7 cells (e.g. Ref. 24), yet in COS-7 cells MsGC-I is cytoplasmically located. Thus, it seems more likely that, in vivo, MsGC-I is localized to the membrane through an interaction with another protein. One possible candidate for this protein is a receptor guanylyl cyclase. To form homodimers, receptor guanylyl cyclases can also form heterodimers (8), indicating that MsGC-I could be localized to membranes by forming a heterodimer with an endogenous receptor guanylyl cyclase. Another possibility is that the unique C-terminal domain of MsGC-I interacts with an unknown membrane protein.

The photoreceptor-specific receptor guanylyl cyclases, GC-E and GC-F (RetGC-I and RetGC-2 in humans) are regulated by their interactions with a heterologous class of proteins—the guanylyl cyclase activating proteins (GCAPs, Ref. 25), which interact with the cyclases at some part of their intracellular domain (11). At high calcium concentrations, the GCAPs inhibit the retinal guanylyl cyclases, and when calcium concentrations drop within the photoreceptors, this inhibition is relieved and the cyclases are activated (11). Recently, GCAPs have also been shown to regulate olfactory cell-specific guanylyl cyclases in a similar manner (26). MsGC-I could also be regulated by a GCAP, inhibiting its activity while also localizing it to the membrane. When the cells containing MsGC-I are stimulated, this inhibition could be relieved. This could also cause dissociation of MsGC-I from the membrane, allowing it to form highly active, cytoplasmically localized homodimers.

In summary, these findings identify MsGC-I as a member of a new class of guanylyl cyclase. Based on its structure and enzyme activity in heterologous cells, we have shown that it cannot be directly activated by either extracellular peptide ligands or NO. This suggests a novel mechanism for the regulation of intracellular cGMP.

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